Engineered Zinc-Finger Proteins Can Compensate Genetic Haploinsufficiency by Transcriptional Activation of the Wild-Type Allele: Application to Williams-Beuren Syndrome and Supravalvular Aortic Stenosis

Pei Zhang,^{1,2} Angela Huang,^{1,3} Manuel Morales-Ruiz,⁴ Barry C. Starcher,⁵ Yan Huang,^{1,2}
William C. Sessa,^{1,6} Laura E. Niklason,^{1,7} and Frank J. Giordano^{1,2}

Abstract

Williams-Beuren syndrome (WBS) and supravalvular aortic stenosis (SVAS) are genetic syndromes marked by the propensity to develop severe vascular stenoses. Vascular lesions in both syndromes are caused by haploinsufficiency of the elastin gene. We used these distinct genetic syndromes as models to evaluate the feasibility of using engineered zinc-finger protein transcription factors (ZFPs) to achieve compensatory expression of haploinsufficient genes by inducing augmented expression from the remaining wild-type allele. For complex genes with multiple splice variants, this approach could have distinct advantages over cDNA-based gene replacement strategies. Targeting the elastin gene, we show that transcriptional activation by engineered ZFPs can induce compensatory expression from the wild-type allele in the setting of classic WBS and SVAS genetic mutations, increase elastin expression in wild-type cells, induce expression of the major elastin splice variants, and recapitulate their natural stoichiometry. Further, we establish that transcriptional activation of the mutant allele in SVAS does not overcome nonsense-mediated decay, and thus ZFP-mediated transcriptional activation is not likely to induce production of a mutant protein, a crucial consideration. Finally, we show in bioengineered blood vessels that ZFP-mediated induction of elastin expression is capable of stimulating functional elastogenesis. Haploinsufficiency is a common mechanism of genetic disease. These findings have significant implications for WBS and SVAS, and establish that haploinsufficiency can be overcome by targeted transcriptional activation without inducing protein expression from the mutant allele.

Introduction

ELASTIN IS AN ESSENTIAL EXTRACELLULAR matrix protein that confers the property of elasticity to specific tissues, including lung, skin, elastic cartilage, and blood vessels (Briones *et al.*, 2010; Rosenbloom *et al.*, 1993; Sherratt 2009). While homozygous loss of elastin gene (*ELN*) expression is incompatible with life, loss of function of a single *ELN* allele, through mutation or deletion, occurs clinically and is the basis of two well described genetic haploinsufficiency syndromes; supravalvular aortic stenosis (SVAS) and Williams-Beuren Syndrome (WBS) (Li *et al.*, 1997; Pober, 2010; Pober *et al.*, 2008a). Although the molecular genetics of these two syndromes is quite distinct, they both feature very similar vascular abnormalities, typified by segmental vascular narrowing in the aorta, pulmonary vasculature, renal arteries,

and other vessels, and specifically by a characteristic narrowing of the aorta just above the aortic valve. That these vascular abnormalities are caused by a deficiency of elastin has been corroborated in genetic models (Li *et al.*, 1998a; Li *et al.*, 1998b).

In humans, the *ELN* gene is located on chromosome 7 in a region (7q11.23) designated the WBS locus. WBS is caused by heterozygous non-allelic homologous recombination at this locus, resulting in deletion of the entire *ELN* gene and 25–27 additional genes (Pober *et al.*, 2008a). The intact *ELN* allele is noncompensatory in WBS, and expression is actually often lower than explainable on the basis of pure haploinsufficiency alone (e.g., 30–40% of normal levels). There are at least 50 identified heterozygous *ELN* mutations that can result in SVAS. These mutations generally cause frame-shifts that create premature termination codons (PTC), targeting the

¹Vascular Biology and Therapeutics Program, ²Department of Medicine, Section of Cardiovascular Medicine, ³Biomedical Engineering, ⁶Pharmacology, and ⁷Anesthesiology, Yale University School of Medicine, New Haven, CT 06520.

⁴Department of Biochemistry and Molecular Genetics, Hospital Clinic of Barcelona, IDIBAPS, CIBERehd, 08036 Barcelona, Spain.

⁵University of Texas Health Science Center at Tyler, Tyler, TX 75708.

mutant transcripts for destruction by the nonsense-mediated decay pathway (Micale *et al.*, 2010; Urban *et al.*, 2000). As in WBS, the intact *ELN* allele is noncompensatory, thus resulting in elastin haploinsufficiency.

Replacement therapy for haploinsufficiency syndromes can be preclusively difficult, either by protein replacement or gene therapy. For genes encoding intracellular proteins or complex extracellular structural proteins like elastin, replacement by providing the mature protein product is not generally possible. For all complex genes, such as those encoding multiple potential splice variants, cDNA-based gene replacement therapy is also highly problematic. The *ELN* gene is structurally and functionally complex. The coding region contains at least 34 exons in the human *ELN* gene, 17 of which have been shown to undergo alternative splicing, resulting in a substantial number and variety of elastin splice variants (Heim *et al.*, 1991; Mecham, 2008; Parks *et al.*, 1992). Although it has been reported that exons 26A and 32 are splice deleted in human skin fibroblasts (Fazio *et al.*, 1988; Holzenberger *et al.*, 1993), exon 22 participates in intramolecular and intermolecular cross-linking (Dyksterhuis and Weiss, 2010; Wise *et al.*, 2005), and the c-terminus is important for binding microfibrils (Kozel *et al.*, 2003), relatively little is known about the individual functions of each exon and splice variant. This not only limits understanding of elastin biology, but this complexity is a crucial issue in the development of therapeutic strategies to promote elastogenesis and to rescue *ELN* haploinsufficiency.

One potential alternative therapeutic approach in WBS, SVAS, and other haploinsufficiency syndromes is to increase expression of the intact wild-type allele to full compensatory levels. Theoretically, this approach should promote expression of all naturally occurring splice variants of a targeted gene. Further, by activating the endogenous expression program for that specific gene, this approach might also promote normal posttranslational processing and assembly of complex proteins, like elastin. Elastogenesis, the formation and maturation of new elastin fibers, involves *ELN* expression combined with an array of complex coordinated events (Czirok *et al.*, 2006; Wagenseil and Mecham, 2007). In mammals, *ELN* encodes a 60–70 kDa protein called tropoelastin, comprised of alternating hydrophobic and lysine-rich cross-linking domains (Mecham, 2008). After synthesis, soluble tropoelastin is secreted into the extracellular space and undergoes cross-linking by lysyl oxidase (LOX) and lysyl oxidase-like 1 (LOXL1) to form insoluble mature elastin fibers that also contain microfibril proteins, such as fibrillin-1 and fibulin-5 (Kielty *et al.*, 2002). While elastogenesis involves multiple genes and additional proteins, it is clear that *ELN* expression is a major driver of this process.

Augmenting transcription from the intact wild-type allele could conceptually achieve compensatory expression of *ELN*. One way to achieve this would be to exploit endogenous *ELN* regulatory mechanisms. Unfortunately, how *ELN* gene expression is regulated is poorly understood, though of high developmental and clinical relevance. *ELN* expression begins around mid-gestation, peaks near birth and the early neonatal period (P7–P14 in mice), then drops sharply to low levels that persist into adulthood. It is also tightly tissue and cell-type restricted. The *ELN* 5' flanking region is G+C rich (66%), does not have a canonical TATA box, and though containing a large number of potential binding sites for

various transcription factors, it remains unknown how temporal and cell type-specific expression is maintained (Oleggini *et al.*, 2007; Rosenbloom *et al.*, 1991). While factors that can regulate *ELN* expression have been identified, such as TGF β 1 and IGF1 (activation), and bFGF (negative regulation), these factors have broad biological effects that preclude exploiting them to therapeutically modulate *ELN* expression (Carreras *et al.*, 2002; Oleggini *et al.*, 2007).

Herein we explore the feasibility of using engineered zinc-finger protein (ZFP) transcription factors to achieve compensatory expression from the normal *ELN* allele in WBS and SVAS. We (and others) have previously shown that ZFPs can be engineered to direct highly specific gene expression *in vivo* and *in vitro*, and that activation of an endogenous gene using this approach can lead to normal stoichiometric expression of that gene's natural splice variants—a distinct advantage for a complex gene like *ELN* (Klug, 2010a; Mandell and Barbas, 2006; Rebar *et al.*, 2002; Tan *et al.*, 2003; Yu *et al.*, 2006). The ZFP approach exploits cumulative knowledge of how specific amino acids within the alpha-helix of a zinc-finger protein can direct binding to targeted DNA sequences with high specificity (Christensen *et al.*, 2011; Gonzalez *et al.*, 2010; Pabo *et al.*, 2001). Each finger binds a specific 3-bp DNA sequence. Building multi-finger ZFPs allows longer sequences to be targeted. By combining the DNA-binding fingers with an appropriate functional domain, highly specific transcription factors can be constructed. A number of papers and excellent reviews describe the principles involved in designing engineered ZFPs (Christensen *et al.*, 2011; Klug 2010b; Miller *et al.*, 2007; Pabo *et al.*, 2001; Rebar *et al.*, 2002).

We describe here the successful construction of an *ELN*-targeted ZFP that achieves compensatory expression of elastin from the intact allele in both WBS and SVAS, and explore how transcriptional activation of the mutant allele in SVAS affects the surveillance mechanism of nonsense-mediated decay. We further show the utility of this approach by demonstrating ZFP-induced *ELN* expression and elastogenesis in bioengineered blood vessels. We contend that this approach to force compensatory expression from the remaining intact allele in haploinsufficiency syndromes may have significant clinical applicability.

Materials and Methods

Generation of ZFP transcription factors for human *ELN* gene

A library of $n > 10^6$ 3-finger ZFPs was screened for specific binding to a 10 bp target sequence (GGGAAAGAGa, 292 bp upstream translation initiation site) on human elastin promoter using a bacterial one-hybrid system (B1H) as described (Meng *et al.*, 2005; Meng and Wolfe, 2006). The resulting human elastin gene targeted zinc finger protein (hELN-ZFP) clones were then inserted to pcDNA3.1 (Clontech) containing cytomegalovirus (CMV) promoter, the nuclear translocation signal from simian virus 40 (SV40) large T antigen, the herpes simplex virus herpes simplex virus virion protein 16 (VP16) transactivation domain spanning amino acids 413–490, and a FLAG peptide (Rebar *et al.*, 2002). The plasmid without ZFP was referred to as VP16 alone and used as control. The uppercase letters in the binding sites denote bases directly contacted by the ZFP, and the extra base shown in lowercase denotes an extra overlapping target site.

Lipofectamine transfection and electroporation

Human dermal fibroblast (HDF) cells were electroporated with ELN-ZFP plasmids using Amaxa nucleofactor kit for primary fibroblasts (Lonza). Human embryonic kidney 293 cells (HEK293) were transiently transfected with ELN-ZFP plasmids using lipofectamine 2000 (Invitrogen). Messenger RNA (mRNA) or protein were harvested 48 h later for analysis.

Retroviral or lentiviral constructs containing ELN-ZFPs

Retroviral constructs containing ELN-ZFP were made and used in transduction as described (Zhang *et al.*, 2010). Briefly, ELN-ZFP or VP16 were inserted into LZRSpBMNZ retroviral vectors through the multiple cloning sites. The correct orientation and the DNA sequence of the insertion were verified by sequencing. The retroviral constructs were transfected into the Phoenix-Ampho packaging cell line by using Lipofectamine 2000. Puromycin-resistant Phoenix-Ampho packaging cells were selected and used to condition the medium. Retrovirus-conditioned medium was passed through 0.45 μ m filters (Millipore), supplemented with 8 μ g/mL polybrene (Sigma-Aldrich), and then added on target cells for 15 hr. Lentiviral constructs were generated by subcloning ELN-ZFP or VP16 into pLVX-Puro (Clontech). Forty-eight hours after 293 T cell were transfected with lentiviral plasmids, fresh lentivirus-containing media were collected and used to infect target cells at 5U MOI (multiplicity of infection of 5). mRNA or protein were harvested 72 hours later after infection.

Cell culture

HDF cells, isolated from human foreskin or adult skin, were grown in low glucose Dulbecco's modified Eagle's medium (DMEM; JRH) with 10% heat-inactivated fetal bovine serum (FBS; Lonza), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin (Invitrogen). Additional HDF cells were purchased from Coriell cell repositories. WBS dermal fibroblast and age-matched HDF were purchased from NICHD Brain and Tissue Bank for Developmental Disorders (University of Maryland). Human vascular smooth muscle cells (VSMC) and SVAS pulmonary VSMC were explanted by outgrowth as described (Zhang *et al.*, 2010) and cultured in 20% FBS in DMEM with supplements and antibiotics.

Cell proliferation assay and scratch assay

Transduced and nontransduced cells of the same number were seeded on plates in complete media. Cell numbers at selected time points were determined by hemocytometer readings. Scratch wounds were created by a p-200 pipet tip on confluent cell monolayers as described (Liang *et al.*, 2007). Wounds were rinsed with phosphate buffered saline (PBS) and cultured in 0.25% FBS media. Wound closure areas 15 hours later were stained by 0.2% crystal violet and quantified with ImageJ software (National Institutes of Health [NIH]).

qPCR

Primers for quantitative polymerase chain reaction (qPCR) were designed by PerlPrimer and experimentally validated by qPCR melting curve and product agarose gel electropho-

resis. Primer sequences include human ELN (non-splicing exon 18 unless specified otherwise) forward aggggtgtgtcaccagaag, reverse cagctccaaccccgtaagta; ELN exon 3 forward cctggggccattcctgggtg, reverse cctccgggaactggcttaa; ELN exon 10 forward ctgccaggtgtataccagg, reverse ccactacacctggagcc tt; ELN exon 13 forward ggctatggactgcctacac, reverse gggcca acccctgtccctgt; ELN exon 23 forward gttagtctgtgtcggcg, reverse tgcaactccaccagggccaa; ELN exon 25 forward agctgcag ctgggcttggtg, reverse gccagggtccaggtactgcc; human COL1A1 forward aaggacacagaggtttcag, reverse gtagcaccatcattccacg; human COL3A1 forward ctctaagtgtcaaggacct, reverse gaga atagtctgaggaccag. Primers for vascular endothelial growth factor A (VEGFA), hypoxia-inducible factor 1 α (HIF-1 α), and 18S ribosomal RNA (18S) were designed as described (Huang *et al.*, 2004). Cycle threshold (Ct) values of the target mRNA were normalized by subtracting 18S Ct value to achieve Δ Ct.

Western blot

To measure tropoelastin expression in the media, cells were grown in 0.5% FBS DMEM media for the final 24 hr, and media proteins were precipitated by 10% trichloroacetic acid (TCA) and resolved on 10% SDS-PAGE. To measure elastin protein associated with cell membrane, equal number of cells were lysed in 6 M urea-containing sample-loading buffer and loaded onto 10% SDS-PAGE. Western membranes were probed with a polyclonal antibody recognizing human elastin (a gift from Dr. Robert Mecham, Washington University, St. Louis, MO). Ponceau red staining or probing with monoclonal antibody against β -actin (Sigma) were used to normalize protein loading among the lanes. ImageJ (NIH) software was used to quantitate the intensity of bands.

Emetine treatment

After lentiviral transduction, SVAS pulmonary VSMC were treated with 100 μ g/mL emetine. mRNA were collected at indicated time points and qPCR analyzed by Taqman probes for ELN and COL1A1 (Applied biosystem). Specific primer pairs used to generate PCR products covering the mutation site in SVAS were ctgccaggtgtataccagg (forward) and ccactacactggagcctt (reverse).

Culture and analyses of tissue-engineered vessels

Engineered arteries having dimensions of 1 mm in diameter and 3 cm in length were grown as previously described (Niklason *et al.*, 1999). Briefly, human VSMC (0.8×10^6 cells/ml) were seeded onto a polyglycolic acid (PGA) scaffold (Albany International) around a silicone tube for support. Vessels were cultured in a bioreactor for 6 weeks in low-glucose DMEM with 10% FBS, 10% human serum, 2.6 mg/ml hydroxyethyl piperazineethanesulfonic acid (HEPES), 100 U/ml penicillin, 50 μ g/ml proline, 20 μ g/ml alanine, 50 μ g/ml glycine, 3 ng/ml CuSO₄, 10 ng/ml basic fibroblastic growth factor (bFGF), and 10 ng/ml platelet-derived growth factor (PDGF-bb), as previously described (Dahl *et al.*, 2007). A pulsatile pump forced PBS through the silicone tubing at a rate of 90 beats per minute to simulate an adult pulse rate.

Light and transmission electron microscopy

Vessel samples were also fixed in 10% formalin, embedded in paraffin, cut into 5 μ m sections, and stained with

hematoxylin and eosin (H&E) and Masson's trichrome stain, which stains collagen blue. For transmission electron microscopy (TEM), tissues were fixed in 2.5% glutaraldehyde, 1% formaldehyde in sodium cacodylate buffer, and embedded in Epon. Images were taken at Yale transmission electron microscope (TEM) center.

Desmosine and hydroxyproline analysis

Desmosine and hydroxyproline, demonstrating cross-linked elastin and collagen levels respectively, were measured as described (Starcher and Conrad, 1995).

Microarray analysis

Total RNA from *ELN*-ZFP3-transduced or VP16-transduced HDF cells in triplicates were isolated as described and hybridized to GeneChip Human Gene 1.0 ST Array that covers 28,869 genes (Affymetrix) at Yale Center for Genome Analysis.

Statistical analysis

Comparisons between two groups were by unpaired student t test. Statistical analyses were performed using Prism 4 software (GraphPad). *P* values were two-tailed and values <0.05 were considered to indicate statistical significance. For microarray analysis, normalized expression signals were calculated from Affymetrix CEL files using RMA (Irizarry *et al.*, 2003). Statistical differences were calculated using the Limma package of linear model analysis from the Bioconductor project (Smyth, 2005). The *p* values were obtained from moderated t statistics using empirical Bayesian methods. *P* values were adjusted for multiple testing with Benjamini and Hochberg's (Klipper-Aurbach *et al.*, 1995).

Results

Design and in vitro testing of *ELN*-targeted ZFPs

A 9-bp target sequence in the promoter of the *ELN* gene was selected by screening the 5' regulatory region for known transcription factor-binding sites and identifying a sequence without significant overlap with these sites, but with features that have been previously shown to facilitate effective ZFP targeting. The 9-bp sequence chosen, GGGAAA GAG, is located ~200-bp upstream of the major translational start site. Three three-finger *ELN*-ZFP DNA binding domains were selected by a bacterial one-hybrid screening method (Meng *et al.*, 2005). Individual cDNAs encoding these DNA-binding domains were cloned in front of the VP16 transactivation domain to generate CMV-driven expression cassettes encoding these *ELN*-ZFP transcription activators, as shown in Figure 1. Their ability to activate the endogenous *ELN* gene was first tested by transient transfection into HEK293 cells, followed by qRT-PCR analysis of elastin mRNA. In comparison to the vehicle (lipofectamine), a control plasmid containing VP16 alone, and a plasmid encoding a previously validated VEGF-ZFP (Rebar *et al.*, 2002), all three *ELN*-ZFPs directed a >6-fold induction of elastin mRNA, with *ELN*-ZFP3 having the highest activation (more than 58-fold) (Fig. 2A). *ELN*-ZFPs had little effect on VEGF-A and HIF-1 α mRNA in HEK293 cells, whereas the VEGF targeted ZFP-activated VEGF expression, as expected. Next, we

FIG. 1. *ELN*-ZFP design. Three-finger zinc-finger proteins (ZFPs) targeting a 9-bp target sequence in the *ELN* promoter were selected from a random three-finger library using a bacterial one-hybrid screening approach. Each finger binds a specific 3-bp sequence on the sense DNA strand, based on the sequence of specific amino acids within the alpha helix of that finger. The sequences of the DNA-binding amino acids within each finger of the three most highly represented clones are shown. cDNA expression cassettes encoding the *ELN*-ZFP transcription factors were created by cloning cDNA encoding the ZFP DNA-binding fingers in front of sequences encoding the VP-16 transactivation domain, as depicted. The *ELN*-ZFP expression cassettes were driven by a CMV promoter (not shown). *ELN*, loss of elastin gene; ZFP, zinc-finger protein, CMV, cytomegalovirus. Color images available online at www.liebertpub.com/hum

electroporated HDF with *ELN*-ZFP plasmids. As seen (Fig. 2B and C), *ELN*-ZFP1 and *ELN*-ZFP3 increased elastin mRNA 12- or 32-fold respectively, and *ELN*-ZFP treatment dramatically increased the levels of secreted tropoelastin protein from these cells (Fig. 2C).

VSMC express *ELN* and play a crucial role in elastogenesis within the blood vessel wall. To evaluate the effects of our ZFP constructs more efficiently in these cells, we generated lentiviral constructs encoding *ELN*-ZFP constructs one and three. As shown (Fig. 3A), transduction with *ELN*-ZFP3 lentivirus increased elastin mRNA >8-fold over basal expression levels in VSMC, as compared to transduction with a viral vector encoding VP16 alone (no DNA-binding fingers) or vehicle alone (no virus). There were no effects on the expression of collagens COL1A1 and COL3A1, demonstrating that gene activation by the *ELN*-ZFPs was not a generalized effect involving all matrix components. *ELN*-ZFP3 also induced marked increases in both cell-associated elastin protein and soluble tropoelastin protein (Fig. 3B). *ELN*-ZFP1 was less effective in these cells, a finding that may reflect the ~10-fold lower expression of this ZFP in these cells when compared to *ELN*-ZFP3 (based on quantitative reverse transcriptase polymerase chain reaction [q-RT-PCR] analysis of VP16 expression as a marker of *ELN*-ZFP expression levels). The reason for this reduced expression of *ELN*-ZFP1 is unclear, but for experiments evaluating the effects of *ELN*-ZFP on splice variant expression and nonsense mediated

FIG. 2. Validation of *ELN*-ZFP clones. **(A)** HEK293 cells were transiently transfected with plasmids encoding select *ELN*-ZFPs and the indicated controls. Total RNA was harvested 48 hours later and q-RT-PCR performed for elastin, VEGF-A, and HIF-1 α . q-RT-PCR values were normalized to 18S and comparisons were made to the vehicle group (vehicle=LF). All three *ELN*-ZFP clones induced *ELN* expression, to different degrees (left panel). A VEGF- targeted ZFP (VEGF-ZFP) induced VEGF mRNA, but the *ELN*-ZFPs had no effect on either VEGF expression (middle panel) or HIF-1 α expression (right panel). q-RT-PCR, quantitative reverse transcriptase polymerase chain reaction; LF, lipofectamine. **(B)** Human dermal fibroblasts were electroporated with expression plasmids encoding either the *ELN*-ZFP constructs, a ZFP targeted to VEGF (VEGF-ZFP), or the VP16 transactivation domain without the DNA-binding fingers (control). Forty-eight hrs later, q-RT-PCR revealed marked induction of *ELN* in *ELN*-ZFP-treated cells, but not in controls or VEGF-ZFP-treated cells. q-RT-PCR values were normalized to 18S, and increases compared to the no-DNA group. Data are mean $\pm$ SEM; * p < 0.05 vs control; n.s., not significant. All data are representative of at least three independent experiments using cells from three different human donors. **(C)** Western blot analysis of conditioned media from experiment B revealed marked induction of tropoelastin protein (soluble elastin) expression and secretion from *ELN*-ZFP-treated cells (lane 1=no DNA, 2=VP16 alone, 3=VEGF-ZFP, 4=*ELN*-ZFP1, 5=*ELN*-ZFP3). VEGF, vascular endothelial growth factor.

decay (see below) we used *ELN*-ZFP3, the construct that demonstrated the highest efficacy in our initial testing.

One of our expectations was that endogenous activation of the *ELN* gene would lead to expression of all the major splice variants and not to a selected population of these variants. To test this we used exon-specific primers to determine by q-RT-PCR the expression levels of key *ELN* exons that are spliced out in specific *ELN* variants. As seen in Figure 3C and D, elastin mRNAs containing the known exons 3, 10, 13, 23, and 25 were significantly increased in *ELN*-ZFP3 transduced cells, and the stoichiometric relationship between them maintained.

Rescue of *ELN* haploinsufficiency in WBS and SVAS cells

Next, we examined the ability of *ELN*-ZFP3 to induce compensatory expression from the intact wild-type allele in dermal fibroblast cells isolated from a patient with WBS. Using comparative genomic hybridization (CM-CGH) testing through the Yale Genetics Core, we verified that these cells have a typical heterozygous $\sim$ 1.5Mb microdeletion at 7q11.23 encoding $\sim$ 25 genes, including *ELN* (Pober, 2010). These elastin-haploinsufficient cells express only 26–36% of elastin mRNA compared to age-matched normal fibroblast

cells from the same bank. *ELN*-ZFP3 increased elastin mRNA close to 7-fold and cell-associated elastin protein by 4.9-fold, levels close to that seen in normal cells (Fig. 4A and B). *ELN*-ZFP1 increased elastin mRNA significantly, but to a much lesser extent in these cells. Both *ELN*-ZFPs appeared to increase COL3A1 mRNA levels to a small extent, although not COL1A1 in these WBS cells. Strong augmentation of *ELN* mRNA by *ELN*-ZFP3 was also seen in dermal fibroblasts from another WBS patient in comparison to levels in matched normal cells (12.2 ± 2.2 vs 1.5 ± 0.7 , p < 0.05, graph not shown).

We also examined the ability of *ELN*-ZFP to increase *ELN* expression in pulmonary VSM cells isolated from a patient with SVAS. In this particular patient, *ELN* haploinsufficiency is caused by a haploid 4-bp insertion (GTAT) in exon 9 of the *ELN* gene, causing a frameshift and a consequent premature termination codon (PTC) in exon 10. This PTC targets the mutant transcript to nonsense-mediated decay (NMD), thus leading to the *ELN* haploinsufficiency. NMD of the mutant transcripts is the major mechanism causing *ELN* haploinsufficiency in most cases of SVAS. Transduction of these SVAS cells with lentivirus encoding *ELN*-ZFP3 induced an $\sim$ 5-fold increase in elastin mRNA from these cells as compared to cells transduced with a control virus, while inducing no significant changes in collagen synthesis (Fig. 4C).

FIG. 3. *ELN*-ZFP-mediated induction of *ELN* expression in vascular smooth muscle cells (VSMC). (A) Early passage human VSMC were transduced with lentivirus constructs encoding the *ELN*-ZFP constructs or the VP16 control, or were treated with transduction vehicle. Seventy-two hours later, total RNA was collected for q-RT-PCR analysis using primers for VP16, collagens COL1A1 and COL3A1, and elastin. q-RT-PCR values were normalized to 18S rRNA. *ELN*-ZFP3 induced a marked increase in *ELN* expression, but no changes in COL1A1 or COL3A1. q-RT-PCR for VP16 is a marker of ZFP or control plasmid expression. (B) Western blot analysis of conditioned media and cell lysates from cells treated under identical conditions as in (A) demonstrated a commensurate marked increase in cell-associated elastin (top panel) and soluble tropoelastin (bottom panel) in response to *ELN*-ZFP3 treatment. (C, D) q-RT-PCR analysis of total RNA from cells transduced with either *ELN*-ZFP3 or VP16 control was performed using exon-specific primer pairs targeted to different *ELN* splice variants. *ELN*-ZFP3 induced increases in all exons assessed (top panel) and maintained the stoichiometric balance between exon levels (bottom panel), suggesting that the major splice variant of *ELN* are induced in a normal stoichiometric balance in response to *ELN*-ZFP treatment. Data are mean $\pm$ SEM and representative of at least three independent experiments using cells from three different human donors. * $p < 0.05$ vs. control.

Effects of *ELN*-ZFP on nonsense mediated decay of mutant *ELN* transcripts

A major mechanism preventing the expression of potentially deleterious mutant proteins in many genetic syndromes, including the majority of patients with SVAS, is nonsense-mediated decay (NMD). In NMD, mutant genes are transcribed, but gene mutations that cause a reading frameshift and a PTC result in rapid clearance of the mutant transcripts—before translation into mutant proteins. In haploid genetic syndromes in which whole genes are deleted, such as WBS, mutant transcripts are not made and thus NMD is not required. Conversely, for many *ELN* mutations that result in SVAS, and in many other genetic syndromes, NMD is essential to prevent mutant protein production.

The DNA sequence targeted by the *ELN*-ZFPs is present in the regulatory region of the mutant as well as the wild-type

allele, thus, the *ELN*-ZFPs could conceivably increase expression of the mutant *ELN* transcripts (Tassabehji *et al.*, 1997; Urban *et al.*, 2000). This raises the question whether *ELN*-ZFP-mediated increases in transcription of mutant *ELN* mRNA could overwhelm clearance by NMD and lead to production of a mutant *ELN* protein. This possibility is a crucial consideration in assessing the feasibility of any strategy to treat haploinsufficiency by augmenting transcription when there is a mutant allele present. To investigate this, we evaluated the effects of *ELN*-ZFP3 on levels of the mutant *ELN* transcript in SVAS cells, in the presence and absence of the translation inhibitor emitine. NMD occurs when a PTC is encountered during translation. Thus, blocking translation also blocks NMD (Noensie and Dietz, 2001). SVAS cells were transduced with either *ELN*-ZFP3 or control lentivirus constructs and then treated with emitine (100 μ g/mL) 72 hours later. At various time points after blocking translation with emitine, levels of

FIG. 4. *ELN*-ZFP3 induces compensatory *ELN* expression from the wild-type allele in haploinsufficient WBS and SVAS cells. **(A)** Human dermal fibroblasts from a WBS patient were transduced with lentivirus constructs encoding the *ELN*-ZFP constructs or the VP16 control. Seventy-two hours later, total RNA was collected for q-RT-PCR analysis. As shown, both *ELN*-ZFP constructs were capable of inducing compensatory *ELN* expression from the remaining *ELN* allele, with the effect of *ELN*-ZFP3 much greater than *ELN*-ZFP1. Both constructs had a small effect on *COL3A1* expression, but none on *COL1A1* expression. VP16 expression is shown as a marker of ZFP or control plasmid expression. **(B)** Western blot analysis revealed a commensurate induction of protein in *ELN*-ZFP3-treated cells to levels approaching wild-type human fibroblasts. **(C)** Under identical conditions as used for experiments in (A), *ELN*-ZFP3 transduction was able to markedly induce *ELN* expression in pulmonary smooth muscle cells from a patient with SVAS with elastin haploinsufficiency caused by the 4-bp addition in Exon 9, shown schematically. For all q-RT-PCR, data are mean $\pm$ SEM, are normalized to 18S, and * $p < 0.05$ vs. VP16 alone. WBS, Williams-Beuren syndrome; SVAS, supravalvular aortic stenosis. Color images available online at www.liebertpub.com/hum.

total elastin mRNA and of the mutant *ELN* transcript were evaluated using a combination of q-RT-PCR and acrylamide gel electrophoresis.

Compared to VP16 alone, *ELN*-ZFP3 transduced SVAS cells had a >4.5 fold increase in elastin mRNA prior to treatment with emetine. Interestingly, 7 hr post-emetine treatment, there was a further 63% increase in total elastin mRNA (Fig. 5A). Both *ELN*-ZFP3 and emetine treatments had negligible effect on *COL1A1* mRNA in SVAS, although there appeared to be a slight increase (40%) in *COL1A1* mRNA in *ELN*-ZFP3 transduced cells 7hr. post-emetine treatment. Standard agarose gel electrophoresis of PCR products using a specific primer pair around the mutation site after 30 cycles of amplification showed that both *ELN*-ZFP3 and emetine increased elastin mRNA in SVAS cells, although the mutant and wild-type alleles could not be resolved with this approach (Fig. 5B, top). After longer amplification (35 cycles) and electrophoresis on 12% acrylamide, the wild-type and mutant *ELN* mRNAs were well separated, allowing assessment of the effects of *ELN*-ZFP3 and emetine (Fig. 5B, middle panel). At baseline (72 hr post-transduction and pre-emetine), there was a barely perceptible mutant elastin mRNA band present, with no significant differences

apparent between the *ELN*-ZFP and control virus transduced cells. Progressively, at incremental time points after addition of emetine, there was an increase in mutant *ELN* mRNA levels in both the *ELN*-ZFP and control-treated cells, although the increase was markedly greater in cells treated with the *ELN*-ZFP. These data demonstrate that the *ELN*-ZFPs do indeed increase transcription from the mutant *ELN* allele, but that this does not overcome the surveillance mechanism of NMD and is thus unlikely to result in the expression of a mutant elastin protein.

ELN-ZFP induction of elastogenesis in bioengineered blood vessels

To determine whether transcriptional activation of *ELN* could enhance elastogenesis we examined the effects of *ELN*-ZFP3 in bioengineered blood vessels being developed for clinical use (Niklason *et al.*, 1999; Solan *et al.*, 2009). These vessels are grown in bioreactors by seeding cells onto a PGA scaffold and exposing to pulsatile flow for extended periods (Niklason *et al.*, 1999). Although they exhibit many crucial properties of human arteries, they do not express sufficient amounts of elastin and do not develop elastic lamellae. We

FIG. 5. *ELN*-ZFP-treatment does not bypass nonsense-mediated decay (NMD). A 4-bp insertion in one *ELN* allele causes destruction of the mutant transcript by NMD, resulting in elastin haploinsufficiency and consequent SVAS. To determine if transcriptional activation of the mutant allele with an *ELN*-ZFP could bypass NMD, leading to an accumulation of mutant transcript, cells were transduced with *ELN*-ZFP3 and then 72 hours later treated with the translation and NMD inhibitor emetine. qRT-PCR of RNA collected at sequential time points (**A**) revealed the expected induction of elastin mRNA by *ELN*-ZFP3, with further increases after emetine treatment, and only a slight increase in COL1A1 expression in the *ELN*-ZFP cells 7 hours after emetine treatment. (**B**) In cells treated identically as above, analysis of RT-PCR products by agarose gel electrophoresis after 30 amplification cycles revealed the expected ZFP-induced increase in elastin mRNA, with greater accumulation seen after emetine treatment (top panel). To resolve mutant transcripts from wild-type transcripts, RT-PCR products were run on 12.5% acrylamide gels after 35 cycles amplification. As shown (middle panel), in the absence of emetine treatment, *ELN*-ZFP3 did not induce an increase in the mutant elastin transcript (higher band), suggesting that ZFP-mediated transcription of the mutant allele does not overcome the surveillance of NMD. When NMD was inhibited by emetine, accumulation of the mutant transcript in response to *ELN*-ZFP was seen. At 35 cycles, the wild-type elastin mRNA levels (lower band) are in the plateau PCR phase and do not show the normal ZFP-induced increases.

transduced early passage human VSMC with lentivirus encoding either *ELN*-ZFP3 or the VP16 control, seeded $\sim 1 \times 10^6$ cells onto each PGA scaffold, and grew vessels under identical conditions in a bioreactor for 8 weeks.

After this 8-week period, immunofluorescence analysis demonstrated that *ELN*-ZFP3 treated vessels contained more of the matrix proteins fibronectin and fibrillin-1, and more elastin than controls (Fig. 6). This increase in elastin was corroborated at the ultrastructural level with transmission electron microscopy. Quantitatively, *ELN*-ZFP3-treated vessels had $\sim$ twice the amount of desmosine, a marker of cross-linked elastin content, vs. controls (Fig. 6K). Thus mature elastin appeared to have been generated in these vessels. There was no concomitant increase in the amount of hydroxyproline, a marker of collagen content, and no difference in cell number in *ELN*-ZFP3 treated vessels vs. controls (Fig. 6L and M).

ELN-ZFP3 does not affect cellular proliferation or migration in vitro

Since we did not see a difference in cellular proliferation between *ELN*-ZFP3-treated vs. VP16-treated bioengineered

vessels (Fig. 6M), we also examined the effects of *ELN*-ZFP3 on normal fibroblasts (NF) or WBS fibroblasts on the proliferative and migratory characteristics of these cells in culture (Fig. 7). Neither proliferation (measured by cell counts and by a WST-1 assay) or cellular migration (determined by scratch assay and corroborated by ECIS cell impedance experiments) was significantly altered by *ELN*-ZFP3 transduction, as compared to VP16 transduced and nontransduced cells (Fig. 7).

ELN-ZFP3 is highly specific

Having established that *ELN*-ZFP3 can drive significant *ELN* activation in both wild-type and *ELN* haploinsufficient cells, we next evaluated its functional specificity. Independent microarray experiments were performed to compare the genome-wide expression changes after *ELN*-ZFP3 transduction in human dermal fibroblasts. We found that among the 30K genes (764K probes) on the Affymetrix chip, only four (*ELN*, *SERPINA3*, *PRSS35*, and *PTPRN*) had significant ($p < 0.05$) changes in gene expression after *ELN*-ZFP3 treatment, and only one (*SERPINA3*) showed a fold change greater than *ELN* (5.14-fold vs 2.07-fold; Table I).

FIG. 6. *ELN-ZFP*-mediated elastogenesis in bioengineered blood vessels. Low passage human vascular smooth muscle cells (VSMC) were transduced with lentivirus encoding *ELN-ZFP3*. Bioengineered blood vessels were grown in a bioreactor for 8 weeks after seeding these cells onto a polyglycolic acid (PGA) scaffolding. As shown by immunofluorescence, vessels grown from cells transduced with *ELN-ZFP3* (A, C, E) had markedly greater deposition of matrix proteins fibronectin and fibrillin 1, and of elastin vs. vessels grown with VP16 transduced (control) cells (B, D, F) (elastin = white arrowheads, remnant PGA = white arrows). This increase in elastin was also seen by TEM (G vs. H; asterisk = collagen bundles; black arrowheads = elastin). There were no gross histological differences noted by standard H & E (I, J). Commensurate with these findings was an increase in desmosine levels (a marker of cross-linked elastin) in the *ELN-ZFP* treated vessels (K), while there were no differences in hydroxyproline (a marker of collagen deposition) or cell number between the *ELN-ZFP* and VP16 vessels (L, M). Scale bars represent 50 μm for (A)–(F), 1 μm for (G) and (H), and 100 μm for (I) and (J). TEM, transmission electron microscopy; H & E, hematoxylin and eosin.

Interestingly, the human *SERPINA3* gene also contains the same 10bp DNA sequence targeted by *ELN-ZFP3* (between exons 1 and 2; chr14:94133491-94133500; GRCh37 assembly of the human genome).

Discussion

Here we show that targeted transcriptional activation of the intact wild-type allele with engineered ZFPs is a viable strategy to induce compensatory expression of *ELN* and rescue haploinsufficiency of this gene in two distinct genetic syndromes, WBS and SVAS. In SVAS, we provide evidence that concomitant transcriptional upregulation of the mutant allele does not bypass surveillance by nonsense mediated decay, and is thus not likely to lead to augmented expression of a mutant elastin protein. These are important preliminary steps in the potential development of targeted transcriptional strategies to treat haploinsufficiency syndromes. It remains

unclear why heterozygous loss of specific genes leads to haploinsufficiency, while heterozygous loss of other genes is compensated by the remaining allele. It has also remained unclear whether the pathology of haploinsufficiency is primarily due to an absolute reduction in amount of the encoded protein, or is due to an altered stoichiometry of that protein relative to other proteins with which it interacts. This has raised concerns that attempted therapeutic overexpression of a haploinsufficient gene might lead to toxicities based on an inability to correct this stoichiometric imbalance. Work on haploinsufficiency in yeast supports the contention that haploinsufficiency is primarily a consequence of reduced protein production, but it is likely that stoichiometric balance is also important for specific haploinsufficient proteins (Deutschbauer *et al.*, 2005). Targeted transcriptional activation of the wild-type endogenous allele with engineered ZFPs engenders several potential advantages in this context. First, the levels of induced expression are potentially

FIG. 7. *ELN*-ZFP does not affect cellular proliferation or migration *in vitro*. Normal fibroblast cells (NF) or WBS fibroblast were transduced with lentivirus encoding *ELN*-ZFP3 or VP16. (A) Transduced cells and non-transduced cells were plated at the same number in complete media. Cell numbers at selected time points were determined by hemocytometer readings. (B) Scratch wounds were created in cell monolayers prepared as in (A), and the area of closure (index of cell migration and proliferation) was examined 15 hours later.

titratable, either by using weaker or stronger transcriptional activation domains, altering the regulatory sequence targeted, or by altering specific properties of the ZFPs such as half-life or target sequence binding affinity. Second, if stoichiometry is indeed important, it is reasonable to assume that splice variant stoichiometry is also important, and endogenous gene activation by the engineered ZFP approach can recapitulate normal splice variant stoichiometry (Rebar *et al.*, 2002).

In the case of *ELN* haploinsufficiency, the argument for targeted endogenous gene activation as a therapeutic approach is particularly strong, given the complexity of this gene and the number of potential splice variants. In a mouse model in which the entire human *ELN* gene was inserted, expression from this transgene partially reversed the cardiovascular phenotype associated with heterozygous deletion of the mouse *Eln* gene and rescued the lethality of the homozygous mouse *Eln* deletion (Hirano *et al.*, 2007), supporting our *ELN* compensation strategy. Although these and other published findings strongly suggest that rescuing elastin haploinsufficiency can prevent WBS vascular pathology, further preclinical work in elastin haploinsufficient animal models (Hirano *et al.*, 2007; Li *et al.*, 1998a) will be

required prior to making a leap to clinical testing of our ZFP approach or other approaches to increase elastin expression.

Indeed, several issues remain to be addressed before any attempted clinical application of engineered *ELN*-ZFPs, including how to deliver them to essential cells and tissues at specific developmental stages. A majority of SVAS and WBS patients develop vascular lesions, often requiring surgical intervention (Pober, 2010; Pober *et al.*, 2008b). Despite the propensity to develop focal aortic stenosis just above the aortic valve, severe vascular stenosis can occur in many different arterial territories and can be recurrent. Thus, an effective therapeutic strategy would arguably necessitate an approach that achieves compensation for *ELN* haploinsufficiency throughout the susceptible vasculature, likely requiring systemic delivery. Expressing ZFPs from cDNA cassettes is effective, but *in vivo* systemic delivery of these cassettes to the vasculature is a significant hurdle, even with advanced generation viral vectors.

To prevent the development or recurrence of vascular lesions at specific locations, catheter-based local *ELN*-ZFP gene delivery to specific arterial segments could be accomplished, potentially with existing technology. However, a broader delivery strategy that achieves rescue of elastin

TABLE 1. MICROARRAY ANALYSIS OF *ELN*-ZFP3-INDUCED CHANGES IN GENE EXPRESSION

Accession	Name	Symbol	Log change	Adjusted p-value	Fold change
NM_001085	Serpin peptidase inhibitor, clade A, member 3	SERPINA3	2.36147	0.006236	5.138937
NM_001170423	Serine protease 35	PRSS35	1.12309	0.020116	2.17813
NM_000501	Elastin	ELN	1.04935	0.020116	2.069597
NM_002846	Protein tyrosine phosphatase, N-type receptor	PTPRN	1.03433	0.040398	2.048162

Global gene expression in *ELN*-ZFP3-transduced human dermal fibroblasts (HDF) were compared to those in VP16-transduced HDF. Out of close to 30K probes on the GeneChip Human gene array, only four gene expressions had significant ($p < 0.05$) changes.

haploinsufficiency throughout the susceptible vasculature would likely be significantly more effective, and is thus more appealing. One potential approach would be to deliver the ZFP-encoding cassettes during the prenatal or early postnatal period using a gene delivery method that achieved genomic integration and thus *ELN*-ZFP expression in progeny cells (e.g., lentivirus transduction). The goal would be to transduce enough cells such that after developmental proliferation there would be a sufficient number to rescue the elastin haploinsufficiency and to substantively decrease the propensity to develop vascular lesions. The potential risks of genomic integration would, however, have to be carefully considered. Another possible approach would be to stably transduce pluripotent cells from an affected fetus or early postnatal child (e.g., cord blood-derived stem cells) with the *ELN*-ZFP construct, and then deliver these cells back with hopes that they would integrate, differentiate, and express normal amounts of elastin. Although both of these strategies present challenging technical hurdles, the potential to modify genetic diseases with early intervention using these approaches is attractive and could be applicable to other genes in the WBS locus and to a significant number of additional genetic syndromes marked by haploinsufficiency.

Although the prenatal and early postnatal approaches described above could theoretically have a significant positive impact, a major consideration for any such approach is the ability to replicate the concise temporal and cell-specific expression patterns that normally occur during the fetal or early postnatal periods. Arguably, this is particularly important for elastogenesis, which is a complex process with tight spatial and temporal control. Although *ELN*-ZFP expression could be restricted to specific cell types by using tissue-specific promoters, replicating specific temporal patterns of *ELN* expression will be difficult. For point mutations more readily amenable to gene correction approaches (e.g., gene correction using ZFP endonucleases) (Urnov *et al.*, 2010), correction of mutations in pluripotent cells in the fetal or early postnatal periods would have significant theoretical advantages, including intact temporal and cell-type specific gene expression control. For deletion syndromes, including those in which whole genes are lost (e.g. *ELN* deletion in WBS), gene correction would require whole gene replacement, and thus transcriptional activation (e.g., our ZFP approach) would be a more viable option. Despite the substantial complexities and technical hurdles, we maintain that transcriptional upregulation of the wild-type allele in WBS and other genetic syndromes that cause haploinsufficiency remains appealing. Advancements in technology, for example, delivering ZFPs as cell permeable proteins, may help to address specific hurdles to clinical implementation.

Effective treatment of adults, and children who are beyond the major developmental periods of elastogenesis, is dependent upon whether increasing *ELN* expression, by an *ELN*-ZFP or any means, will result in the effective production of new incorporated elastin, and whether this will have significant functional effects on the vasculature. On the positive side, we and others have shown that antagomirs to microRNA miR29 can increase elastin expression (Zhang *et al.*, 2011), and one study demonstrated that this approach alters aortic aneurysm formation in a mouse model (Boon *et al.*, 2011). This suggests that functionally significant elastin production can occur in an established vasculature. None-

theless, whether induction of *ELN* expression with ZFPs in a child or adult can prevent or modify WBS or SVAS vascular lesions remains unclear. Underscoring the biological complexity and thus clinical challenge is the broad phenotypic variability in patients with WBS or SVAS. Although it is now well established that *ELN* loss or mutation is the primary driver of the vascular lesions in these genetic syndromes, there are likely multiple modifiers that contribute to the phenotype variation. A recent study using quantitative trait locus analysis in mice identified several potential genetic modifiers of the *ELN* deficiency vascular phenotype, including genes involved in reactive oxygen species generation (Kozel *et al.*, 2011). We and others have identified several additional factors that can modify *ELN* expression at transcriptional and post-transcriptional levels, including specific miRNA species, growth factors, and control of the cell cycle (Kuang and Goldstein, 2003; Sen *et al.*, 2011; Shi *et al.*, 2012; Zhang *et al.*, 2011). The *ELN* promoter also contains a CpG island, and it is possible that methylation can epigenetically modify *ELN* expression.

Another necessary consideration is that the precise mechanism whereby elastin haploinsufficiency leads to vascular stenosis remains unclear. Nonstenotic areas of aorta in WBS show medial thickening, and areas of supravalvular aortic stenosis often demonstrate areas of increased smooth muscle cell proliferation along with fragmented elastic lamellae. *Ex vivo* examination of cultured arterial segments from elastin-deficient mice revealed increased rates of smooth muscle cell proliferation (Li *et al.*, 1998a; Li *et al.*, 1998b), and *in vitro* studies have shown that adding exogenous soluble elastin to the media can reduce the proliferation rate of cells from individuals with WBS and SVAS (Urban *et al.*, 2002). These data have led to a widely held hypothesis that elastin insufficiency in the vessel wall leads to an increase in smooth muscle proliferation, thus leading to stenosis. However, the pathology of SVAS lesions can be variable, and this variability in vascular pathology, along with the identification of potential genetic modifiers, suggests that the mechanisms underlying vascular lesion formation involve more than this inverse relationship between elastin levels and smooth muscle proliferation. In our studies, despite marked induction of elastin mRNA and protein, we did not see a reduction in cellular proliferation in normal fibroblasts, or those from a WBS patient when transduced with a lentivirus encoding *ELN*-ZFP. Whether this is a function of the specific cells we used, the fact that we relied upon endogenous tropoelastin production rather than exogenous application, or perhaps relates somehow to the specific pattern of *ELN* expression induced by the *ELN*-ZFP (e.g., specific splice variants) is unclear and will require further study in additional cell lines and conditions.

To be effective in treating heterozygous genetic haploinsufficiency syndromes, it is important that ZFP-mediated transcriptional activation not induce the expression of a potentially deleterious mutant protein. Our findings suggest that transcriptional activation of the mutant *ELN* allele by *ELN*-ZFP in SVAS does not overwhelm the capacity of the NMD mechanism to target destruction of the mutant transcripts. In WBS, with complete deletion of one *ELN* allele and consequently no mutant allele, ZFP-mediated transcriptional overriding of NMD is not a concern. For most SVAS mutations, however, NMD is the major mechanism preventing production of a mutant protein. Thus the possibility that

transcriptional activation of the mutant allele can overcome NMD and result in production of a potentially pathological mutant protein is a significant and legitimate concern. Although our results demonstrate that this does not occur with the current batch of *ELN*-ZFPs in the specific SVAS cells we tested, the possibility that transcriptional activation could bypass NMD in cells with other SVAS mutations, or in different cell types, has not been ruled out. Further, the effects of transcriptional activation on NMD surveillance may be different for mutations in other genes, and this will have to be investigated further before concluding that NMD is generally unaffected by the ZFP approach. Whether strategies to increase mRNA half-life, such as our miR29 antagomir approach, will bypass NMD and lead to mutant protein production is also an unanswered question, and strategies to treat haploinsufficiency in this manner will also require clarification of this issue.

One prominent advantage of engineered ZFPs is that they are designed or selected for high affinity binding to specific target DNA sequences. For this study, we used a 3-finger ZFP targeted to a specific 9-bp DNA sequence, but the modularity of ZFP design allows us to make even longer ZFPs (Wright *et al.*, 2006). The most effective *ELN*-targeted ZFP we constructed, *ELN*-ZFP3, significantly increased the expression of only four genes two-fold or greater out of ~30,000 genes on an Affymetrix array, including *ELN*. One of the other 3, *SERPINA3*, contains our targeted sequence in the intron between exons 1 and 2. Another of these 3, *PTPRN*, contains a 9-bp sequence in its promoter that differs from our target sequence by one base. It is possible that binding of our ZFP to these sites directs the increased expression of these genes. The other method of *ELN* upregulation we have investigated, stabilization of elastin mRNA by an anti-miR29 antagomir approach, has potential advantages regarding the deliverability in a clinical context. Conversely, current antagomirs generally alter the expression of a significant number of genes and thus lack the specificity of the ZFP approach. Likely, technological advances will improve the clinical potential of each of these approaches. Further, given that ZFPs increase mRNA synthesis while the antagomirs stabilize mRNA transcripts, these approaches may prove to be therapeutically synergistic.

The ability to induce elastogenesis has broad clinical implications. The integrity of elastin is of crucial biological importance (Kielty, 2006), and degradation/loss of elastin is a feature of emphysema (Pierce *et al.*, 1995; Snider, 2000), decreased skin elasticity with aging (Sherratt, 2009; Uitto, 2008), vascular aneurysms and other vascular pathologies (Hellenthal *et al.*, 2009; Lee and Oh, 2010; Sawabe, 2010), and numerous additional clinical entities (Sherratt, 2009; Word *et al.*, 2009). As elastogenesis is a complex process, involving both *ELN* expression and crucial post-translational processing, it was important to determine whether transcriptional upregulation of *ELN* could lead to the deposition of mature elastin in the extracellular matrix. The clinical use of bioengineered vessels as arterial conduits (e.g., coronary bypass surgery) is limited because of their paucity of elastin and consequent weak mechanical properties (Kielty *et al.*, 2007). Our data establishes that increased deposition of cross-linked elastin in these vessels can be achieved by ZFP-induced *ELN* transcription. While an important first step toward the goal of achieving stronger bioengineered vessels, a significant

amount of additional studies and optimization steps will be necessary before concluding that the *ELN*-ZFP methodology should be incorporated into the production of bioengineered vessels. Currently, we did not observe mature elastin lamella in these vessels and transduction efficiency in these experiments was suboptimal, but with optimization in transduction efficiency, and with combined efforts to improve other steps involved in elastogenesis, such as increasing the activity of cross-linking enzymes (LOX) and the expression of microfibril proteins, more natural patterns of mature elastin deposition may be achieved.

In summary, this study establishes that compensation for elastin haploinsufficiency in WBS and SVAS can be achieved by augmenting expression from the wild-type allele using engineered transcription factors. Further, this approach can be very highly specific and is unlikely to induce mutant protein production by overwhelming NMD surveillance. Finally, induction of *ELN* expression in this manner appears capable of stimulating elastogenesis. These findings have significant implications specific to WBS and SVAS and generally suggest that treating haploinsufficiency states by inducing expression of the wild-type endogenous allele may eventually prove to be a viable clinical strategy.

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There are no conflicts of interest to report.

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Address correspondence to:

Frank J. Giordano, M.D.

Yale University School of Medicine

Amistad Research Building

10 Amistad Street

New Haven, CT 06520

E-mail: frank.giordano@yale.edu

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