

## SHORT COMMUNICATION

# Restoration of normal BMP signaling levels and osteogenic differentiation in FOP mesenchymal progenitor cells by mutant allele-specific targeting

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Fibrodysplasia ossificans progressiva (FOP) is a rare autosomal dominant disorder of progressive heterotopic ossification for which there is presently no cure. FOP is caused by a recurrent heterozygous activating mutation (c.617G>A; R206H) of Activin receptor type IA/Activin-like kinase-2 (ACVR1/ALK2), a bone morphogenetic protein (BMP) type I receptor that occurs in all classically affected individuals. The FOP mutation dysregulates BMP signaling and initiates the formation of a disabling second skeleton of heterotopic bone. We generated allele-specific siRNA (ASP-RNAi) duplexes capable of specifically suppressing the expression of the mutant c.617A allele in mesenchymal progenitor cells from FOP patients and showed that this ASP-RNAi approach decreased the elevated BMP signaling that is characteristic of patient cells to levels similar to control cells and restored enhanced osteogenic differentiation to control levels. Our results provide proof-of-principle that ASP-RNAi has potential therapeutic efficacy for the treatment of FOP.

Gene Therapy advance online publication, 20 October 2011; doi:10.1038/gt.2011.152

**Keywords:** Allele-specific RNAi; fibrodysplasia ossificans progressiva; FOP; heterotopic ossification; bone morphogenetic protein; ACVR1/ALK2

## INTRODUCTION

Fibrodysplasia ossificans progressiva (FOP), a rare autosomal dominant disorder of progressive heterotopic ossification, is caused by a recurrent heterozygous mutation in all classically affected individuals.<sup>1</sup> This mild constitutively activating mutation (c.617G>A; R206H) of Activin receptor type IA/activin-like kinase-2 (ACVR1/ALK2), a bone morphogenetic protein (BMP) type I receptor, dysregulates BMP signaling in connective tissue progenitor cells<sup>2,3</sup> and initiates the formation of a disabling second skeleton of heterotopic bone. Presently, there is no effective treatment for FOP. Small chemical inhibitors of BMP type I receptor activation exist;<sup>4</sup> however, their lack of specificity and unwanted global suppression of BMP signaling would adversely affect many organs including the normotopic skeleton.

RNA interference (RNAi) is a powerful tool to silence gene expression. Allele-specific RNAi (ASP-RNAi) is an advanced method of RNAi that allows suppression of disease-causing alleles without inhibition of the corresponding normal alleles.<sup>5–7</sup> Therefore, ASP-RNAi has the potential to suppress mutant genes in dominant human diseases in which one of the two alleles is mutated. ASP-RNAi has been successfully applied both *in vitro* and in mouse models to suppress mutant target gene expression in dominant diseases such as Huntington's,<sup>8,9</sup> Alzheimer's<sup>10,11</sup> and Amyotrophic lateral sclerosis.<sup>12–14</sup> In classic FOP, the same single nucleotide substitution causes the disease in all patients, making this condition particularly amenable to targeted RNAi therapeutic strategies.

In this study, we designed ASP-RNAi duplexes to target suppression of the mutant (c.617A) ACVR1/ALK2 allele as a

necessary proof-of-principle to determine whether targeted suppression of the mutant allele is capable of suppressing the mild constitutive receptor signaling activity and the enhanced osteogenic differentiation of mesenchymal progenitor cells from FOP patients. Our results demonstrate that ASP-RNAi can mediate selective suppression of the mutant c.617A allele and can restore the elevated BMP pathway signaling and osteogenic differentiation of connective tissue progenitor cells from FOP patients to control levels.

## RESULTS AND DISCUSSION

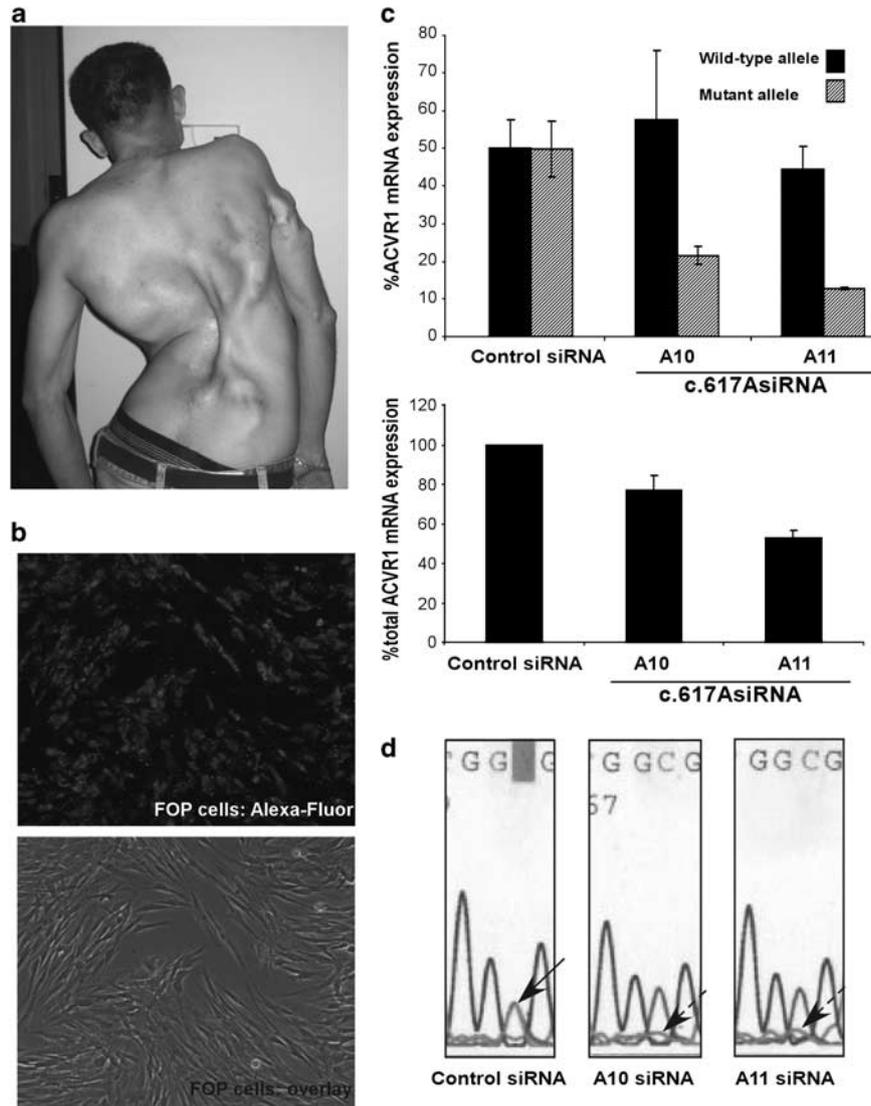
Primary FOP SHED cells are transfected efficiently with ASP-RNAi. FOP is an autosomal dominant genetic disorder of progressive heterotopic endochondral ossification that is characterized by the formation of extensive heterotopic bone that severely impairs movement and diminishes quality of life (Figure 1a). Allele-specific RNAi provides an opportunity to selectively decrease signaling from the mutant allele while permitting signaling from the normal allele.

Primary dental pulp of human exfoliated deciduous teeth (SHED) cells<sup>15–17</sup> were chosen as our model system to evaluate ASP-RNAi. These cells are patient-derived cells that endogenously express the c.617A mutant allele and are capable of differentiating into osteoblasts upon BMP stimulation. Importantly, SHED cells can be safely obtained from FOP patients without the risk of biopsy-related trauma that could induce heterotopic endochondral ossification in the patients.

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Received 25 July 2011; accepted 25 August 2011



**Figure 1.** Specific inhibition of the mutant c.617A allele expression in FOP SHED cells. **(a)** Large areas of heterotopic bone protrude from the back and right arm of an FOP patient, causing substantial deformity. **(b)** Top panel, FOP SHED cells that were transfected with Alexa-fluor red-labeled control siRNA for 48 h show efficient siRNA delivery (intense red fluorescence). Bottom panel, the fluorescent image is overlaid with the phase contrast image of the FOP SHED cells. **(c)** Top panel, FOP SHED cells were transfected with two different ASP-RNAi (A10 and A11) or control (scramble) siRNA and show selective suppression of the mutant c.617A allele expression (hatched bars); the expression of the wild-type allele is minimally affected. Bottom panel, total ACVR1 mRNA expression was reduced in siRNA-transfected cells. **(d)** DNA sequencing of cDNA isolated from cells transfected with control (scrambled) and mutant ASP-RNAi (A10 or A11) demonstrate specificity of targeting with reduction of the T-nucleotide peak (sequenced in reverse and corresponding to c.617A), as depicted by hatched arrows. Two overlapping peaks characteristic of heterozygous allele expression are visualized in control siRNA transfected cells (arrow).

To evaluate transfection efficiency, FOP SHED cells were transfected with 40 nM control scrambled Alexa-fluor red 555-labeled siRNA for 48 h. The FOP SHED cells showed a very high level of transfection efficiency as visualized by red fluorescence staining of all cells (Figure 1b). Furthermore, as shown in Figure 1c, we consistently found similar expression of both wild-type and mutant c.617A alleles in FOP SHED cells demonstrating that the pathogenesis of FOP is a result of a mildly activating mutation (not dysregulated mRNA expression) of one allele. Collectively, these data demonstrate that SHED cells represent an informative model system to evaluate our ASP-RNAi.

#### ASP-RNAi specifically inhibits the mutant c.617A allele

We generated a series of chemically unmodified synthetic siRNA duplexes containing the c.617G > A mutation tiled throughout the

duplex and also included mismatches at the 5'-end of the guide strand to favor loading of the guide strand into the RNA-induced silencing complex (see Supplementary Figure S1).<sup>6,18</sup> To test whether these siRNA duplexes were capable of selectively knocking-down the mutant c.617A allele, FOP SHED cells were transfected with 10 nM of an siRNA duplex, and the level of wild-type c.617G and mutant c.617A mRNA was assessed using a qPCR assay designed to selectively detect the endogenous expression of the wild-type c.617G or the mutant c.617A allele. In FOP SHED cells that were transfected with control (scrambled) siRNA, allele-specific qPCR consistently demonstrated approximately equal expression of wild-type and mutant c.617A ACVR1 alleles (Figure 1c, upper panel). In FOP SHED cells transfected with siRNA duplexes containing the mutant sequence, allele-specific targeting of the mutant c.617A mRNA was observed with most of the siRNA duplexes in our series (Figure 1c, data not shown); we selected

duplexes A10 and A11 for further detailed analysis. To confirm that total ACVR1 mRNA expression was decreased in ASP-RNAi transfected cells, an independent primer set amplifying both wild-type c.617G and mutant c.617A alleles indiscriminately demonstrated decreased levels of total ACVR1 expression following transfection of ASP-RNAi duplexes in FOP SHED cells (Figure 1c, lower panel).

Further verification of allele-specific targeting of the mutant c.617A allele was demonstrated by sequence analysis of cDNA. FOP SHED cells transfected with mutant ASP-RNAi duplexes A10 or A11 show the nearly complete absence of the mutant allele (T nucleotide peak) with predominant expression of the wild-type allele (C nucleotide peak) (Figure 1d). These DNA sequencing data further confirm the specificity and validity of both the ASP-RNAi and our ASP-qPCR detection system.

Our data demonstrate that ASP-siRNA duplexes are capable of specifically and selectively suppressing the endogenous expression of the mutant c.617A allele in primary mesenchymal stem cells obtained from FOP patients.

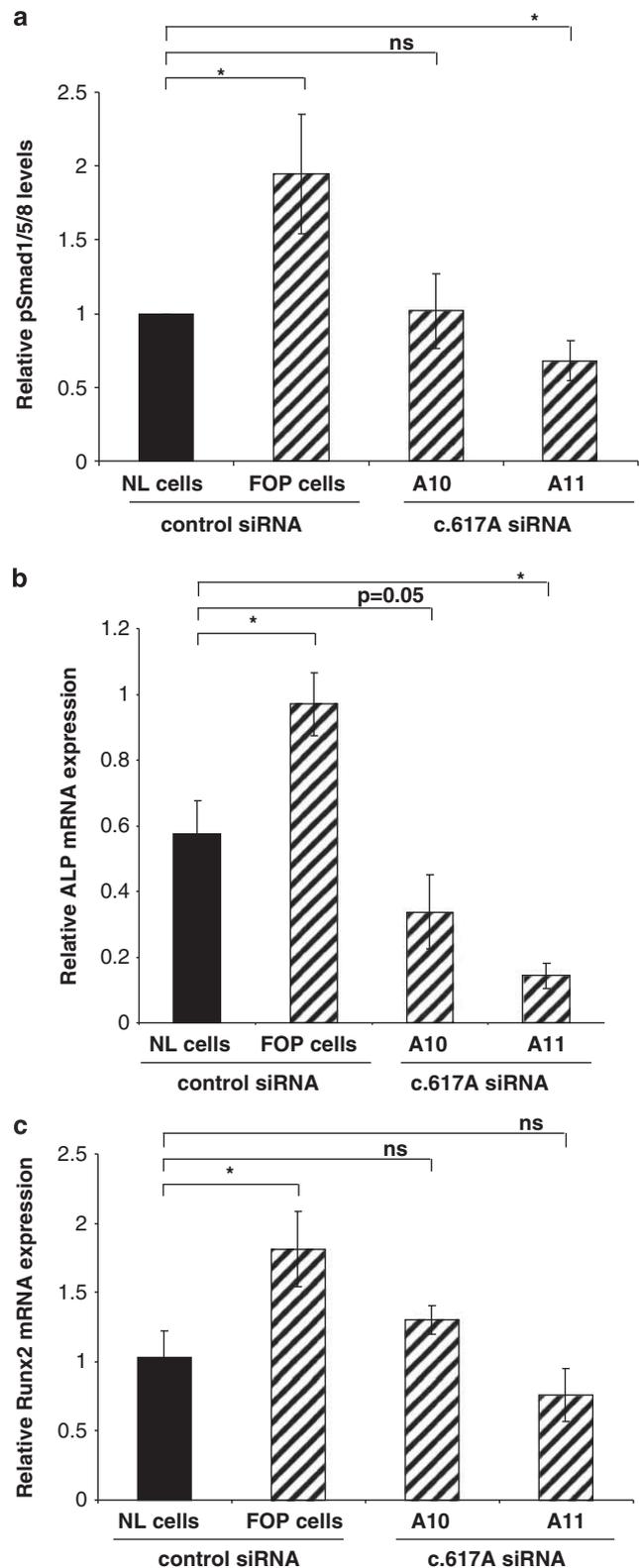
Phospho-Smad1/5/8 signaling and osteogenic differentiation are restored to control levels in FOP SHED cells transfected with mutant ASP-RNAi

We next examined whether selective suppression of the mutant c.617A allele in these cells is sufficient to reduce the elevated BMP pathway signaling from the mutant receptor to levels comparable to normal SHED cells in response to BMP ligand.

FOP SHED cells were transfected with ASP-RNAi duplexes A10 and A11 for 48 h then treated with  $10 \text{ ng ml}^{-1}$  BMP4 for 1 h. Proteins were extracted and detected for phosphorylated Smad1/5/8 (pSmad1/5/8) by immunoblotting. In response to BMP4, FOP SHED cells treated with control RNAi showed approximately twofold higher pSmad1/5/8 compared with normal SHED cells (Figure 2a). Following transfection with mutant ASP-RNAi duplexes, this increased pSmad1/5/8 in BMP-treated FOP cells was reduced to levels similar to normal SHED cells. The reduced or 'restored' BMP signaling levels demonstrated for the first time that allele-specific RNAi is a potential therapeutic tool to suppress the enhanced signaling driven by the expression of the mutant c.617A allele in FOP patient cells.

To determine whether mutant allele-specific knockdown would reduce the enhanced osteogenic potential of osteogenic precursor cells containing the ACVR1 R206H mutation, we cultured FOP SHED cells in the presence of mutant or wild-type ASP-RNAi duplexes under osteogenic induction conditions. Because of the nature of these chemically unmodified siRNA duplexes and, consequently, their short half-life in cell culture experiments, we evaluated the effect of ASP-RNAi duplexes during short-term osteogenic differentiation of FOP SHED cells. In response to osteogenic media, after 3 days, FOP SHED cells demonstrate significantly higher levels of early osteoblast differentiation markers, alkaline phosphatase and Runx2, compared with normal SHED cells (Figure 2b). Transfection of FOP SHED cells with mutant

ASP-RNAi duplexes was capable of reducing the expression of alkaline phosphatase and Runx2 to levels similar to normal SHED cells. These results confirm the effectiveness of targeted suppression of the mutant c.617A allele and demonstrate that enhanced



**Figure 2.** Decreased BMP signaling and osteogenic differentiation in FOP cells in response to mutant ASP-RNAi. (a) FOP SHED cells (hatched bars) were transfected with ASP-RNAi duplexes A10 and A11 and treated with BMP4 ( $10 \text{ ng ml}^{-1}$ ) for 1 h. pSmad1/5/8 was detected by immunoblotting as an assay for BMP signaling activity. pSmad1/5/8 was quantified and compared with BMP4-treated control SHED cells ( $*P < 0.05$ , compared with normal SHED cells). Relative mRNA expression in normal and FOP SHED cells (transfected with mutant ASP-RNAi duplexes A10 or A11) of the osteogenic markers alkaline phosphatase (ALP) (b) and Runx2 (c) following 3 days in osteogenic media ( $*P < 0.05$ , compared with normal SHED cells).

4 osteogenic differentiation in FOP cells is mediated through expression of the mutant c.617A allele.

Improvements in RNAi design, such as chemical modifications of siRNA duplexes, are advancing at a rapid rate<sup>19–22</sup> and will enhance the stability, potency and specificity of RNAi allowing for longer-term experiments both *in vitro* and *in vivo*. We used chemically unmodified siRNA duplexes to provide proof-of-principle that targeted suppression of the c.617A allele in human FOP cells can restore BMP-induced signaling to similar levels detected in control cells. These ASP-RNAi knockdown experiments can be applied to emerging mouse models of FOP providing hope that inhibition of the mutant c.617G>A allele will be a therapeutic strategy to abrogate the catastrophic formation of heterotopic bone in FOP patients.

In summary, we used chemically unmodified siRNA duplexes in primary FOP mesenchymal progenitor cells to specifically target suppression of the mutant c.617A allele and restored BMP signaling and osteogenic differentiation to control levels. Our results provide proof of-principle that ASP-RNAi has potential therapeutic efficacy for the treatment of FOP.

## MATERIALS AND METHODS

### Materials

Trizol,  $\alpha$ MEM and DMEM culture media, High Capacity RNA to cDNA reagents, and Lipofectamine RNAiMAX were obtained from Invitrogen (Carlsbad, CA, USA).

Human recombinant BMP4 was obtained from R&D Systems (Minneapolis, MN, USA); stock solutions (100 ng  $\mu$ l<sup>-1</sup>) were prepared as recommended by the manufacturer.  $\beta$ -glycerophosphate, ascorbic acid sodium salt, and type II collagenase were from Sigma (St Louis, MO, USA). Sense strand of siRNAs are as follows: A10 (5'-CAGTGGCTC**ACC**AGATTAC-3') and A11 (5'-ACAGTGGCTC**ACC**AGATTA-3'). Bold nucleotide indicates the position corresponding to nucleotide c.617. All were synthesized (Sigma) as complementary sequences (except were noted) with 3'UU overhangs. Control scrambled Alexa-fluor red 555-labeled siRNA was from Invitrogen.

### SHED cell isolation, culture and treatments

Exfoliated teeth were obtained from normal and FOP pediatric patients according to the Institutional Review Board-approved protocols at the University of Pennsylvania. Cells were isolated as previously reported<sup>2,23</sup> and used up to passage 10. In brief, dental pulp was digested with 3 mg ml<sup>-1</sup> type II collagenase for 1 h at 37 °C, then the collagenase activity was neutralized with the addition of growth media ( $\alpha$ MEM with 10% FCS) and filtered through a 100- $\mu$ m cell strainer (BD Falcon, Franklin Lakes, NJ, USA). Cells were recovered by centrifugation (1200 rpm, 10 min) and plated in growth media containing antibiotics.

For transfection, cells were seeded at a density of  $2.5 \times 10^4$  cells cm<sup>-2</sup>, allowed to attach and transfected with siRNA duplexes (10 nM), using RNAiMax reagent (Invitrogen) according to manufacturer's instructions in serum-free containing media for 48 h. For pSmad1/5/8 detection, transfected cells were serum starved for 2 h then treated with 10 ng ml<sup>-1</sup> BMP4 for 1 h. For osteogenic differentiation, at 48 h following transfection, cells were cultured with osteogenic medium ( $\alpha$ MEM, 10% FCS, 10 mM  $\beta$ -glycerophosphate, 50  $\mu$ g ml<sup>-1</sup> ascorbic acid, and 10 ng ml<sup>-1</sup> BMP4). Media was replenished every 3 days. A Nikon eclipse TE2000-U microscope (Nikon Instruments Inc., Melville, NY, USA) was used for visualization of Alexa-fluor red 555-labeled control scrambled siRNA in transfected cells.

### RNA isolation, real-time PCR, and DNA sequencing

Cells were harvested at indicated times and RNA was isolated using TRIzol (Invitrogen), according to manufacturer's instructions. Following phase separation, 10  $\mu$ g of glycogen (Roche Applied Science, Indianapolis, IN, USA) was added to facilitate precipitation of RNA. RNA quantification was performed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). cDNA was synthesized using High Capacity RNA-to-cDNA reagents (Applied Biosystems, Foster City, CA, USA).

Real-time quantitative PCR reactions contained forward and reverse primers, cDNA (1:10 dilution), and Fast SYBR Green PCR Master Mix (Applied Biosystems). For wild-type ACVR1 allele detection: forward: 5'-TGGTACAAAGAACAGTGGCTAG-3', mutant allele detection: forward: 5'-TGGTACAAAGAACAGTGGCTTA-3' and common reverse primer: 5'-CCA TACCTGCCTTTCCCGA-3'. PCR reactions for allele-specific detection was performed at extension temperature of 63 °C and primers diluted 1:4 from a 5- $\mu$ M stock. Primers for detection of Runx2 forward: 5'-GGCATCAAACA GCCTTTCAG-3', reverse: 5'-gggtgctcgatccaaaag-3' and for Alkaline Phosphatase forward: 5'-ACCATTCCCACGCTTCACATTTG-3', reverse: 5'-AGACATTCTCTCGTTCACCGCC-3'. Each sample was analyzed in triplicate (Applied Biosystems 7500 Fast Real-Time PCR Systems) and target gene mRNA levels quantified from standard curves and normalized to GAPDH.

cDNA from transfected FOP SHED cells was PCR amplified (30 cycles of 94 °C 1 min, 66 °C 1 min and 72 °C 2 min) spanning the region containing nucleotide c.617. PCR products were gel-purified and the DNA sequence determined (DNA Sequencing Facility, Department of Genetics, University of Pennsylvania).

### Protein isolation and immunoblotting

Cells were harvested and lysed in two packed cell volumes of 1  $\times$  lysis buffer (150 mM NaCl, 100 mM EDTA, 1% NP-40, 40 mM Tris, pH 7.9, 10% glycerol, 0.1% SDS, and 1  $\times$  protease inhibitor cocktail). Protein concentration determined by BCA Protein Assay (Pierce Biotechnology, Rockford, IL, USA) using BSA as a standard.

Proteins were electrophoresed through 4–12% gradient SDS-polyacrylamide gels (Invitrogen) and transferred to nitrocellulose membranes. Membranes were blocked in LI-COR blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) for 1 h and incubated with primary antibodies: 1:1,000 dilution of pSmad1/5/8 antibody (Cell Signaling Technology, Danvers, MA, USA) and 1:4,000 dilution of  $\beta$ -actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in LI-COR blocking buffer overnight at 4 °C. Bound primary antibodies were detected with species matched IRDye-labeled secondary antibodies (LI-COR) diluted 1:40,000 (in LI-COR blocking buffer) for 1 h at room temperature (protected from light). Imaging and quantification of blots were performed using the Odyssey Infrared Detection System (LI-COR). Data are presented as ratios of pSmad1/5/8 intensity normalized to  $\beta$ -actin and results plotted relative to normal cells treated with BMP4 (set = 1).

### Statistics

The unpaired two-tailed Student's *t*-test was used to determine the significant difference between means. All relevant comparisons were significantly different at  $P < 0.05$  unless otherwise indicated. All experiments were performed at least in triplicate.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### ACKNOWLEDGEMENTS

We thank the members of our research laboratory for their helpful discussions during the development of these studies. This work was supported in part by the Center for Research in FOP and Related Disorders, the International FOP Association (IFOPA), the Ian Cali Endowment, the Weldon Family Endowment, the Isaac and Rose Nassau Professorship of Orthopaedic Molecular Medicine, the Rita Allen Foundation, and the Penn Musculoskeletal Center, and by grants from the NIH (R01-AR41916 and R01-AR046831).

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Supplementary Information accompanies the paper on Gene Therapy website (<http://www.nature.com/gt>)