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CRISPR-Cpf1 Activation of Endogenous *BMP4* Gene for Osteogenic Differentiation of Umbilical-Cord-Derived Mesenchymal Stem Cells

Jaehoon Choi,[1,10] Taegeun Bae,[2,10] Ninj Byambasuren,[3] Seong-Ho Park,[6] Chris H. Jo,[4] Dokyoung Kim,[5,6,7,8] Junho K. Hur,[6,9] and Nathaniel S. Hwang[1,3]

[1]School of Chemical and Biological Engineering, Institute of Chemical Processes, Seoul National University, Seoul, Korea; [2]Department of Medicine, Graduate School, Kyung Hee University, Seoul, Korea; [3]Interdisciplinary Program in Bioengineering, Institute of Bio Engineering, Seoul National University, Seoul, Korea; [4]Department of Orthopedic Surgery, SMG-SNU Boramae Medical Center, Seoul National University College of Medicine, Seoul, Korea; [5]Department of Anatomy and Neurobiology, College of Medicine, Kyung Hee University, Seoul, Korea; [6]Department of Biomedical Science, Graduate School, Kyung Hee University, Seoul, Korea; [7]Center for Converging Humanities, Kyung Hee University, Seoul, Korea; [8]Medical Research Center for Bioreaction to Reactive Oxygen Species and Biomedical Science Institute, Kyung Hee University, Seoul, Korea; [9]Department of Pathology, College of Medicine, Kyung Hee Unversity, Seoul, Korea

The CRISPR systems provide powerful genome-editing tools for wide applications in biological and medical research fields. However, the safety issue due to off-target effects of CRISPR has been one of the major hindrances of its application to regenerative medicine. The conventional CRISPR system has the intrinsic danger of inducing unpredictable mutations at non-targeted genomic loci via erroneous double-strand DNA breaks (DSBs). In this study, we demonstrate a safety-enhanced application of a recently discovered CRISPR-Cpf1 for targeted gene activation, without DNA double-strand break, to facilitate osteogenic differentiation of human umbilical-cord-derived mesenchymal stem cells (UC-MSCs). To this end, we developed a catalytically inactive AsCpf1 fused to tripartite transcription activator domain (dAsCpf1-VPR) that can induce upregulation of targeted gene expression in mammalian cells. We observed that the CRISPR-dAsCpf1-VPR activator can be applied to enhance the osteogenic differentiation of human UC-MSCs, via increasing the expression level of endogenous *BMP4* gene. The results suggested that the CRISPR-Cpf1 activator provides versatile methods applicable for bone regeneration and regenerative medicine.

INTRODUCTION

CRISPR is a robust genome-editing tool that has been widely applied in various research areas.[1] However, many conventional CRISPR systems introduce double-strand breaks (DSBs) at target loci, and the unwanted mutations in mammalian cells by CRISPR are major safety concerns of their applications for therapeutic purpose.[2–4] In order to circumvent the DSB issues, several types of catalytically inactive forms of a CRISPR molecule are utilized for regulating endogenous target gene expression without DNA mutations.[5–8] Previous studies showed that catalytically inactive CRISPR proteins could be fused with transcriptional activators or repressors: CRISPR activation (CRISPRa) or CRISPR interference (CRISPRi), respectively.[9,10] These transformative techniques demonstrated that target-specific transcriptional regulation could be achieved by combining the specific gene targeting by CRISPR and the regulatory activities of the fused transcriptional effectors.[9,10]

Among the diverse CRISPR types, CRISPR-SpCas9 has been one of the most widely applied systems for precise genome engineering. Notably, some recent studies showed that CRISPR-Cpf1, a more recently discovered CRISPR effector,[11] potentially has even lower off-target effects compared to SpCas9, suggesting that Cpf1 could be applied for CRISPRa with increased specificities.[12–14] Two previous studies showed that CRISPR-Cpf1 could be applied to regulate gene expression in mammalian cells.[15,16] Chemically inducible dLbCpf1 could be fused to a transcriptional activator for multiplex gene perturbation via CRISPR RNA (crRNA) maturation in human embryonic kidney (HEK293) cells.[15] In addition, programmable ligand-controlled dAsCpf1 systems could be applied for regulating the gene expression.[16] The control of dAsCpf1 activation in HeLa and HEK293T cells was conducted by using crRNAs with engineered riboswitches and dAsCpf1 proteins fused to G protein-coupled receptors. While the previous studies suggested that Cpf1 has the potential for useful biomedical tool, the application of dCpf1-based CRISPRa to regenerative medicine has not been demonstrated.

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[10]These authors contributed equally to this work.

Correspondence: Dokyoung Kim, Department of Anatomy and Neurobiology, College of Medicine, Kyung Hee University, Seoul, Korea.
E-mail: dkim@khu.ac.kr
Correspondence: Junho K. Hur, Department of Pathology, College of Medicine, Kyung Hee Unversity, Seoul, Korea.
E-mail: jhur@khu.ac.kr
Correspondence: Nathaniel S. Hwang, School of Chemical and Biological Engineering, Institute of Chemical Processes, Seoul National University, Seoul, Korea.
E-mail: nshwang@snu.ac.kr

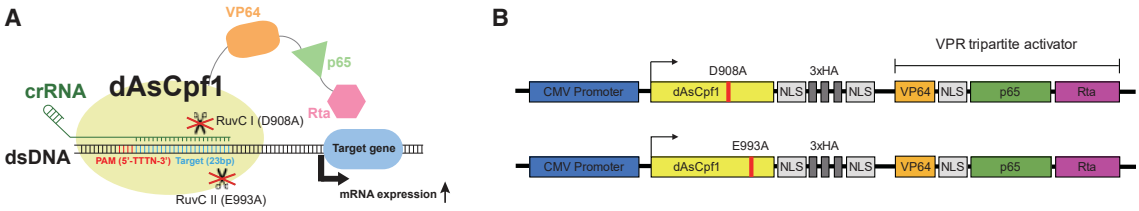

Figure 1. A Schematic Representation of the CRISPR Activator (dAsCpf1-VPR) Construct

(A) A model of the dAsCpf1-VPR system on the target genomic location. The ribonucleoprotein complex, comprised of catalytically inactive dAsCpf1 fused to the VP64-p65-Rta transcriptional activators (VPR) and crRNA, recognizes and binds at the promoter region of the target gene. The localized VPR enhances the mRNA expression of the target gene. (B) The domain architectures of dAsCpf1-VPR. Two catalytically inactive dAsCpf1 constructs were generated by alanine substitution of D908 or E993. The dAsCpf1 constructs were fused with VPR transcriptional activator for expression by CMV promoter.

To assay the applicability of CRISPR-Cpf1 activation system, we sought to utilize the system to osteogenic differentiation of mesenchymal stem cells (MSCs), specifically derived from human umbilical cords. MSCs comprise a key element in tissue regeneration since their facile expandability and plasticity provide extensive potential for tissue engineering.[17] Osteogenic differentiation of MSCs is one of the most important cell differentiations in regenerative medicine, contributing to bone formation and regeneration.[18] Previous studies have demonstrated that one of the key factors in osteogenic differentiation is *bone morphogenetic protein 4* (BMP4), a member of signaling molecules in the transforming growth factor β (TGF-β) pathway that plays a significant role in activating the SMAD signal pathway and upregulating osteoblast transcription factors.[19,20] Consistently, introducing the exogenous *BMP4* gene was shown to facilitate bone regeneration.[21] Hence, in this study, we sought to apply CRISPR-Cpf1 activation to the *BMP4* gene for osteogenic differentiation of hMSCs without exogenous gene expression.

RESULTS

Application of CRISPR-Cpf1 for Targeted Gene Activation

We sought to construct and utilize a CRISPR-Cpf1-based gene activation system, named dAsCpf1-VPR, for increasing target gene mRNA expression levels in mammalian cells. To this end, we first generated a catalytically inactive form of CRISPR-Cpf1 from *Acidaminococcus sp. BV3L6* (dAsCpf1) by conducting site-directed mutagenesis on AsCpf1 to induce mutations on the endonuclease catalytic residues (D908A or E993A) (Figure 1A). Mutation of either catalytic site of Cpf1 resulted in complete loss of endonuclease activity.[11] Then we utilized dAsCpf1 as a recruiting factor for a tri-partite transcriptional activator consisting of VP64, p65, and Rta (VPR), which was shown to achieve a high level of gene activation.[7] To this end, we fused a VPR tripartite activator on the C-terminal of dAsCpf1 and named the fusion construct dAsCpf1-VPR (Figures 1A and 1B). We next sought to assay the activity of both D908A and E993A dAsCpf1-VPR for inducing activation of endogenous genes. To this end, we designed crRNAs to localize dAsCpf1-VPR within the promoter region of *interleukin-1 receptor antagonist* gene (IL1RN) (Figure 2A).[7,15] We searched genome data on the UCSC genome browser (http://genome.ucsc.edu/). For *IL1RN* endogenous gene activation by dAsCpf1-VPR, we utilized the genomic sequence of IL1RN (ENSG00000136689) and selected the binding regions for crRNAs that were between 1 and 300 bp upstream of the transcriptional start site (TSS). We designed four crRNAs targeting the *IL1RN* gene to activate the gene expression. We assayed the efficiency of dAsCpf1-VPR-mediated gene activation of endogenous *IL1RN* in a human cell line by transfecting dAsCpf1-VPR and crRNAs targeting the *IL1RN* gene into HEK293T cells (Figure 2B). CRISPRa of dAsCpf1(E993A)-VPR and dAsCpf1(D908A)-VPR resulted in increased mRNA expression levels of the *IL1RN* gene by ∼500-fold and ∼1,700-fold, respectively (Figure 2B). Therefore, we selected dAsCpf1(D908A)-VPR (dAsCpf1-VPR, hereafter) in the subsequent gene-activation experiments.

CRISPR-Cpf1 Activator Induces Greater Increase of Endogenous BMP4 Gene Expression Level Compared to CRISPR-Cas9

We next sought to apply dAsCpf1-VPR to increasing the expression levels of endogenous *BMP4* gene, an important factor of osteoblast differentiation. For BMP4 endogenous gene activation by dAsCpf1-VPR and dSpCas9-VPR, we selected a BMP4 (ENSG00000125378) gene variant and designed two crRNAs targeting ∼300 bp upstream of the TSS on BMP4 and other two crRNAs targeting a region between exons 1 and 2 (Figure 3A). The latter region is a putative promoter region of a BMP4 transcript variant and also has a higher expression level of H3K27Ac, one of the well-known promoter markers. We also sought to compare the efficiency of activation by dAsCpf1-VPR to CRISPR-Cas9-based activation, for which we prepared dSpCas9-VPR activation by designing four sgRNAs targeting the promoter region of endogenous BMP4 gene.[7]

We first assessed the CRISPRa of *BMP4* gene expression by transfection in HEK293T cells (Figure 3B). We found that positioning dAsCpf1-VPR at the canonical promoter regions (crRNA 3&4) increased the BMP4 mRNA expression by ∼15-fold, while localizing dAsCpf1-VPR between exons 1 and 2 (crRNA 1&2) resulted in insignificant gene activation. Notably, the gene activation of *BMP4* by dAsCpf1-VPR with crRNA 3&4 was more efficient than dSpCas9-VPR, which showed only a 3- to 4-fold increase (Figures 3C and 3D). Next, we asked whether the dSpCas9-VPR-mediated gene activation using crRNA 3 and crRNA 4 may be subject to steric hindrance between the two crRNAs binding at the target DNA. To this end, we conducted dSpCas9-VPR activation of *BMP4* gene using crRNA

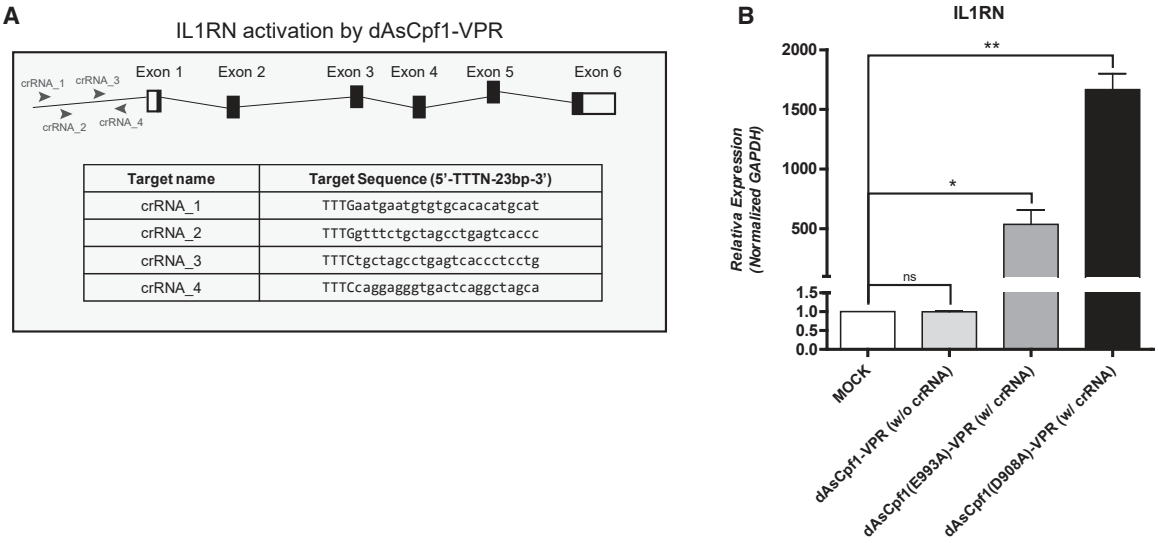

Figure 2. CRISPR Activation of Endogenous *IL1RN* Gene by dAsCpf1-VPR in HEK293T

(A) A diagram of positions and sequences of crRNAs targeting the *IL1RN* genomic locus. (B) CRISPR activation of *IL1RN* gene by dAsCpf1-VPR. Shown are the changes of mRNA levels of *IL1RN* in HEK293T. Data shown are results from three biological replicates (error bars indicate the SEM). p values less than 0.05 were considered significant (*$p < 0.05$, **$p < 0.01$), and no significance between groups was indicated as ns.

3, crRNA 4, or crRNA 3&4. Interestingly, activation of *BMP4* by either crRNA did not result in significant increase in the target gene expression levels. On the contrary, activation using crRNA 3 and crRNA 4 simultaneously induced an almost 15-fold increase. The results suggested that the gene activation using crRNA 3 and crRNA 4 in combination showed a synergistic effect, rather than steric hindrance (Figure S1A). We also sought to assay whether dAsCpf1-VPR retained catalytic activity and could induce insertions or deletions (indels) at the target site. To this end, we conducted genome editing of the *BMP4* locus using dAsCpf1-VPR and wild-type AsCpf1, the latter of which served as a positive control. The deep sequencing quantification showed that dAsCpf1-VPR was unable to induce a detectable indel rate, while the indel rate of AsCpf1 was around 38%. Together, the results suggested that dAsCpf1-VPR was unable to induce indels at the *BMP4* locus and had insignificant endonuclease activity (Figure S1B).

We then assessed whether dAsCpf1-VPR could also increase the levels of endogenous *BMP4* levels in human MSCs derived from umbilical cord (UC-MSCs). To this end, we transfected UC-MSCs that were maintained in growth medium, with dAsCpf1-VPR and single guide RNA (sgRNA) to activate the endogenous *BMP4* gene (Figures 4A and S2A). We found that dAsCpf1-VPR increased the endogenous *BMP4* gene expression levels of UC-MSC by ~2-fold in both osteogenic differentiation medium (OM) and in growth medium (GM). The upregulation of *BMP4* levels were accompanied by an increase in expression levels of genes related to osteogenic differentiation such as *osteocalcin* (OCN), *runt-related transcription factor 2* (RUNX2), and *type 1 collagen* (COL1) (Figures 4B, 4C, and S2B).[22,23] Based on the above results, we hypothesized that increasing the expression level of endogenous *BMP4* gene in UC-MSC by dAsCpf1-VPR may enhance the cellular differentiation from MSCs to osteoblasts.

Activation of Endogenous BMP4 Gene by CRISPR-Cpf1 Facilitates Osteogenic Differentiation

Next, we sought to test whether dAsCpf1-VPR-mediated activation of endogenous *BMP4* gene levels could facilitate cell differentiation (Figures 4D, 4E, S3A and S3B). For quantitative analyses, we conducted *in vitro* osteogenic differentiation assay. In brief, we performed *BMP4* gene activation of UC-MSCs by dAsCpf1-VPR ($crRNA^+$), and then cultured the UC-MSCs for 3 weeks in OM. The progress of osteogenic differentiation was then assessed by Alizarin red S (ARS) staining. We observed that, after culturing UC-MSCs for 3 weeks with dAsCpf1-VPR activation of endogenous BMP4 gene, the signal of ARS stain was significantly increased, indicative of enhancement in osteogenic differentiation. We also sought to assess whether increments of BMP4 alone could enhance osteogenic differentiation. To this end, we cultured UC-MSCs for 3 weeks in OM with addition of exogenous recombinant human BMP4 protein (rhBMP4) and observed that addition of BMP4 in OM ($rhBMP4^+$) also resulted in increased ARS staining levels. The results indicated that activation of *BMP4* by itself is sufficient for facilitating osteogenic differentiation of UC-MSCs in OM. Together, the data suggested that dAsCpf1-VPR-mediated activation of the endogenous *BMP4* gene led to increased BMP4 protein levels that in turn facilitated the commitment of UC-MSCs to osteogenic differentiation without other external gene products. We also found that transfection of dAsCpf1-VPR without crRNA ($crRNA^-$) did not result in increased ARS staining, indicating that the facilitation of osteogenic differentiation was not triggered by non-specific gene activation. In

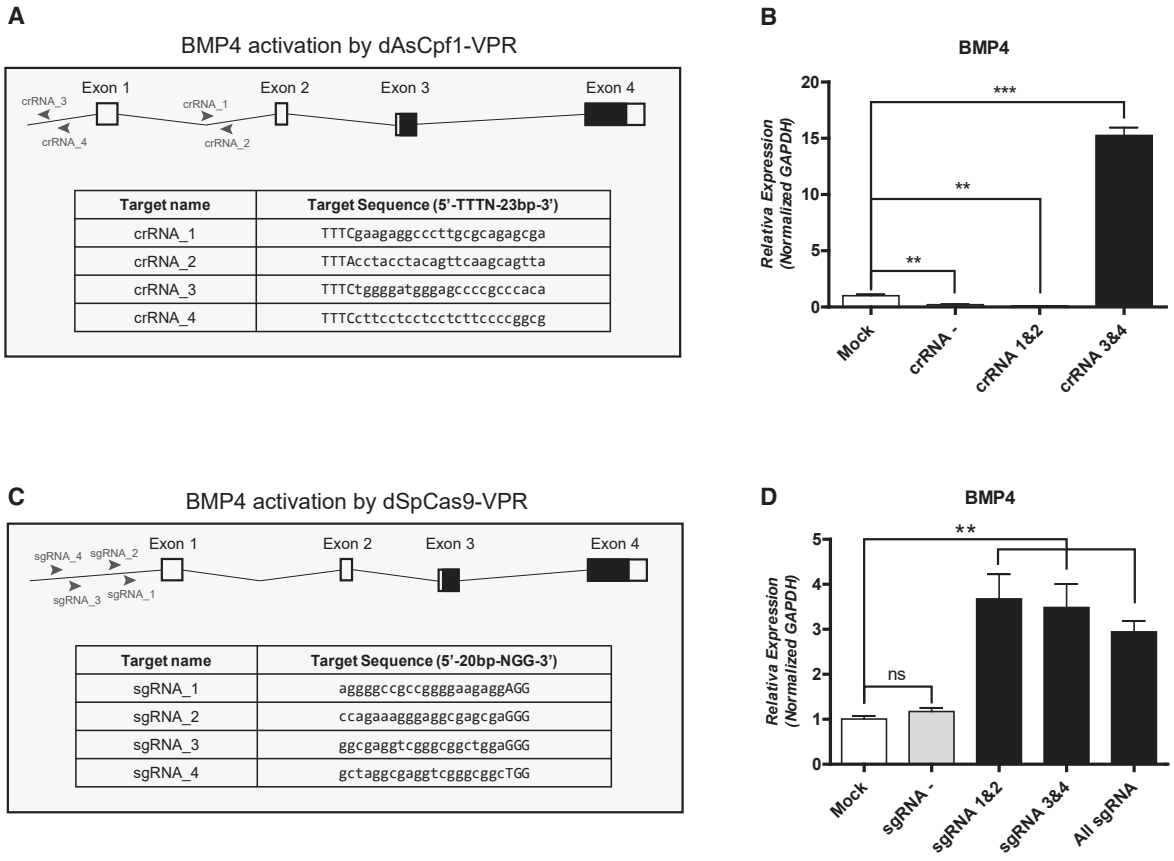

Figure 3. CRISPR Activation of Endogenous *BMP4* Gene in HEK293T by dAsCpf1-VPR and dSpCas9-VPR

(A) A schematic of positions and sequences of four crRNAs targeting different locations within the *BMP4* gene. (B) The changes in mRNA levels of endogenous *BMP4* gene by dAsCpf1-VPR and four crRNAs. (C) A schematic of positions and sequences of sgRNAs targeting the BMP4 gene. All sgRNAs are located on the proximal region of the promoter of the BMP4 gene. (D) The changes in mRNA levels of endogenous BMP4 gene by dSpCas9-VPR and four different sgRNAs. Mock, no transfection; crRNA^{-}, transfection of only dAsCpf1-VPR without crRNAs; crRNA 1&2, transfection of dAsCpf1-VPR with crRNA 1&2 targeting the *BMP4* gene; crRNA 3&4, transfection of dAsCpf1-VPR with crRNA 3&4 targeting the *BMP4* gene; sgRNA^{-}, transfection of only dSpCas9-VPR without sgRNAs; sgRNA 1&2, transfection of dSpCas9-VPR with sgRNA 1&2 targeting the *BMP4* gene; sgRNA 3&4, transfection of dSpCas9-VPR with sgRNA 3&4 targeting the *BMP4* gene; all sgRNA, transfection of dSpCas9-VPR with all sgRNAs targeting the *BMP4* gene. Data shown are combined triplicate qRT-PCR results (error bars indicate the SEM). p values less than 0.05 were considered significant (**$p < 0.01$, ***$p < 0.001$).

contrary, the precise targeted activation of *BMP4* by dAsCpf1-VPR via designing the crRNA to the promoter region is critical for the boosting osteogenic differentiation of UC-MSC. The results together suggested that the increased endogenous *BMP4* levels, by dAsCpf1-VPR, can enhance osteogenic differentiation of human MSCs in OM without adding exogenous gene products.

DISCUSSION

To our best knowledge, this is the first study to apply CRISPR-Cpf1 activation to osteogenic differentiation of human stem cells. As CRISPR-Cpf1 can target genomic loci with sequences that are incompatible with CRISPR-Cas9, Cpf1 can be an attractive alternative of Cas9 for targeted gene activation of endogenous genes. In addition, we found that dAsCpf1-VPR-mediated activation of endogenous *BMP4* gene was even more efficient than the conventional CRISPRa by dSpCas9-VPR. The high efficiency of dAsCpf1-VPR activation could be partially explained by the potentially synergistic effect of using two crRNAs simultaneously. Such cooperative effect between crRNAs was consistent with the results of previous studies.[15,16]

We anticipate that, in addition to *BMP4*, there will be more genes related with osteogenic differentiation that could be activated by CRISPR for bone regeneration. Notably, in the *in vitro* differentiation assay, culturing the UC-MSCs in GM efficiently suppressed osteogenic differentiation. The results suggested that the differentiation of UC-MSCs to osteoblasts is regulated by BMP4 levels and the culture medium. We anticipate that the two-layered regulation of modulating the gene expression level and the culture medium composition could be utilized for versatile control of osteogenic differentiation in cell engineering and gene therapy. However, it is important to note that generalization of our CRISPR-Cpf1 activation system to the MSCs derived from other than umbilical cords might not be efficient. This

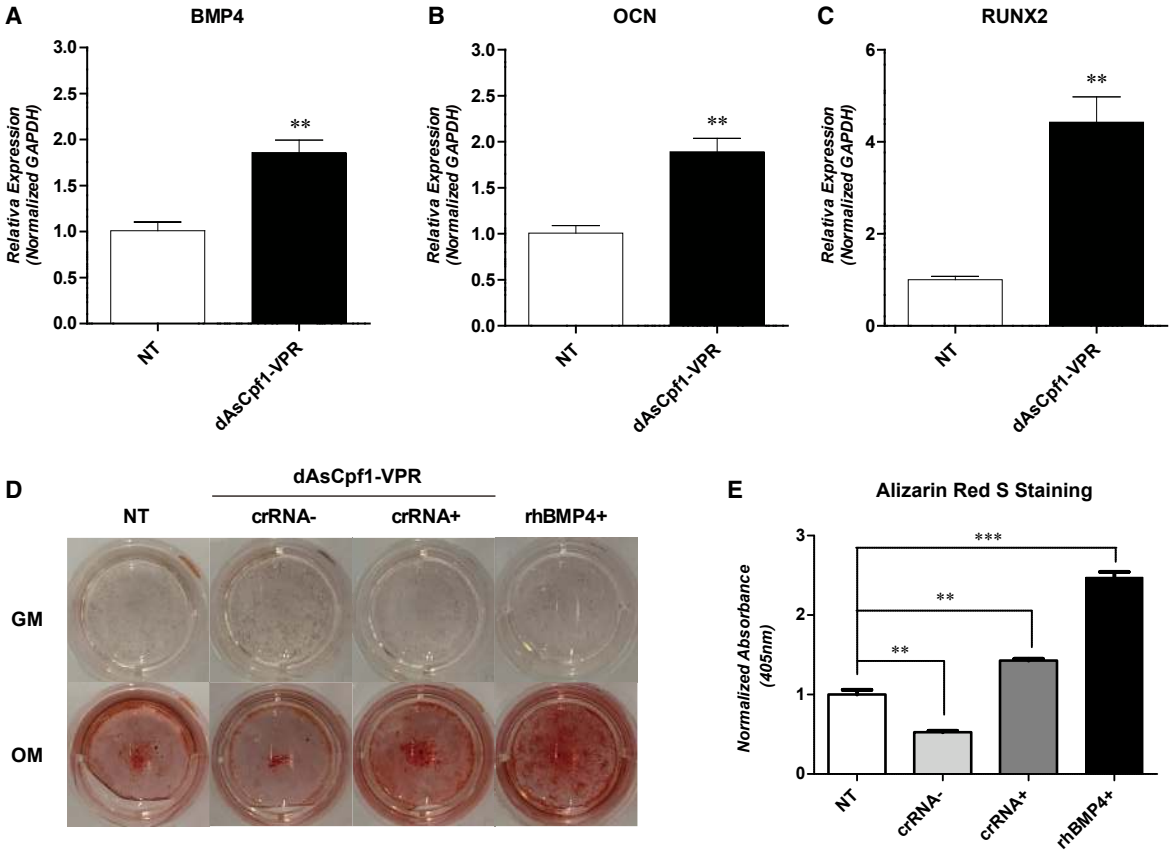

Figure 4. dAsCpf1-VPR Mediated Activation of Endogenous *BMP4* Gene Expression in UC-MSC Facilitates Osteogenic Differentiation

(A) Shown are the changes in mRNA expression levels of endogenous *BMP4* in UC-MSC by dAsCpf1-VPR activation using crRNA 3&4. (B and C) Changes in mRNA expression levels of *OCN* (B) and *RUNX2* (C). (D) Alizarin red S staining of UC-MSC cultured in growth medium (GM) or osteogenic differentiation medium (OM). (E) Quantification of the intensities of ARS staining of UC-MSC cultured in OM. NT, no transfection; crRNA^{-}, transfection of dAsCpf1-VPR without crRNAs; crRNA^{+}, transfection of dAsCpf1-VPR and crRNA 3&4 targeting *BMP4*; rhBMP4^{+}, supplement of recombinant human BMP4 protein (10 ng/mL). Data shown are combined triplicate qRT-PCR results (error bars indicate the SEM). p values less than 0.05 were considered significant (**$p < 0.01$, ***$p < 0.001$).

is because only a single type of UC-MSC was tested, though characteristics of MSCs are likely dependent not only on source tissue types but also on MSC donors' features, including age, sex, and types or stages of disease. We also note that the dAsCpf1-VPR activation of BMP4 showed a significant but lower rate of osteogenic differentiation compared to addition of exogenous rhBMP4. The difference of rates could be potentially caused because we applied single transient transfection of dAsCpf1-VPR to UC-MSCs, and the expression levels of BMP4 gradually decreased during the 3 weeks of differentiation. We anticipate that the osteogenic efficiency could be increased by applying repeated transfection of dAsCpf1-VPR and using long-term viral delivery.

With the advancement of molecular biology tools, the application of gene therapy to stem cells has been gaining more importance in regenerative medicine.[24,25] Researchers have been working on cell-fate engineering to enhance the functionality of stem cells.[26,27] Some sought to develop methods to determine the cell lineage and enhance the cellular function by adding exogenous cDNAs of genes of interest into the cells.[21,28–30] The development of CRISPRa enabled increasing the expression levels of endogenous target genes without changing the DNA sequences or introducing exogenous genes into cells.[5,31,32] The CRISPRa technology provides a flexible tool for cell-fate engineering, as any endogenous gene can be targeted for activation by designing crRNAs to match the promoter sequences. CRISPRa can be especially useful for activating the expression of large genes, as their long DNA length can prohibit exogenous gene delivery via vectors such as adeno-associated virus (AAV).[33] We anticipate that the advantages of CRISPRa can be utilized for precise and safe biomedical applications for cellular and *in vivo* systems.

In summary, we showed that CRISPR-Cpf1 activation of the *BMP4* gene can facilitate the osteogenic differentiation of human MSCs. The dAsCpf1-VPR system provides a novel and flexible technique for cell engineering and eliminates the safety concerns of conventional CRISPR methods that can induce erroneous DNA mutations

by off-target effects. We anticipate that the CRISPR-Cpf1 method could be further applied for regenerative medicine, including development of therapeutics for bone-related disorders.

MATERIALS AND METHODS

Plasmid Construction

dAsCpf1-VPR plasmids were cloned from pCMV-hAsCpf1 plasmid (Addgene #69982) and dSpCas9-VPR plasmid (Addgene #68497) using NEBuilder HiFi DNA assembly master mix (NEB, E2621). The pCMV-hAsCpf1 plasmid, which has a cytomegalovirus (CMV) promoter, was cut by EcoRI-HF restriction enzyme (NEB, R3101), which is located in the C terminus of the plasmid, and the VPR domain is amplified by PCR from dSpCas9-VPR plasmid. The VPR domain is inserted into EcoRI cut-AsCpf1 plasmid. After that, the catalytic residues of wild-type AsCpf1 (D908 or E993) were substituted by alanine via site-directed mutagenesis to make the dAsCpf1-VPR construct.

Cell Culture

A HEK293T cell line cultured on 0.1% gelatin-coated culture dishes was purchased from the ATCC and maintained in a GM that consists of high-glucose DMEM (Corning), 10% fetal bovine serum (FBS) (Corning), 100 U/mL penicillin and streptomycin (Gibco), and 1% Gibco GlutaMAX supplement. For gelatin coating, 0.1% gelatin on culture dishes was incubated for 15 min and was carefully removed. UC-MSCs were extracted in the Stem Cell & Orthopedic Research Laboratory at SMG-SNU Boramae Medical Center and maintained in UC-MSC GM (low-glucose DMEM [Corning], 10% FBS [Corning], 100 U/mL penicillin and streptomycin [Gibco], and 1% Gibco GlutaMAX supplement) supplemented with 5 ng/mL recombinant human fibroblast growth factor (FGF)-basic (100-18B, PeproTech). At 70%–90% confluency, cells were treated with 0.25% trypsin (Gibco) and subcultured at certain seeding densities (HEK293T, 40,000 cells/cm^2; UC-MSC, 5,000 cells/cm^2) after counting. For optimal balance of cell number and differentiation potency, UC-MSCs were expanded to passage 6, and their response to BMP4 gene activation via dAsCpf1-VPR system was gauged. Mycoplasma contamination was not tested.

Transfection for CRISPRa

As for lipofection to deliver dAsCpf1-VPR and crRNAs targeting the *IL1RN* gene, HEK293T cells were seeded into 24-well plates at 70%–80% confluency and were transfected using Lipofectamine 2000 (Invitrogen), guided by the manufacturer's instruction. In brief, the cells were transfected with dAsCpf1 expression plasmids (500 ng per well) and crRNA expression plasmids (500 ng per well). The transfected cells were cultured for 48 h, and total RNA was isolated with Invitrogen TRIzol reagent for subsequent analyses. All electroporation tests were performed in a Neon transfection system using 100-μL tips. For *BMP4* gene activation confirmation test by dAsCpf1-VPR and dSpCas9-VPR, HEK293T cells were transfected by electroporation (1,100 V, 20 ms, two pulses). 0.8 M cells were resuspended in 120 μL resuspension buffer containing 2 μg dAsCpf1-VPR or dSpCas9-VPR and 2 μg total crRNA or sgRNA plasmids. 0.2 M of transfected cells were seeded into a well in 24-well plates. After 48 h incubation, cells were harvested and lysed for RNA extraction. For primary cell transfection, UC-MSCs were electroporated at 1,600 V, 20 ms, and one pulse. 3.5 M cells were resuspended in 630 μL R buffer containing 29.2 μg dAsCpf1-VPR and same masses of total crRNA plasmids. Transfected cells were seeded on a 150 mm cell culture dish per group and incubated for 1 day after transfection in order to remove dead cells. After 24 h incubation, cells were detached, counted, and seeded with 35,000 cells/cm^2 seeding density in a well in 24-well plates.

RNA Extraction to qRT-PCR

Invitrogen TRIzol reagent was used for RNA extraction. On the day of RNA extraction, sample cells were harvested and treated with 200 μL of the TRIzol reagent. RNA extraction was performed through instructions from manufacturers. 1 μg (HEK293T) or 200 ng (UC-MSC) of isolated total RNAs were converted to cDNA in 20-μL reaction volumes by using an M-MLV cDNA synthesis kit from Enzynomics, according to the manufacturer's instructions. For quantitative RT-PCR (qRT-PCR), the cDNA samples were diluted five times, and 3 μL of the diluted cDNA (30 ng or 6 ng) was put in each well of a 96-well reaction plate (Applied Biosystems). TOPreal qPCR 2× PreMIX (SYBR green with low ROX, Enzynomics) served as fluorescence signals to detect target cDNA amounts. Relative mRNA expressions were determined by ΔΔCt method, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as endogenous control. Reactions were run on a StepOnePlus real-time PCR system (Applied Biosystems). All primers for qRT-PCR were listed in Table S1.

Targeted Deep Sequencing

The target sites of the *BMP4* gene were amplified by PCR using Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific). The PCR amplicons were subjected to paired-end read sequencing using an Illumina iSeq 100 instrument. The sequencing data were analyzed by a Cas-Analyzer. The frequencies of indels located 17 bp downstream of the protospacer adjacent motif (PAM) were considered to be the mutations induced by CRISPR-Cpf1.

In Vitro Osteogenic Differentiation

Osteogenic differentiation of UC-MSCs was achieved in OM. OM was composed of high-glucose DMEM, 10% FBS, 100 U/mL penicillin and streptomycin, 0.1 μM dexamethasone, 1 mM ascorbic acid-2-phosphate, and 10 mM glycerol-2-phosphate. All chemical components were from Sigma-Aldrich. One day after 24-well seeding, UC-MSC GM was changed to OM, and day counting started. rhBMP4$^+$ groups were cultured GM and OM supplemented 10 ng/mL rhBMP4 protein (314-BP, R&D Systems). 150 μL of suitable media for each group was added to a well every 2 days. Media containing rhBMP4 were used for only a week for maximum effect and discarded after a week. UC-MSCs were cultured in GM or OM for 21 days and assayed to confirm osteogenic differentiation.

ARS Staining

40 mM ARS solution from ScienCell was used for this assay. OM-cultured UC-MSCs were fixed by 4% paraformaldehyde for 15 min. To detect calcium deposits from osteo-differentiated cells, samples

were treated with 300 μL of ARS solution in a well for 45 min with shaking. Next, the samples were washed five times with distilled water for in order to completely remove the residual dyes. After taking pictures, quantification steps were performed according to the manufacturer's directions. The absorbance of ARS was read at 405 nm with a plate reader.

Statistical Analysis

Data is shown as mean ± SEM (standard error of the mean). qRT-PCR data combined triplicates from one independent experiment. Differentiation experiments were replicated in two independent experiments with triplicate samples. Two-tailed Student's t test was performed to explain whether two groups are from same population or not with statistical significance (p values). Asterisks were used to indicate statistical significance between two groups. p values of less than 0.05 were considered significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2019.12.010.

AUTHOR CONTRIBUTIONS

Conceptualization, D.K., J.K.H., and N.S.H.; UC-MSC Preparation, C.H.J.; Methodology, J.C., T.B., S.-H.P., N.B., D.K., J.K.H., and N.S.H.; Investigation, J.C., T.B., S.-H.P., and N.B.; Writing – Original Draft, J.C., T.B., and N.B.; Writing – Review & Editing, D.K., J.K.H., and N.S.H.; Resources, D.K., J.K.H., and N.S.H.; Supervision, D.K., J.K.H., and N.S.H.

CONFLICTS OF INTERESTS

The authors declare no competing interests.

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