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CRISPR-Cpf1 activation of endogenous BMP4 gene for osteogenic differentiation of umbilical cord-derived mesenchymal stem cells

CRISPR-Cpf1 발현증가시스템을 이용한 제대혈 유래 중간엽 줄기세포의 BMP4 내생 유전자의 발현 증가를 통한 골분화 증진

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ABSTRACT

CRISPR-Cpf1 activation of endogenous BMP4 gene for osteogenic differentiation of umbilical cord-derived mesenchymal stem cells

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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 hindrance 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, I 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, I developed a catalytically inactive AsCpf1 fused to

tripartite transcription activator domain (dAsCpf1-VPR) that can induce up-regulation of targeted gene expression in mammalian cells. I 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.

Keyword: CRISPR-Cpf1, CRISPR activation, Genetic Engineering, Gene Regulation, Osteogenic Differentiation, Umbilical Cord-derived Mesenchymal Stem Cell (UC-MSC), Bone Morphogenetic Protein 4 (BMP4), Endogenous Gene Activation

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1. 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 (DSB) at target loci, and the unwanted mutations in mammalian cells by CRISPR are major safety concerns of their applications for therapeutic purpose[2, 3]. In order to circumvent the DSB issues, several types of catalytically inactive form of a CRISPR molecule are utilized for regulating endogenous target gene expression without DNA mutations[4-7]. Scientists worldwide have repurposed this nuclease-free CRISPR protein for further applicability by fusing it to transcriptional activators or repressors: CRISPR activation (CRISPRa) or CRISPR interference (CRISPRi), respectively[8, 9]. These transformative techniques ensure target-specific transcriptional regulation on the basis of functional combination of base-pairing recognition from a CRISPR protein-RNA complex and gene expression regulation from a fused transcriptional effector[8, 9].

While utilizing catalytically dead form of CRISPR-SpCas9, a highly efficient and canonical Cas protein for CRISPR application, for activation of target gene expression was shown as a useful tool, some recent studies should that CRISPR-Cpf1, a more recently-discovered CRISPR effector[10], has even lower off-target effects compared to SpCas9, suggesting that Cpf1 could be applied for more precise CRISPRa[11-13]. The two major achievements in CRISPR-Cpf1 activators have been earned. In HEK293, a human embryonic kidney cell, Y Esther Tak, et al.[14] developed the chemical-inducible dLbCpf1 system fused to transcriptional activation domains (VPR) to enable multiplex gene perturbation via crRNA maturation, one of key utilities in Cpf1 differed from SpCas9. Yuchen Liu, et al.[15] constructed the programmable ligand-induced dAsCpf1 linking to transcriptional activating (VPR) or repressing domains (KRAB) with a crRNA revised for a riboswitch, working in human cells including Hela and HEK293T cells. Although those engineerings have not only created functional CRISPR-Cpf1 activation systems but also provided them with inducibility and RNA-processing, the results are limited in scope of human cell lines. It reveals that application of Cpf1-based CRISPR activation to

regenerative medicine has not been demonstrated, despite the potential usefulness of CRISPR-Cpf1 based CRISPRa as a biomedical tool[14, 15].

To assay the applicability of CRISPR-Cpf1 activation system, I 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[16]. Osteogenic differentiation of MSCs is one of the most important cell differentiations in regenerative medicine, contributing to bone formation and regeneration[17]. Previous studies have demonstrated that one of the key factors in osteogenic differentiation is bone morphogenetic protein 4 (BMP4), a member of signalling molecules in the TGF-β pathway that plays a significant role in activating SMAD signal pathway and upregulating osteoblast transcription factors[18, 19]. Consistently, introducing exogenous BMP4 gene was shown to facilitate bone regeneration[20]. Hence, in this study, I sought to apply CRISPR-Cpf1 activation to BMP4 gene for osteogenic differentiation of hMSCs without exogenous gene expression.

2. Materials and Methods

2.1. 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 was cut by EcoRI-HF® restriction enzyme (NEB, R3101) which is located in C-terminal 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 dAsCpf1-VPR construct.

2.2. Cell culture

HEK-293T cell line cultured on 0.1%-gelatin coated culture dishes was purchased from ATCC, and maintained in a growth medium which consists of high-glucose DMEM(Corning®), 10% FBS(Corning®), 100U/ml Penicillin and Streptomycin(Gibco™), and 1% Gibco® GlutaMAX™ supplement. For gelatin coating, 0.1% Gelatin on culture dishes incubated for 15min and was carefully removed. UC-MSC was extracted in Stem Cell & Orthopedic Research laboratory at SMG-SNU Boramae Medical Center and maintained in UC-MSC growth medium (Low-glucose DMEM(Corning®), 10% FBS(Corning®), 100U/ml Penicillin and Streptomycin (Gibco™), and 1% Gibco® GlutaMAX™ supplement) supplemented with 5ng/ml recombinant human FGF-basic(100-18B, PeproTech). At 70-90% confluency, cells were treated with 0.25% trypsin(Gibco™) and subcultured at certain seeding densities (HEK-293T: 40,000 cells/cm2, UC-MSC: 5,000 cells/cm2) 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.

2.3. Transfection for CRISPR activator

As for lipofection to deliver dAsCpf1-VPR and crRNAs targeting IL1RN gene, HEK-293T were seeded into 24-well plates at 70-80% confluency and were transfected using Lipofectamine 2000 (Invitrogen), guided by the manufacturer's instruction. Briefly, the cells were transfected with dAsCpf1 expression plasmids (500 ng) and crRNA expression plasmids (500 ng) per well. The transfected cells were cultured for 48 hours, and total RNA was isolated with Invitrogen™ TRIzol™ Reagent for subsequent analyses. All electroporation tests were performed in Neon® Transfection System using 100ul-tips. For BMP4 gene activation confirmation test by dAsCpf1-VPR and dSpCas9-VPR, HEK-293T were transfected by electroporation (1,100V, 20ms, two pulses). 0.8M cells were resuspended in 120ul Resuspension buffer containing 2ug dAsCpf1-VPR or dSpCas9-VPR and 2ug total crRNA or sgRNA plasmids. 0.2M of transfected cells were seeded into a well in 24-well plates. After 48h incubation, cells were harvested and lysed for RNA extraction. For primary cells transfection, UC-MSCs were electroporated at 1,600V, 20ms, and one pulse. 3.5M cells were resuspended in 630ul R buffer containing 29.2ug dAsCpf1-VPR and same masses of total crRNA plasmids. Transfected cells were seeded on a 150pi-dish per group and incubated for 1 day after transfection in order to remove died cells. After 24h incubation, cells were detached, counted, and seeded with 35,000 cells/cm2 seeding density in a well in 24-well plates.

2.4. RNA extraction to quantitative RT-PCR (qRT-PCR)

Invitrogen™ TRIzol™ Reagent was used for RNA extraction. On the day of RNA extraction, sample cells were harvested and treated with 200ul of the TRIzol reagent. RNA extraction was performed through instructions from manufacturers. 1ug (HEK-293T) or 200ng (UC-MSC) of isolated total RNAs were converted to cDNA in 20ul reaction volumes by using M-MLV cDNA Synthesis Kit from enzynomics, according to the manufacturer's instructions. For quantitative RT-PCR (qRT-PCR), cDNA samples were diluted by 5 times and 3ul of the diluted cDNA (30ng or 6ng) was put in each well

of a 96-well Reaction Plate (Applied Biosystems). TOPreal™ qPCR 2X 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 quantitative RT-PCR were listed on Table S1.

2.5. Targeted deep sequencing

The target sites of 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 wellumina iSeq 100 instrument. The sequencing data were analyzed by Cas-Analyzer. The frequencies of indels (insertions and deletions) located 17bp downstream of the PAM were considered to be the mutations induced by CRISPR-Cpf1.

2.6. In vitro osteogenic differentiation

Osteogenic differentiation of UC-MSCs was achieved in osteogenic differentiation medium (OM). OM was composed of high-glucose DMEM, 10% FBS, 100U/ml Penicillin and Streptomycin, 0.1μM dexamethasone, 1mM ascorbic acid-2-phosphate, and 10mM glycerol-2-phosphate. All chemical components were from Sigma-Aldrich. On one day after 24-well seeding, UC-MSC growth medium (GM) was changed to OM and day counting started. rhBMP4+ groups were cultured GM and OM supplemented 10ng/ml recombinant human BMP4 protein (rhBMP4, 314-BP, R&D Systems). 150ul of suitable mediums for each group was added to a well every two days. Mediums 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 21days and assayed to confirm osteogenic differentiation.

2.7. Alizarin Red S staining

40mM Alizarin Red S (ARS) solution from ScienCell was used for this assay. OM-cultured UC-MSCs were fixed by 4% paraformaldehyde for 15min. To detect calcium deposits from osteo-differentiated cells, samples were treated with 300ul of ARS solution into a well for 45min with shaking. Over 5 times of distilled water washing were followed to completely remove additional dyes. After taking pictures, quantification steps were performed according to the manufacturer's directions. The absorbance of ARS was read at 405nm with a plate reader.

2.8. Statistical analysis

Data is shown as mean ± s.e.d. RT-qPCR data combined triplicates from one independent experiment. Differentiation experiments were replicated in two independent experiments with triplicate samples. Two-tailed students 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 (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

3. Results

3.1. Application of CRISPR-Cpf1 for targeted gene activation

I 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, I 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-activity10. Then I utilized dAsCpf1 as a recruiting factor for a tri-partite transcriptional activator consisting of VP64, p65, and Rta (VPR), which was shown to achieve high-level of gene activation6. To this end, I fused VPR tripartite activator on the C-terminal of dAsCpf1 and named the fusion construct dAsCpf1-VPR (**Figure 1a and b**). I next sought to assay the activity of both D908A and E993A dAsCpf1-VPR for inducing activation of endogenous genes. To this end, I designed crRNAs to localize dAsCpf1-VPR within the promoter region of interleukin-1 receptor antagonist gene (IL1RN) (**Figure 2a**)6, 14. I searched genome data on The UCSC Genome Browser (http://genome.ucsc.edu/). For IL1RN endogenous gene activation by dAsCpf1-VPR, I utilized the genomic sequence of IL1RN (ENSG00000136689) and selected the binding regions for crRNAs were between 1 and 300bp upstream of the transcriptional start site (TSS). I designed four crRNAs targeting IL1RN gene to activate the gene expression. I assayed the efficiency of dAsCpf1-VPR mediated gene activation of endogenous IL1RN in human cell line, by transfecting dAsCpf1-VPR and crRNAs targeting IL1RN gene into human embryonic kidney (HEK293T) cells (**Figure 2b**). CRISPR activation of dAsCpf1(E993A)-VPR and dAsCpf1(D908A)-VPR resulted in increased mRNA expression levels of IL1RN gene by ~500 folds and ~1700 folds, respectively (**Figure 2b**). Therefore, I selected dAsCpf1(D908A)-VPR (dAsCpf1-VPR, hereafter) in the subsequent gene activation experiments.

3.2. CRISPR-Cpf1 activator induces greater increase of endogenous BMP4 gene expression level compared to CRISPR-Cas9

I 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, I selected BMP4 (ENSG00000125378) gene variant and designed two crRNAs targeting ~300bp upstream of the TSS on BMP4 and other two crRNAs targeting a region between exon 1 and 2 (**Figure 3a**). The latter region is a putative promoter region of a BMP4 transcript variant and also has higher expression level of H3K27Ac, one of well-known promoter markers. I also sought to compare the efficiency of activation by dAsCpf1-VPR to CRISPR-Cas9 based activation, for which I prepared dSpCas9-VPR activation by designing four sgRNAs targeting the promoter region of endogenous BMP4 gene[6].

I first assessed the CRISPR activation of BMP4 gene expression by transfection in HEK-293T cells (**Figure 3b**). I 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 exon 1 and 2 (crRNA 1&2) resulted in insignificant gene activation. Interestingly, both crRNA 3&4 were needed to activate BMP4 and sole crRNA were not able to induce activation of the gene. It means that there could not be steric hindrance between those dual crRNAs but synergistic effect in the process of endogenous gene activation by dAsCpf1-VPR (**Figure 4a**). To confirm catalytically inactivity of dAsCpf1-VPR, I tested indel rate of dAsCpf1-VPR at BMP4 targeted loci by deep sequencing, compared to Wild-type AsCpf1. No indel induction from dAsCpf1-VPR should that the endonuclease-activity of dAsCpf1-VPR was completely removed (**Figure 4b**). Notably, the gene activation of BMP4 by dAsCpf1-VPR, with crRNA 3&4, was more efficient than dSpCas9-VPR, which should only 3-4 fold increase (**Figure 3 c,d**).

Next, I assessed if dAsCpf1-VPR could also increase the levels of endogenous BMP4 levels in human MSCs derived from umbilical-cord (UC-MSCs). To this end, I transfected UC-MSC that were maintained in growth medium, with dAsCpf1-VPR and

sgRNA to activate the endogenous BMP4 gene (**Figure 5a and Figure 6a**). I 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 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) (**Figure 5 b,c and Figure 6b**). Based on the above results, I hypothesized that increasing the expression level of endogenous BMP4 gene in UC-MSC by dAsCpf1-VPR may enhance the cellular differentiation from mesenchymal stem cells to osteoblasts.

3.3. Activation of endogenous BMP4 gene by CRISPR-Cpf1 facilitates osteogenic differentiation

Next, I sought to test whether dAsCpf1-VPR mediated activation of endogenous BMP4 gene levels could facilitate the cell differentiation (**Figure 5 d,e and Figure 7 a,b**). For quantitative analyses, I conducted in-vitro osteogenic differentiation assay. Briefly, I performed BMP4 gene activation of UC-MSC by dAsCpf1-VPR (crRNA+), and then cultured the UC-MSC for 3 weeks in osteogenic medium (OM). The progress of osteogenic differentiation was then assessed by Alizarin Red S (ARS) staining. I observed that, after culturing UC-MSC 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. I also sought to assess whether increment of BMP4 alone could enhance osteogenic differentiation. To this end, I cultured UC-MSC 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-MSC in OM. Together, the data suggested that dAsCpf1-VPR mediates activation of the endogenous BMP4 gene led to increased BMP4 protein levels, that in turn facilitated the commitment of UC-MSC to osteogenic differentiation without other external gene products. I also found that

transfection of dAsCpf1-VPR without crRNA (crRNA-) did not results in increased ARS staining, indicating that the facilitation of osteogenic differentiation was not triggered by non-specific gene activation. In 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.

4. Discussion

To my 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 is incompatible with CRISPR-Cas9, Cpf1 can be an attractive alternative of Cas9 for targeted gene activation of endogenous genes. In addition, I found that dAsCpf1-VPR mediated activation of endogenous BMP4 gene was even more efficient than the conventional CRISPR activation by dSpCas9-VPR. I 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-MSC cells in growth medium (GM) efficiently suppressed osteogenic differentiation. The results suggested that the differentiation of UC-MSC to osteoblasts are regulated by BMP4 levels and the culture medium. I anticipate that the two-layered regulation could be utilized versatile control of osteogenic differentiation in cell engineering and gene therapy. However, it is important to note that generalization of this CRISPR-Cpf1 activation system to the MSCs derived from other than umbilical cords might not be efficient. This is because only 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.

With the advancement of molecular biology tools, the application of gene therapy to stem cells has been gaining more importance in regenerative medicine[21, 22]. Researchers have been working on cell-fate engineering to enhance the functionality of stem cells[23, 24]. 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[20, 25-27]. The development of CRISPR activation enabled increasing the expression levels of endogenous target genes without changing the DNA sequences or adding introducing exogenous genes into cells[4, 28, 29]. The CRISPR activation 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. CRISPR activation 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)[30]. I anticipate

that the advantages of CRISPR activation can be utilized for precise and safe biomedical applications for cellular and in vivo systems.

5. Conclusion

In summary, I should that CRISPR-Cpf1 activation of 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. I anticipate that the CRISPR-Cpf1 method be further applied for regenerative medicine including development of therapeutics for bone-related disorders.

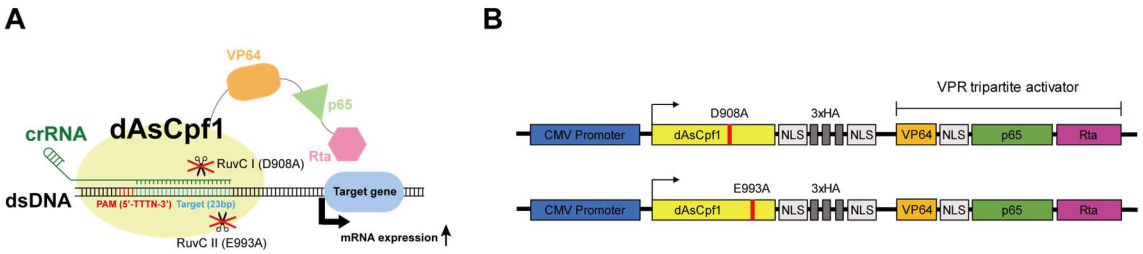

Figure 1. A schematic representation of the CRISPR activator (dAsCpf1-VPR) construct. (a) A model of dAsCpf1-VPR system on the target genomic location. The ribonucleoprotein complex, comprised of catalytically inactive dAsCpf1 fused to the VP64-p65-Rta transcriptional activators (VPR) and crRNA, recognizes 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.

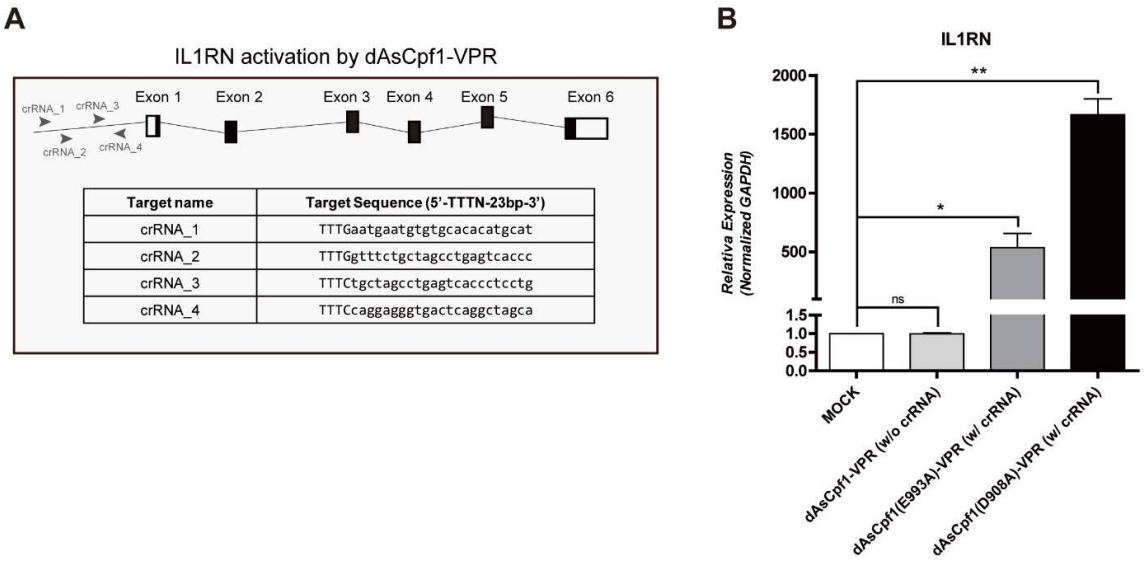

Figure 2. CRISPR activation of endogenous IL1RN gene by dAsCpf1-VPR in HEK-293T. (a) A diagram of positions and sequences of crRNAs targeting IL1RN genomic locus. (b) CRISPR activation of IL1RN gene by dAsCpf1-VPR. Shown are the changes of mRNA levels of IL1RN in HEK-293T assayed by RT-qPCR, 48 hours after transfection of dAsCpf1-VPR and crRNAs targeting IL1RN. Data shown are combined triplicate qRT-PCR results (error bars indicate the s.e.m.). P-values less than 0.05 were considered significant (*P < 0.05, **P < 0.01) and no significance between groups was indicated as ns.

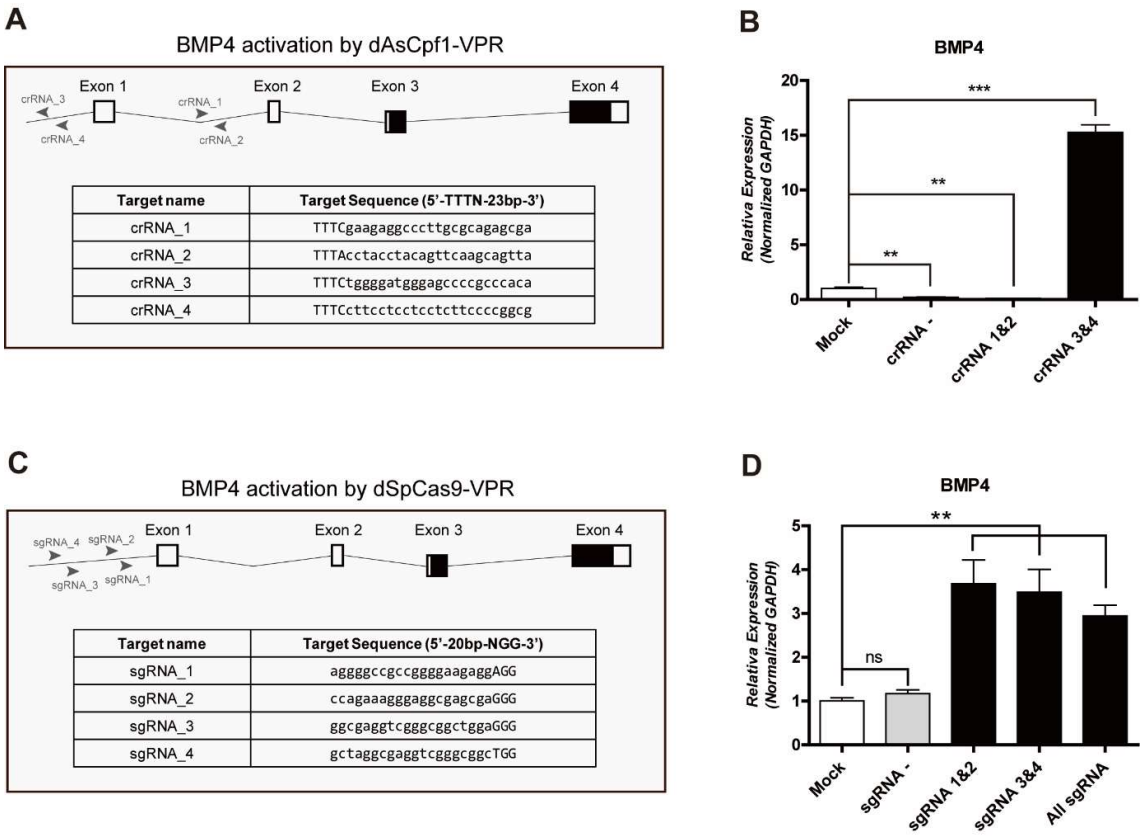

Figure 3. CRISPR activation of endogenous BMP4 gene in HEK-293T by dAsCpf1-VPR and dSpCas9-VPR. (a) A diagram of positions and sequences of four crRNAs targeting different locations within BMP4 gene. (b) The changes in mRNA levels of endogenous BMP4 gene by dAsCpf1-VPR and four different crRNAs. (c) A diagram of positions and sequences of sgRNAs targeting BMP4 gene. All sgRNAs are located on proximal region of the promoter of BMP4 gene. (d) A qRT-PCR result confirming BMP4 gene activation depend on combinations of sgRNAs' positions. Mock, no transfection, crRNA-, transfection of only dAsCpf1-VPR without crRNAs, crRNA 1&2, transfection of dAsCpf1-VPR with crRNA 1&2 targeting *BMP4* gene, crRNA 3&4, transfection of dAsCpf1-VPR with crRNA 3&4 targeting *BMP4* gene, sgRNA-, transfection of only dSpCas9-VPR without sgRNAs, sgRNA 1&2, transfection of dSpCas9-VPR with sgRNA 1&2 targeting *BMP4* gene, sgRNA 3&4, transfection of dSpCas9-VPR with sgRNA 3&4 targeting *BMP4* gene, All sgRNA, transfection of dSpCas9-VPR with All sgRNAs targeting *BMP4* gene. Data shown are combined triplicate qRT-PCR results (error bars indicate the s.e.m.). P-values less than 0.05 were considered significant (**P < 0.01, ***P < 0.001).

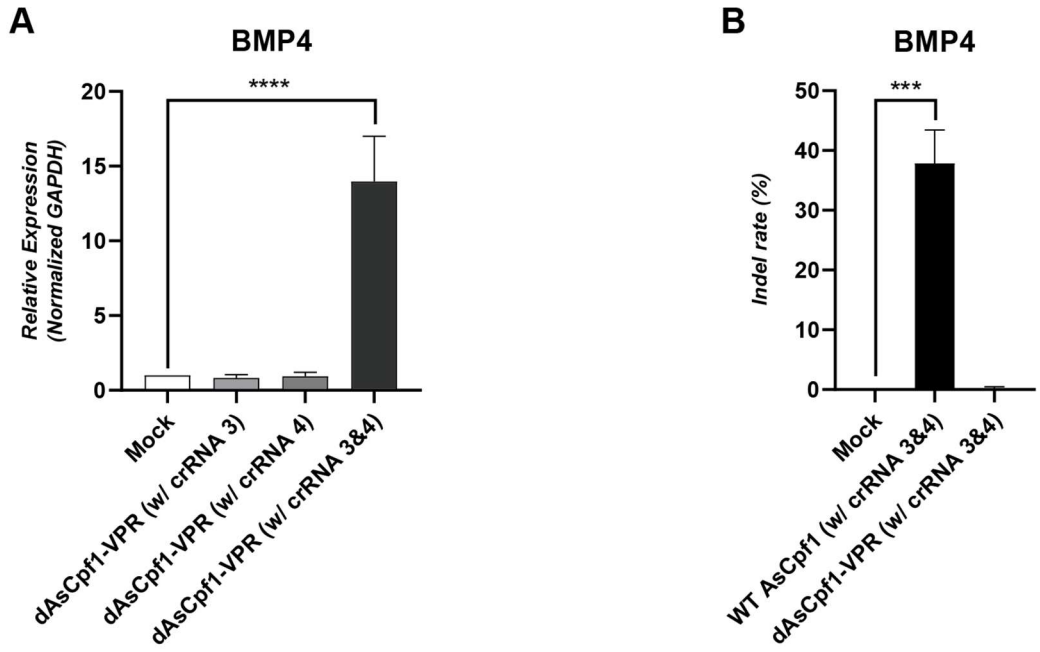

Figure 4. Comparison activation efficiency of endogenous BMP4 gene using single crRNA and dual crRNAs and analysis of indel efficiency of WT AsCpf1 and dAsCpf1-VPR. (a) After I selected the combination of crRNA 3&4, BMP4 gene activation efficiency using each crRNA and dual crRNAs was compared. There is no difference between Mock and dAsCpf1-VPR with each crRNA. On the other hand, dAsCpf1-VPR with crRNA 3&4 should ~14 fold increase for BMP4 gene expression. So, using dual crRNAs not single crRNA for BMP4 resulted in significant gene activation. (b) Whether dAsCpf1-VPR with D908A substitution is inactivated form was checked. WT AsCpf1 with crRNA 3&4 induced ~38% indels on BMP4 endogenous target site. However, dAsCpf1-VPR with crRNA 3&4 was no effect on target site. Therefore, this dAsCpf1-VPR construct is perfectly inactivated form which never induce DSBs on target site.

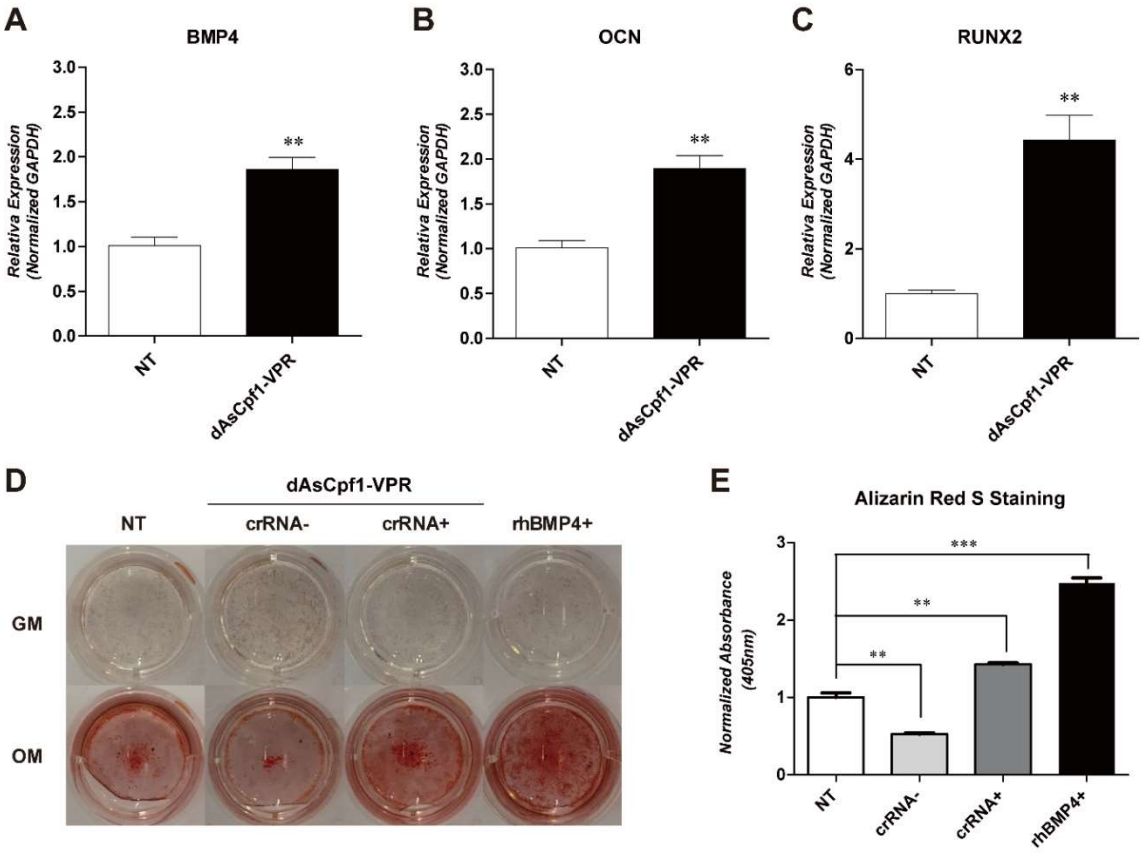

Figure 5. dAsCpf1-VPR mediated activation of endogenous *BMP4* gene expression in UC-MSC facilitates osteogenic differentiation. (a) Shown are the increase of mRNA levels of endogenous *BMP4* in UC-MSC by dAsCpf1-VPR and crRNA 3&4 targeting *BMP4*, assayed by qRT-PCR. (b,c) Concomitant changes of mRNA levels of osteogenic genes, *OCN* and *RUNX2*, assessed by RT-qPCR. (d) Alizarin red S staining of UC-MSC cultured in growth medium (GM) or osteogenic differentiation medium (OM). (e) Intensities of ARS staining of UC-MSC cultured in OM. For (d and e) 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 (10ng/ml). Data shown are combined triplicate RT-qPCR results (error bars indicate the s.e.m.). P-values less than 0.05 were considered significant (**P < 0.01, ***P < 0.001).

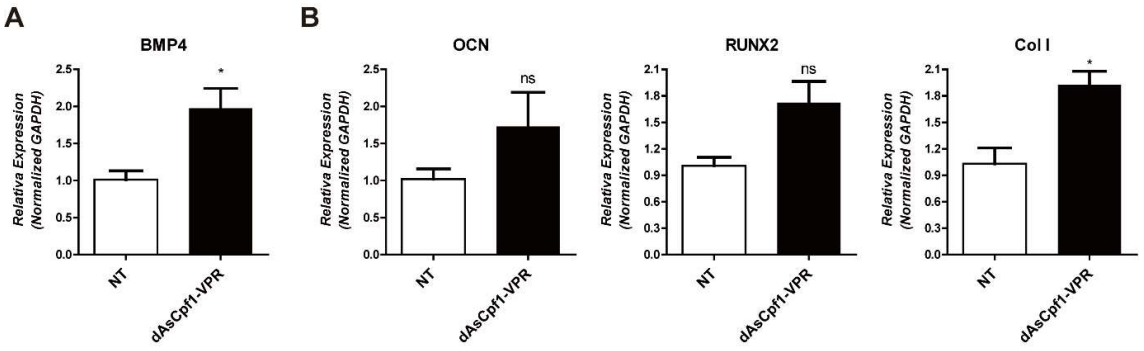

Figure 6. Endogenous gene activation of BMP4 and osteogenic genes in UC-MSC cultured in OM by dAsCpf1-VPR targeting BMP4 gene. (a) A qRT-PCR result of BMP4 gene activation in UC-MSC cultured in OM using dAsCpf1-VPR and crRNA 3&4 targeting BMP4 gene at day 5 after transfection. (b) qRT-PCR results showing activation tendency of osteogenic genes (OCN, RUNX2, and COL I) in same condition of (a) with no statistical significance except for COL I.

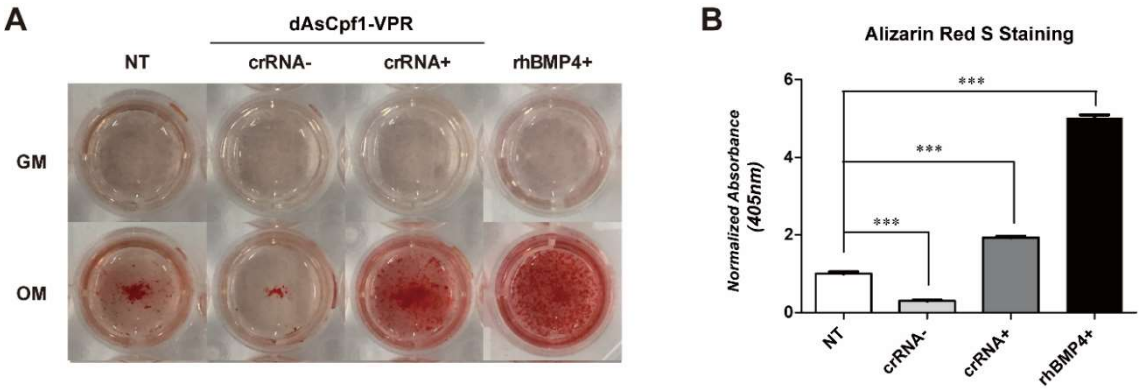

Figure 7. A reproducible test of Figure 5-D identifying boosting effect of osteogenic differentiation by the dAsCpf1-VPR activation system. (a) Representative well images of Alizarin Red S (ARS) staining results of UC-MSC cultured in UC-MSC growth medium (GM) or OM for 3 weeks. (b) Quantification of staining samples shown in (a) in OM. Experiment conditions were the same as the Figure 2.

Target gene	Primer	Sequence (5' to 3')
BMP4	Forward	GACTTCGAGGCGACACTTCT
	Reverse	ATGACGGCACTCTTGCTAGG
COL I	Forward	ATCCAGCTGACCTCCCTGCG
	Reverse	TCGAAGCCGAATTCCTGGTCT
GAPDH	Forward	GGACTCATGACCACAGTCCA
	Reverse	TCAGCTCAGGGATGACCTTG
IL1RN	Forward	GGAATCCATGGAGGGAAGAT
	Reverse	TGTTCTCGCTCAGGTCAGTG
OCN	Forward	CCTTTGTGTCCAAGCAGGAG
	Reverse	CAGCCATTGATACAGGTAGCG
RUNX2	Forward	TGAGCTCCGGAATGCCTCTG
	Reverse	TGTCTGTGCCTTCTGGGTTCC

Table 1. All primer sequences for qRT-PCR performed in this study. All primers were designed to target human genes. Tm values of them were ranging from 57℃ to 60℃.

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국문초록

CRISPR-Cpf1 발현증가시스템을 이용한 제대혈 유래 중간엽 줄기세포의 BMP4 내생 유전자의 발현 증가를 통한 골분화 증진

크리스퍼(CRISPR) 시스템은 강력한 게놈 편집 기술(Genome Editing)로서 생명 및 의료 분야에 폭넓게 활용되고 있다. 하지만, CRISPR의 비특이적 효과로 인한 안전문제는 재생의학(Regenerative Medicine)에 본 기술을 활용하는 데에 있어 주요한 한계이다. 전통적인 CRISPR 시스템은 원하지 않는 게놈 위치에 DNA 절단(double strand DNA breaks, DSBs)을 유도하여 예측이 어려운 돌연변이를 유도할 잠재적 위험을 가지고 있다. 본 연구에서는 최근에 보고된 CRISPR-Cpf1을 활용해 DNA 절단 없이 유전자의 선택적 발현 증진 시스템을 제작해 안전위험을 줄이고 인간 제대혈 유래 중간엽 줄기세포(human umbilical cord-derived mesenchymal stem cells, UC-MSCs)의 골분화를 향상시킬 수 있음을 보였다. 구체적으로, 본 연구에서는 AsCpf1의 핵산가수분해 기능을 비활성화시킨 뒤 세 개의 전사활성인자(VPR)를 결합시켜 동물세포에서 선택적인 내생 유전자 활성을 유도하는 dAsCpf1-VPR 시스템을 제작했다. 본 연

구에서는 CRISPR-dAsCpf1-VPR 전사 활성 시스템을 통해 UC-MSC의 내생 BMP4 유전자의 발현을 증가시켜 골분화를 향상하였다. 본 결과는 CRISPR-Cpf1 전사 활성 시스템이 골재생과 재생의학을 위한 새로운 방법을 제시한다고 할 수 있다.

주요어: CRISPR-Cpf1 시스템, 크리스퍼 유전자 활성 시스템, 게놈 공학, 유전자 활성 조절, 골분화, 제대혈 유래 중간엽 줄기세포, BMP4, 내생 유전자 발현 증진