Genetic correction of adipose tissue-derived mesenchymal stem cells mediated by TALEN targeting the GDF5 gene

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Abstract. Stem cells and other associated cell types may be a potential alternative to treat various genetic disorders that currently do not benefit from traditional approaches. Functional recovery of cells could be induced via directional differentiation or genetic manipulation. In this study, mesenchymal stem cells (MSCs) were obtained from a patient with osteoarthritis (OA) carrying a functional single-nucleotide polymorphism (SNP rs143383, C/T transition) within the 5'-UTR of growth and differentiation factor 5 (GDF5) gene. The SNP causes GDF5 expression to be reduced and thus increases OA susceptibility. Aiming to correct the dysfunctional gene, a pair of transcription activator-like effector nucleases (TALENs) were designed to cleave the DNA around the mutated locus, coupled with a short single stranded DNA complementary to the cleavage site. Following *in vitro* cell colony formation and selection, two genetically corrected MSC colonies were identified out of a total of 142. These MSCs were induced and differentiated into chondrocytes. As a result, genetically corrected chondrocytes exhibited normal morphology and lower levels of apoptosis compared with cells carrying the SNP. In cultured cells, the secretion of matrix metalloproteinases was suppressed and TIMP metallopeptidase inhibitor 1 was increased by correction of the mutation. Furthermore, the expression of GDF5 target genes, cell vitality-associated genes and extracellular matrix degrading genes were returned to normal levels in corrected cells compared with mutation-carrying cells, indicating the functional recovery of these corrected chondrocytes. The present study demonstrated that TALEN-mediated genetic correction can be used to edit genes in adipose-derived MSCs from patients with OA and may have clinical potential.

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Introduction

Osteoarthritis (OA) is the most prevalent degenerative disease in joints characterized by progressive loss of articular cartilage and a major cause of disability. OA has been estimated to impact 5% of adults world wide (1). The number of sufferers is believed to being increasing within the aging population with longer working lives (2). Despite its high prevalence, the underlying molecular mechanisms are still not fully understood. OA is a multifactorial disease caused by both environmental factors and genetic predisposition. It was previously reported that the influence of genetic factors on OA ranged from 39-65% (3). It has been reported that multiple genes are involved in OA (4,5), however, few results have been firmly replicated across multiple populations. Accumulating evidence demonstrates that multiple genes contribute to OA susceptibility, such as asporin (ASPN), bone morphogenetic protein 5 (BMP5) and growth and differentiation factor 5 (GDF5) (6).

The functional integrity of the joint is maintained by a delicate balance between degradation and synthesis of the cartilage extracellular matrix (ECM) through mechanisms controlled by chondrocytes (7). Anabolic and catabolic factors, including dysregulation of the main cartilage ECM degrading enzymes matrix metalloproteinase-13 (MMP-13) and aggrecanases, have crucial roles in the molecular mechanisms involved (8). Studies revealed that GDF5, a member of the BMP family and the transforming growth factor-β superfamily, reduced MMP-13 expression in human chondrocytes and acted extracellularly to promote the development, maintenance and repair of synovial joint tissues, particularly bone and cartilage (9,10). In addition, a causative single-nucleotide polymorphism (SNP) rs143383 located in the 5'-untraslated region (5'-UTR) of GDF5 was demonstrated to affect transcription factor binding, and consequently inactivated GDF5 expression (11). This research indicated that mutant GDF5 gene could be exploited to overcome the OA genetic deficit.

Stem cell-based tissue engineering for cartilage regeneration is a promising alternative pathway in the regeneration of damaged or diseased articular cartilage tissues or both. However, the regeneration of articular cartilage tissues remains a significant clinical challenge. Current medical treatments are effective at reducing pain but ineffective at reversing the course of musculoskeletal degeneration often caused by the low cellularity and vascularity in these tissues. Cartilage tissue engineering using human mesenchymal stem cells (hMSCs) has demonstrated the potential to enhance cartilage healing (12).

Several novel genome-editing technologies have emerged from which the four most studied methods are zinc-finger nucleases (ZFNs) (13), transcription activator-like effector nucleases (TALENs) (14), clustered regularly interspaced short palindromic repeat (CRISPR) (15) and NgAgo (16). Each technique has demonstrated highly efficient and locus-specific genome editing in numerous species. Furthermore, these genome-editing tools have been successfully applied in the correction of genetic mutation causing hereditary tyrosinemia (17).

In this study, MSCs were obtained from a patient with OA carrying a homozygous T allele of polymorphism rs143383 in the *GDF5* gene. The mutated locus was genetically corrected using a pair of TALENs and a short single stranded DNA (ssDNA) in cells *in vitro*. Following differentiation, chondrocytes derived from the modified MSC colony exhibited normal cell morphology and viability. Enzymes responsible for ECM metabolism and the expression of associated genes were returned to normal levels, suggesting the functional recovery of these corrected chondrocytes.

Materials and methods

Isolation and culture of adipose tissue-derived MSCs. The Ethics Committee of Southern Medical University (Guangzhou, China) approved this study and written informed consents were obtained from the patient/volunteer. Adipose tissue was obtained from discharged fat tissue from a male 23-year-old volunteer with OA carrying a homozygous T allele of polymorphism rs143383 in the *GDF5* gene confirmed by Sanger sequencing. Additionally, adipose tissue from a 27-year-old healthy volunteer was collected as the control and his genotype was confirmed by sequencing as well. The methods for isolating and culturing stromal stem cells from human adipose tissue was performed according to previously described procedures with minor modifications (18). Briefly, the tissue was digested with collagenase A type I (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C for 6 h. Cells were collected and then plated at a density of ~1x10^6 cells/dish in 5 ml of low-glucose Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with fetal bovine serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), and maintained at 37˚C in a maximum humidity atmosphere containing 5% CO_2.

Design and construction of TALENs vector. Candidate TALENs were identified using the online tool TAL Effector Nucleotide Targeter 2.0 (https://tale-nt.cac.cornell.edu/node/add/talen) (19). Construction of a TALEN pair targeting rs143383 is shown in Fig. 1. RVD modules were assembled into the pTAL3 vector (Addgene, Cambridge, MA,USA) using the Golden Gate method (20), and were subsequently cloned into the modified backbone-vector pTAL-3.1 (Addgene), in which a cassette encoding a 152aa TALEN-terminal and a 63aa C-terminal segment fused to an enhanced *Fok*I nuclease flanking either side of the insert (21). Additionally, a ssDNA of 160 nt in length spanning the target site was chemically synthesized, in which a C was incorporated at rs143383.

Electrotransfection of MSCs. MSCs were trypsinized, centrifuged at 300 x g for 10 min at room temperature, resuspended in modified minimal essential medium (MEM; ref. 11380037; Invitrogen; Thermo Fisher Scientific, Inc.). Electrotransfection was performed according to the previous description with some modifications (22). Briefly, ~1x10^6 cells in 50 μl S-MEM containing 10 μg TALEN mRNA and 100 ng ssDNA were then transferred to a 4 mm electroporation cuvette, and electroporated using Gene Pulser Xcell electroporation system (both from Bio-Rad Laboratories, Inc., Hercules, CA, USA). The electroporation was performed by applying a pulse magnitude ranging from 80-120 V at a 1 Hz repetition frequency. After electrotransfection, the cells were incubated at room temperature for 5 min and transferred to the tissue culture dishes with 10 ml culture medium.

Mutation identification of the cell colonies. Seven to ten days after electroporation DNA was extracted from the colonies by the phenol-chloroform method. Polymerase chain reaction (PCR) was conducted on the extracted DNA using rTaq polymerase (Takara Biotechnology Co., Ltd., Dalian, China) and primers spanning the target site, generating a 443 bp amplicon (GDF5-forward, 5'-TGTGTGTGTGTGTGAAGTA-3' and GDF-reverse, 5'-TCAGCGGCTGGCCAGAGG-3'; Table I) under the following thermal conditions: 5 min at 95˚C followed by 35 cycles of 30 sec at 95˚C, 30 sec at 95˚C, 30 sec at 72˚C, and a final extension of 3 min at 72˚C, generating a 443-bp amplicon. Genotyping was executed by T7 endonuclease I (T7EI) assay as described by Sakurai *et al* (23) with minor modifications (23). Initially, 10 μl PCR product from the tested colonies was mixed with 10 μl PCR product from the healthy control. Subsequently, a mix containing 10 μl PCR product and 2 μl of 10X NEB buffer 2 (New England BioLabs, Inc., Ipswich, MA, USA) was incubated at 95˚C for 10 min and then incubated at room temperature for ~30 min. Following the annealing reaction, 0.5 μl T7EI (2.5 U/μl; New England BioLabs, Inc.) was added to the sample and the mixture was incubated at 37˚C for 1 h. Finally, the digested product was electrophoresed on a 2.0% agarose gel. Colonies producing cleaved bands at ~192 and 251 bp were subjected to Sanger sequencing to confirm the editing.

MSC differentiation into chondrocytes. Chondrogenic differentiation of MSCs was induced using MSC chondrogenic differentiation medium (PromoCell GmbH, Heidelberg, Germany) according to manufacturer's instructions. MSCs (1x10^5 per 24-well) were plated and incubated for 2 h in MSC growth medium. The medium was changed to MSC chondrogenic differentiation medium (PromoCell) to induce chondrogenesis. The medium was changed every third day and chondrocytes were induced (24). Three chondrocyte lines were

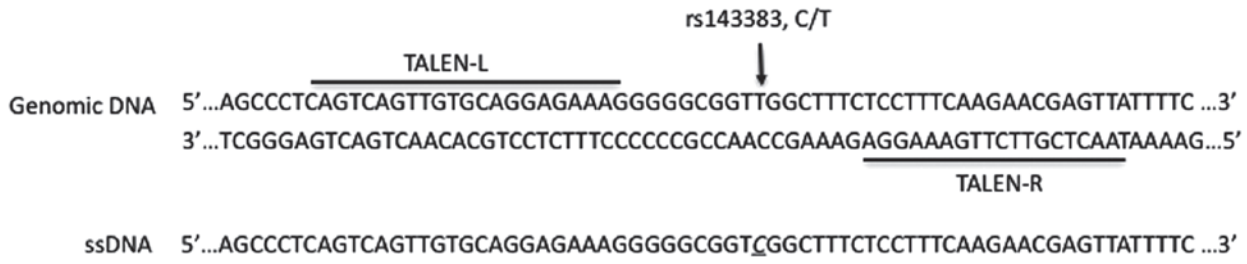

Figure 1. Schematic representation of the designed TALENs and binding site, and short ssDNA in the human growth and differentiation factor 5 gene. TALENs, transcription activator-like effector nucleases; ssDNA, single stranded DNA.

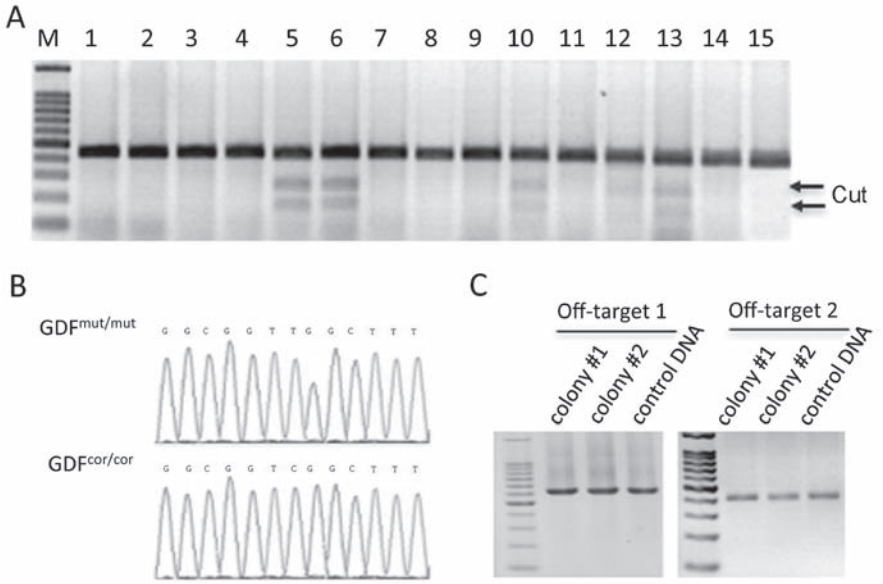

Figure 2. Identification TALEN-induced modifications in the GDF5 gene. (A) Genotyping of mesenchymal stem cell colonies T7EI analysis. The arrowheads indicate the cleaved bands. (B) Sanger sequencing of the colonies of mutant and corrected GDF5 genes. (C) T7EI analysis of the two potential off-target sites hit by TALENs. TALENs, transcription activator-like effector nucleases; GDF5, growth and differentiation factor 5; T7EI, T7 endonuclease I; M, marker; mut, mutation; cor, corrected mutation.

obtained: $GDF5^{con/con}$, chondrocytes from the healthy subject with wildtype GDF5 gene; $GDF5^{mut/mut}$, chondrocytes from the patient with OA with mutant GDF5 gene; $GDF5^{cor/cor}$, chondrocytes from the patient with OA with genetically corrected GDF5 gene.

Cell proliferation assay. The methyl thiazolyl tetrazolium (MTT; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) colorimetric assay was used to detect cell proliferation ability. Formazan was dissolved in dimethyl sulphoxide (DMSO) in the MTT assay. The cells were seeded in 96-well plates at a density of $1x10^3$ cells/well. The cells were trypsinized after 1-12 days of seeding, and suspended in PBS. Cell number was counted every two days for 12 days by measuring the absorbance at 490 nm. The assay was performed in triplicate.

Assessment of apoptosis. Chondrocytes were stained with an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (R&D Systems, Inc., Minneapolis, MN, USA) to determine whether cells are undergoing apoptosis. Propidium iodide (PI) staining was used as a control to differentiate cells undergoing necrosis. Chondrocytes were seeded in tissue culture slides and allowed to attach for 24 h. Then the cells were resuspended in 500 μl binding buffer, 5 μl Annexin V-FITC and 5 μl PI were added and incubated for 5 min at 37˚C in the dark. Flow cytometry analysis was performed to evaluate the apoptosis (25). Chondrocytes were washed with PBS and stained with Annexin V-FITC and PI according to the manufacturer's instruction. Data was processed with FlowJo software version 7.6.1.

Detection of MMPs in culture medium. An aliquot of 50 μl medium supernatant was collected and human MMP-1, MMP-13 and TIMP metallopeptidase inhibitor 1 (TIMP1) ELISA kits (cat. nos. DY901B, DM1300 and DTM100, respectively; R&D Systems, Inc.) were used to quantify the MMPs concentration strictly in accordance with the manufacturer's instructions. Briefly, samples and standards were diluted in 96-well plates and 50 μl conjugate solution was added into each well. After an incubation of 2 h at room temperature, the wells were washed three times. Subsequently, 200 μl substrate solution was added. The plate was incubated for 10-15 min and the color development was stopped. Absorbance of each well was determined at 450 nm in a microplate reader (BioTeke Corporation, Beijing, China). A standard curve was

Table I. Primers sequences.

Gene name	Forward (5'-3')	Reverse (5'-3')	Tm (˚C)	Product (bp)
GDF5	TGTGTGTGTGTGTGTGAAGTA	TCAGCGGCTGGCCAGAGG	60	443
Off-target 1	GCAAGTACTTACTCCAGA	CTACCAGCCTGCCAGCAGT	60	613
Off-target 2	TCCTCACGTGTTCATTTCCTCA	CCCATCGGTACCTATTAGGA	59	421
CD73	CGCAACAATGGCACAATTAC	CAGGTTTTCGGGAAAGATC	60	196
COL2A1	GGGAGTAATGCAAGGACCAA	ATCATCACCAGGCTTTCCAG	62	175
ACAN	GACATCAGGGTGGCGACTCT	GGGTTGAGGTATCAGAGGT	60	152
SOX9	CTGACCCAGTACCCCTTTGA	CAGCTGGACTGGTTGTCTCA	62	213
MMP-13	GCACTGAAGCCAGGTCT	GGGCCTTTTCTCCAGGTAAC	60	177
TIMP1	CCTGCAAGACTATCGACATGGA	CCTCAGCAGACGCAGCTCTG	60	283
IL-1	CCCAGTGAAGATGCAGGT	CAGCCTGAGAGGGTCTTG	60	183
TNF-α	TCTTGGGACTGATGCTGGTG	CATTTCCACGATTTCCCAGA	58	155
TRAIL	ATCCACGAAACTACCTTCAA	TCTTGATCTTCATTGTGCTG	60	166
FZD5	TGGGGGTACTGTGGAAATGC	CCTTCCATTGCCCACTCTGT	60	162
DKK1	AACTTTGCTTCCCAGATGTCC	GCCTCGGTGTCCCTTCATT	58	233
CTNNB1	CCAGGACGGTCATTTACGAG	CGATGGTCTGGGTTCAGGTT	60	217
GAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG	60	185

GDF5, growth and differentiation factor 5; CD73, 5'-nucleotidase ecto; COL2A1, collagen type II α 1 chain; ACAN, aggrecan; SOX9, SRY-box 9; MMP-13, matrix metalloproteinase-13; TIMP1, TIMP metallopeptidase inhibitor 1; IL-1, interleukin-1; TNF-α, tumor necrosis factor-α; TRAIL, TNF superfamily member 10; FZD5, frizzled class receptor 5; DKK1, dickkopf WNT signaling pathway inhibitor 1; CTNNB1, catenin β 1.

constructed by plotting the absorbance of standards against the known concentration and the sample content was deduced from the standard curve.

Transcription analysis. Total RNA was extracted from the three cell lines $GDF5^{con/con}$, $GDF5^{mut/mut}$ and $GDF5^{cor/cor}$ chondrocytes using TRIzol reagent according to manufacturer's instructions. Subsequently, 1 μg RNA was reverse transcribed into cDNA using a First-Strand cDNA synthesis kit (Takara Biotechnology Co., Ltd.). cDNA (1 μl) was used for quantification on an ABI 7500 real-time machine (Thermo Fisher Scientific, Inc.) using SYBR-Green (Takara Biotechnology Co., Ltd.) and gene expression primer pairs (Table I) for the target genes of GDF5, cell viability-associated genes and genes in Wnt/β-catenin signaling. The thermocycling parameters were 5 min at 95˚C followed by 40 cycles of 30 sec at 95˚C, 30 sec at annealing temperature of each primer pair (Table I), 30 sec at 72˚C, and a final extension of 3 min at 72˚C. GAPDH was employed as internal control and gene expression was analyzed by the the comparative ΔΔCt method (26).

Statistical analysis. All the data were presented as the mean ± standard deviation. ANOVA analysis and Dunnett test were performed to evaluate the statistical significance between two means of equal variance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Generation of corrected MSCs. MSCs were collected from the patient with OA and the healthy volunteer, then cultured in cell plates. When the cells reached a confluence of ~70%, they were transfected with 10 μg TALEN mRNA and 100 ng ssDNA. After 2 weeks, cell colonies formed. T7EI assay was performed to screen for cells with the mutation induced by TALENs (Fig. 2A). In all, 142 colonies were tested. Results here showed that 34 colonies were genetically edited: 26 colonies were modified to heterozygous (one allele was indeled and the other was unchanged), and 8 were homozygous. Fortunately, 2 colonies were identified as completely corrected at rs143383 in the *GDF5* gene, which was confirmed by Sanger sequencing (Fig. 2B).

Although inherently site-specific, unsolicited cleavage is a perpetual issue of genome-editing tools. To interrogate possible off-target cleavage by the GDF5 TALENs, BLAST was used to compare the targeting sequence against the human genome, and picked the second and third most complimentary hits to be analyzed for mutations. DNA from the corrected colonies was amplified by two pairs of primers spanning the two off-target sites (Table I). Off-target mutations were then detected by T7EI assay as previously described (23). No cleaved bands were detected, indicating that the TALENs did not target these two sites and presumably no other sites predicted by the primary sequence alone (Fig. 2C). Therefore, these colonies were chosen for chondrocytic differentiation.

MSCs differentiating into chondrocytes. MSCs derived from the healthy control with normal GDF5 gene, and from the OA cells with mutant and with corrected GDF5 gene were cultured for chondrocytic differentiation, designated as $GDF5^{con/con}$ $GDF5^{mut/mut}$ and $GDF5^{cor/cor}$, respectively. The

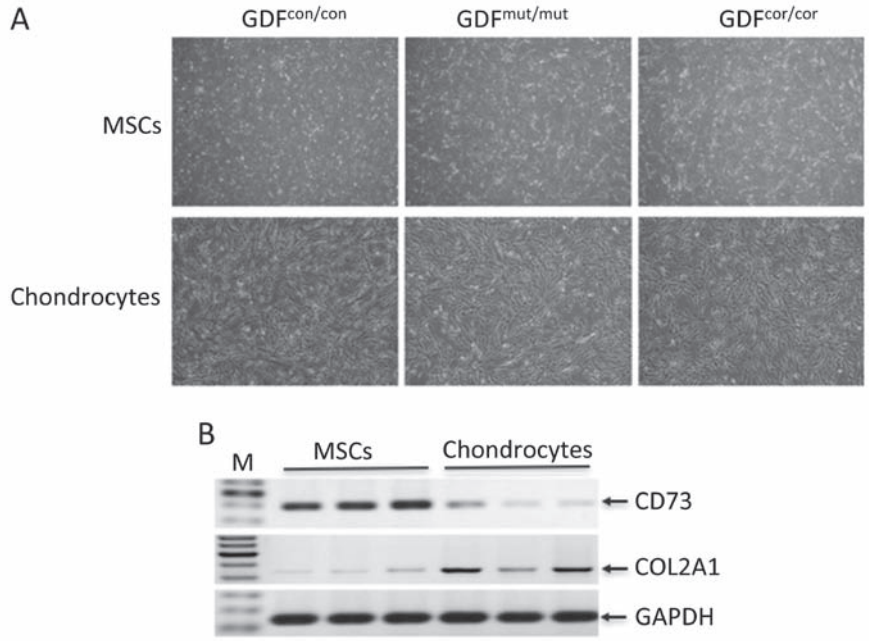

Figure 3. Characterization of the differentiated chondrocytes by (A) morphology and (B) reverse transcription-polymerase chain reaction for molecular markers. GDF5, growth and differentiation factor 5; con, control; mut, mutated; cor, corrected mutation; M, marker; MSCs, mesenchymal stem cells; CD73, 5'-nucleotidase ecto; COL2A1, collagen type II α 1 chain.

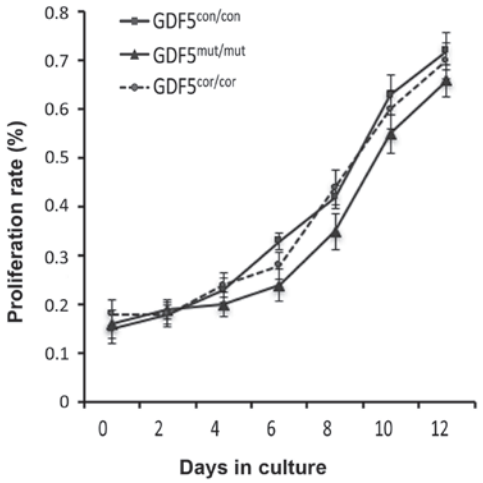

Figure 4. Cell proliferation in corrected and mutant chondrocytes. GDF5, growth and differentiation factor 5; con, control; mut, mutated; cor, corrected mutation.

three cell lines exhibited fibroblast-like morphology as classic MSCs (Fig. 3A). Following differentiation, similar cell morphology was observed among these cells (Fig. 3A). To verify the differentiation, these cells were characterized by lineage-specific markers, CD73 for MSCs and COL2A1 for chondrocytes (27). As shown by reverse transcription PCR, CD73 was highly expressed in MSCs. Following differentiation, chondrocyte-specific marker COL2A1 was observed to be expressed at higher level in differentiated cells compared with in undifferentiated cells (Fig. 3B). These data demonstrated that the MSCs were successfully induced into chondrocytes.

Proliferation of chondrocytes with the corrected GDF5 gene. The defective of chondrocyte function and proliferation would lead to the failure of the articular cartilage and consequently to the progression of OA, thus the proliferation of chondrocytes derived from the cells with mutant ($GDF5^{mut/mut}$) and corrected ($GDF5^{cor/cor}$) GDF5 was determined. The MTT assay showed that the $GDF5^{cor/cor}$ cells propagated at a comparable rate with the $GDF5^{mut/mut}$ cells. In the meantime, no significant difference was observed between these two cell lines ($GDF5^{mut/mut}$ and $GDF5^{cor/cor}$) and the cells from the healthy donor ($GDF5^{con/con}$; Fig. 4).

Decreased apoptosis rate in $GDF5^{cor/cor}$ chondrocytes. As chondrocyte apoptosis has a crucial role in maintaining the homeostasis of articular cartilages (28,29) and GDF5 modulates the apoptosis of the chondrogenic cell line (30), it was determined whether reversing the mutation of the functional SNP rs143383 could restore the cell viability. The chondrocytes were stained with Annexin V-FITC and flow cytometry was performed to assess the proportion of cells undergoing apoptosis. Compared with the control cells, a significant increase of apoptotic cells was detected in the group $GDF5^{mut/mut}$ (1.31±0.12 vs. 14.25±0.68; Fig. 5). Importantly, the number of apoptotic $GDF5^{cor/cor}$ chondrocytes was reduced compared with the $GDF5^{mut/mut}$ group, and the proportions of apoptotic cells were almost equal between the $GDF5^{cor/cor}$ and $GDF5^{con/con}$ groups (Fig. 5). Results here indicated that genetic correction of GDF5 prevented chondrocyte apoptosis.

Decreased MMPs secretion in $GDF5^{cor/cor}$ chondrocytes. MMPs functioning in the degradation of ECM were documented as the most important candidates for the progression of OA, including MMP-1, MMP-2 and MMP-9 (31). Particularly, MMP-13 was reported to be the major collagenase in OA cartilage and had the highest activity (32). In the current study, the secretion of MMP-1, MMP-13 and TIMP1 was measured in

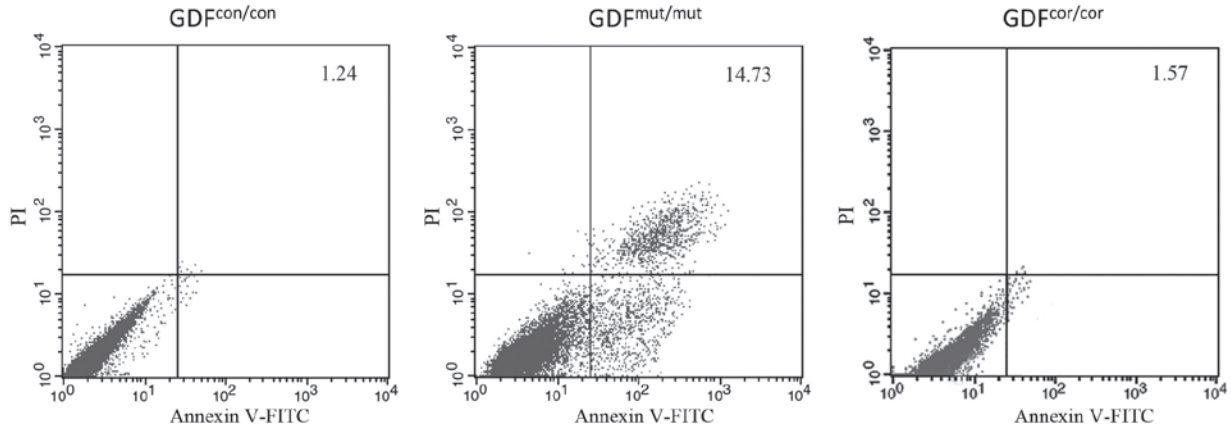

Figure 5. Cell apoptosis in corrected and mutant chondrocytes. The assay was performed in triplicate. GDF5, growth and differentiation factor 5; con, control; mut, mutated; cor, corrected mutation; PI, propidium iodide; FITC, fluorescein isothiocyanate.

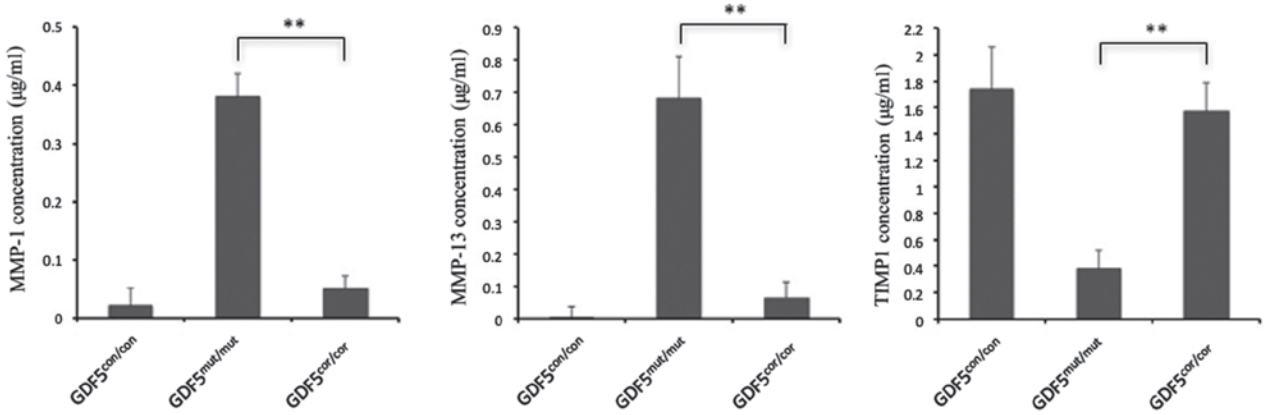

Figure 6. MMP secretion in the supernatants of culture medium of corrected and mutant chondrocytes. *P<0.05, **P<0.01. MMP, matrix metalloproteinase; TIMP1, TIMP metallopeptidase inhibitor 1; GDF5, growth and differentiation factor 5; con, control; mut, mutated; cor, corrected mutation.

the supernatant of culture medium of GDF5$^{con/con}$, GDF5$^{mut/mut}$ and GDF5$^{cor/cor}$ chondrocytes. Generally, the MMPs were at relatively low levels in GDF5$^{con/con}$ cells. However, the concentrations of MMP-1 and MMP-13 were found to be higher in GDF5$^{mut/mut}$ chondrocytes, while in the GDF5$^{cor/cor}$ chondrocytes, the production of these MMPs was reduced compared with in GDF5$^{mut/mut}$ chondrocytes to varying degrees. Maximum suppression was observed for MMP-13, with a 7.2-fold decrease compared with GDF5$^{mut/mut}$ chondrocytes. In addition, the secretion of MMP suppressor TIMP1 was elevated in GDF5$^{cor/cor}$ chondrocytes compared with GDF5$^{mut/mut}$ chondrocytes (Fig. 6). Inhibition of MMP production and increased expression of TIMP indicates that correction of the GDF5 mutation has the potential to inhibit the degradation of ECM.

Restored gene expression in GDF5$^{cor/cor}$ chondrocytes. Having identified the impacts of mutation correction on cell survival and MMP production, the alterations in OA-associated gene expressions caused by GDF5 editing were also determined. In this study, GDF5 expression was observed to be decreased in GDF5$^{cor/cor}$ chondrocytes compared with GDF5$^{mut/mut}$ chondrocytes (Fig. 7A). This was in accordance with the previous findings that the T allele of rs148833 reduces the mRNA expression of GDF5 (33,34). Activation of GDF5 signaling transduced by BMP receptor and downstream Smad1/5/8 regulated the expression of target genes, including COL2A1, ACAN, SOX9, MMP-13, MMP-1 and TIMP1, which encode key catabolic and anabolic proteins in chondrocytes (35). The relative expression of COL2A1, ACAN, SOX9 and TIMP1 were significantly enhanced, while MMP-13 and MMP-1 were suppressed in the GDF5$^{cor/cor}$ chondrocytes compared with GDF5$^{mut/mut}$ chondrocytes. There was no difference in mRNA expression of these targets between GDF5$^{cor/cor}$ and GDF5$^{con/con}$ chondrocytes (P>0.05; Fig. 7A). The restoration of mRNA production of GDF5 itself and its targets indicated that appropriate signaling was rescued by gene correction.

Interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α) and TNF superfamily member 10 (TRAIL) have critical functions in chondrocyte viability (36,37). Expression of TNF-α and TRAIL in GDF5$^{mut/mut}$ cells were significantly higher than that in control cells. By contrast, GDF5$^{cor/cor}$ chondrocytes displayed downregulated gene expressions compared with GDF5$^{mut/mut}$ cells. There was no difference in IL-1 expression among these cells (Fig. 7B).

The canonical Wnt/β-catenin pathway, which is mediated by frizzled (FZD) proteins, inducing nuclear translocation of β-catenin, has important roles in ECM degradation (9). In this

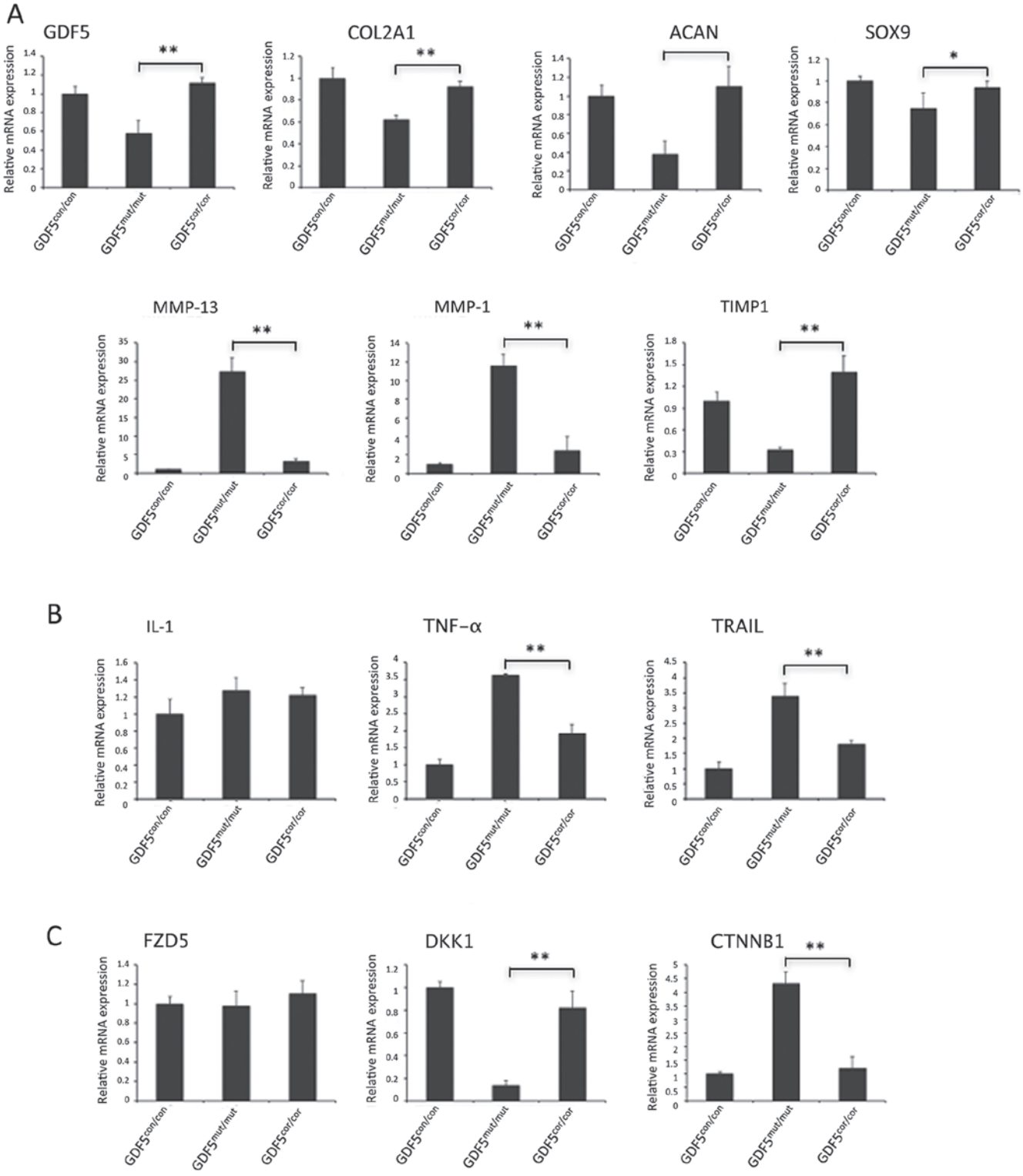

Figure 7. Expression profiling of target genes of (A) GDF5, (B) cell viability-associated genes and (C) genes in canonical Wnt/β-catenin signaling. GAPDH was used as internal control and gene expression of the healthy control was set as 1. *P<0.05, **P<0.01. GDF5, growth and differentiation factor 5; con, control; mut, mutated; cor, corrected mutation; COL2A1, collagen type II α 1 chain; ACAN, aggrecan; SOX9, SRY-box 9; MMP, matrix metalloproteinase; TIMP1, TIMP metallopeptidase inhibitor 1; IL-1, interleukin-1; TNF-α, tumor necrosis factor-α; TRAIL, TNF superfamily member 10; FZD5, frizzled class receptor 5; DKK1, dickkopf WNT signaling pathway inhibitor 1; CTNNB1, catenin β 1.

cascade, the main Wnt receptor, FZD5, was expressed at similar levels in GDF5$^{mut/mut}$ and GDF5$^{cor/cor}$ cells. Dickkopf WNT signaling pathway inhibitor 1 (DKK1), which acts as a Wnt antagonist (38), was upregulated in corrected cells compared with the mutant cells, reaching the same expression level as the controls. Accordingly, the expression of the gene CTNNB1, encoding the core regulator of Wnt pathway, was observed to be notably decreased in GDF5$^{cor/cor}$ chondrocytes (Fig. 7C). The altered gene expression of key regulators in Wnt signaling suggested the inactivation of the pathway.

Discussion

OA is the most common degenerative disease in joints and is predicted to be the single greatest cause of disability by 2030. Its prevalence after the age of 65 years is ~60% in men and 70% in women (39). The etiology of OA is multifactorial, with inflammatory, metabolic and mechanical causes. There is no cure, and current therapeutic strategies are primarily aimed at reducing pain and improving joint function (40). It is now generally accepted that the chondrocyte is the target of these aforementioned causative factors, and genetic factors also contribute to the alterations in the functional activities of these cells (41). Chondrocytes reside in articular cartilages and are responsible for the maintenance of ECM. The compromising of their function and survival would lead to the failure of the articular cartilages (29). Therefore, cell-based functional restore of chondrocytes could be a promising approach to improve OA.

MSCs are multipotent precursor cells originating from several adult connective tissues. MSCs possess the ability to self-renew and differentiate into several lineages, including osteogenic, chondrogenic, cardiogenic and hepatogenic differentiation (42). There has been an increasing interest in the clinical use of MSCs in regenerative medicine. MSCs can be obtained from various sources such bone marrow, umbilical cord, periodontal ligament and adipose tissue. Adipose-derived MSCs, especially autologous cells, are believed to be one of the most promising cell populations identified thus far, because adipose tissue is ubiquitous and easily obtained in large quantities with minimal patient discomfort (43). Therefore, MSCs were isolated from the adipose tissue of a patient with OA and induced these cells into chondrocytes in the present study.

Despite its high prevalence, the etiology of OA is still not fully elucidated. Genetic factors have important roles with estimated heritability ranging from 40-65% (44). In recent years, numerous candidate genes associated with OA have been identified, such as nuclear receptor coactivator 3, ASPN, GDF5 and cadherin 2 (45). A causative SNP rs143383 located in the 5'-UTR of GDF5 alters transcription factor binding and consequently reduces GDF5 expression (11). In chondrocytes, GDF5 was previously reported to reduce MMP-13 expression via DKK1 (9). Thus, genetic correction of the causative mutation may be important for MSCs-based therapy.

In recent years, great advances have been achieved in the ability to make site-specific modifications to the human genome. Currently, the major techniques to mediate genome modification are the ZFN, TALENs and CRISPR/Cas9, all of which are reported to be highly efficient. Each of these techniques has their own potential advantages and disadvantages. It is important to consider the off-target activity when editing the genome, and among the platforms TALENs are highly specific (46). In this study, a pair of TALENs were constructed to target the functional SNP rs143383 in GDF5 and synthesized an ssDNA as the donor template to correct the mutant site. Consequently, a total of 34 colonies were modified out of 142 MSC colonies, and 2 colonies were corrected in both alleles. When determining the potential off-target activity in these 2 colonies, no unpredicted mutations were observed.

Recovery of functionality and viability of chondrocytes has a crucial role in maintaining the homeostasis of articular cartilages (28,29). In corrected cells, the proliferation rate was not changed compared with control and mutation cells, while apoptosis was markedly decreased compared with GDF5-mutant chondrocytes. These results indicated that the TALENs caused enhanced cell viability. MMPs are the major component involved in the degradation of ECM, which is an important factor involved in the progression of OA (31). MMP-1 and MMP-13 secretion was reduced, and TIMP1 was increased, in corrected cells compared with mutation cells, suggesting that ECM degradation was inhibited in modified cells. Expression of GDF5 target genes and genes affecting cell viability and ECM metabolism, including TNF-α, TRAIL and canonical Wnt/β-catenin signaling, were at similar levels in the control and corrected cells. The data suggested that cell viability and functionality were restored by correction of the GDF5 mutation.

In conclusion, the dysfunctional gene GDF5 was successfully corrected in adipose tissue-derived MSCs using a pair of TALENs. Modified MSCs were induced and differentiated into chondrocytes. These cells exhibited normal morphology and enhanced cell vitality. In corrected cells, the secretion of MMPs was suppressed and TIMP1 was increased compared with the mutated cells. The expressions of target genes of GDF5, cell vitality-associated genes and ECM degrading genes were returned to normal levels by correction of the GDF5 SNP, suggesting the functional recovery of these corrected chondrocytes. To the best of our knowledge, this study was the first attempt to generate functional chondrocytes by editing a mutant gene in MSCs, and the results demonstrated that TALEN-mediated genetic correction has the potential to be applied in regenerative medicine. However, further work is required.

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Competing interests

The authors declare there is no competing interest.

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