Molecules and Cells

Genome Edited Sirt1-Overexpressing Human Mesenchymal Stem Cells Exhibit Therapeutic Effects in Treating Collagen-Induced Arthritis

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Even though mesenchymal stem cells (MSCs) are known for cartilage regeneration, their therapeutic efficacy needs to be enhanced. In the present study, we produced genome-edited silent information regulator 2 type 1 (Sirt1)-overexpressing MSCs, and evaluated their therapeutic potential in a damaged cartilage mouse liver fibrosis model. The Sirt1 gene was successfully inserted into a ‘safe harbor’ genomic locus in amniotic mesenchymal stem cells (AMMs), and the chondrogenic properties of the Sirt1 gene overexpressing AMMs (AMM/S) were characterized using quantitative PCR and histology. Therapeutic potentials were investigated in a collagen-induced arthritis (CIA) mouse model. Chondrocyte-differentiated AMM/S expressed cartilage-specific genes and were positive for Safranin O staining. Transplantation of AMM/S attenuated CIA progression and suppressed T helper (Th)-17 cell activation while increasing the Treg cell population in CIA mice. Pro-inflammatory factors, such as interleukin (IL)-1β, IL-6, monocyte chemoattractant protein (MCP)-1, and tumor necrosis factor (TNF)-α were significantly decreased in AMM/S-injected joint tissues. In conclusion, genome-edited AMM/S may represent a safe and alternative therapeutic option for the treatment and repair of damaged cartilage, or in inflammatory joint arthritis.

Keywords: anti-inflammation, cell therapy, genome editing, mesenchymal stem cells, osteoarthritis

INTRODUCTION

Osteoarthritis (OA) is a chronic joint disease in the elderly population due to the destruction of cartilage and other joint tissues. The pathogenesis of OA is known to be related to pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6, IL-17, and tumor necrosis factor (TNF) (Hedbom and Hauselmann, 2002). The prevalence of OA increases with age, and patients require long-term treatment including pain-control drugs, physical therapy, and other surgical procedures.

Tissue engineering using stem cells has been of interest in the treatment of OA. However, obstacles remain regarding the therapeutic potential or control of stem cell dysfunction in the environment of host tissues. Thus, more sophisticated or advanced technologies are required to improve therapeutic efficacy. Recently, genome editing technology has attracted attention for its highly specific cellular genome engineering capability. It is possible to precisely modify the genomes of mammalian cells. Guide RNA directs an endonuclease to a specific genomic target and editing cuts the chromosomal...
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DNA in living cells (Jinek et al., 2012). This process enables the activation of endogenous cellular DNA repair pathways and genome editing, such as the addition or disruption of genes.

Strategies for enhancing the therapeutic effects of stem cells in OA have been applied using various growth factors, chemicals, and scaffolding applications (Qasim et al., 2020). Silent information regulator 2 type 1 (Sirt1) plays a role in cartilage extracellular matrix synthesis and promotes cell survival, even under proinflammatory stress (Dvir-Ginzberg and Steinmeyer, 2013). Sirt1 is an epigenetic regulator of particular relevance to OA and is associated with the modulation of aging and caloric intake (Dvir-Ginzberg and Steinmeyer, 2013). In fact, Sirt1-deficient mice exhibit altered cartilage phenotypes (Gabay et al., 2013). In this study, we investigated the therapeutic properties of Sirt1-overexpressing amniotic mesenchymal stem cells (AMM/S) generated using gene editing in a damaged cartilage during inflammatory process.

MATERIALS AND METHODS

Cell culture and mice
Human amniotic mesenchymal stem cells (AMMs) were purchased from Thermo Fisher Scientific (USA). The AMMs were cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco). Six-week-old male DBA/1 mice were purchased from Orientbio (Korea).

Donor vector construction
Sirt1 was synthesized and inserted into the adeno-associated virus integration site 1 (AAVS1) safe harbor site, targeting the donor vector (System Biosciences, USA) at the NdeI and SalI restriction sites.

Transfection and selection, fluorescence-activated cell sorting (FACS)
AMMs were maintained in DMEM supplemented with 10% FBS. For electroporation, human AMMs were harvested, counted, and 1 × 10^5 cells were resuspended with 0.6 μg of AAVS1 left Transcription activator-like effector nuclease (TALEN) vector (System Biosciences), AAVS1 right TALE-Nuclease vector (System Biosciences), and AAVS1 HR Donor (System Biosciences) in 10 μl of electroporation buffer. The cells were electroporated using the Neon Transfection System (System Biosciences), and AAVS1 HR Donor vector (System Biosciences), AAVS1 right TALE-Nuclease vector (System Biosciences), and AAVS1 left Transcription activator-like effector nuclease (TALEN) vector (System Biosciences) were transfected simultaneously, and 1 × 10^6 plasmid DNA was used. Two days after transfection, Sirt1 knock-in cells were selected by incubation with 5 μg/ml puromycin for 7 days. Puromycin-selected cells were resuspended in FACS buffer and sorted as previously described (Choi et al., 2019).

Genomic DNA extraction and junction polymerase chain reaction (PCR)
Genomic DNA was extracted from the cultured cells using a G-spin™ Total DNA Extraction Mini Kit (InTROM Biotechnologies, Korea) according to the manufacturer’s instructions. Next, 120 ng of genomic DNA was amplified by touch-down PCR (36 cycles) and a second-round PCR, as previously described.

Table 1

| Gene   | Sequence (5’-3’) | Size (bp) |
|--------|-----------------|-----------|
| GAPDH  | Forward TCTTACACTACATGGAGAAG CATGAGTCTCCACGAGTAC | 224       |
|        | Reverse CATGAGAATCGGGTGAAGAAG CAGGGCTACTCTACGAGTAC | 362       |
| SOX9   | Forward GAGGAAGTCTGGTGAAGAAG CAGGGCTACTCTACGAGTAC | 424       |
|        | Reverse CAGGGCTACTCTACGAGTAC CATGAGAATCGGGTGAAGAAG | 424       |
| COMP   | Forward GGAGATCGTGACAGCAATTG CAGGGCTACTCTACGAGTAC | 495       |
|        | Reverse GAATCGCACCCTGTAGTGC CATGAGAATCGGGTGAAGAAG | 495       |
| COL10A1| Forward CACTACCAACCAACACAGAC CAGGGCTACTCTACGAGTAC | 495       |
|        | Reverse GACGACCAGGAGCACCATA CATGAGAATCGGGTGAAGAAG | 495       |

Reverse transcription PCR (RT-PCR)
To confirm gene expression, RNA isolation and cDNA synthesis were conducted as previously described (Han et al., 2020). RT-PCR primers were designed and synthesized by Bioneer (Korea) targeting (Table 1).

Quantitative real-time (qRT)-PCR
qRT-PCR assays were conducted according to previous studies (Choi et al., 2012; Kim et al., 2010). Briefly, total RNA was isolated from cells using RNA-stat (Iso-Tex Diagnostics, USA). The genomic DNA contamination was removed using DNase (Thermo Fisher Scientific). Extracted RNA was reverse-transcribed using TaqMan reagents (Applied Biosystems, USA) according to the manufacturer's specifications. The synthesized cDNA was subjected to qRT-PCR using specific primers and probes. RNA levels were quantitatively measured using an ABI PRISM 7000 instrument (Applied Biosystems). Relative mRNA expression was normalized to that of GAPDH expression. The qRT-PCR primers used were as follows: human Sirt1 (Hs01009006_m1), GAPDH (Hs99999905_m1), and mouse IL-1β (Mm00434228_m1), IL-6 (Mm00446190_m1), MCP-1 (Mm00441242_m1), TNF-α (Mm00443258_m1), and GAPDH (Mm99999915_g1). All primers and probe were purchased from Applied Biosystems.

Safranin O staining
After 3 weeks of culture in chondrocyte differentiation medium (Lonza, USA), which consists of chondrocyte differentiation basal medium, insulin growth factor, transforming growth factor (TGF)-β, Insulin, transferrin and 10% of FBS, the cells were fixed with 4% paraformaldehyde for 10 min and stained using a Safranin O staining kit (ScienCell Research Laboratories, USA) following the manufacturer's instructions.

Splenocyte co-culture and enzyme-linked immunosorbent assay (ELISA)
Splenocyte co-culture assays have previously been reported (Wu et al., 2016). Briefly, spleens from healthy male DBA/1 mice were harvested, and tissues were minced in phosphate-buffered saline (PBS). Splenocytes were isolated using Ficoll-Hypaque density-gradient centrifugation and suspended in RPMI 1640 medium. To determine the effects of AMM/S on T cells, 1 × 10^5 AMMs or AMM/S were treated with or without 10 ng/ml TNF-α for 1 day and then co-cultured with 1 × 10^5 splenocytes in RPMI 1640 containing 10% FBS. After 2
days, supernatants from co-cultures were collected and cytokine levels were measured. The cytokine concentration levels in the supernatant or serum were examined using murine IL-10 or IL-17A ELISA kits (R&D Systems, USA) according to the manufacturer’s specifications.

**Induction of collagen-induced arthritis (CIA) model and treatment**

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Catholic Kwandong University (CKU-01-2020-013). Bovine type II collagen (Chondrex, USA) was emulsified at a ratio 1:1 with complete Freund’s adjuvant (Chondrex) containing 2 mg/ml heat-killed *Mycobacterium tuberculosis*. Six-week-old male DBA/1 mice (OrientBio) received a primary immunization, followed by booster immunization on day 21 using the same concentration of bovine type II collagen and incomplete Freund’s adjuvant (Chondrex). Injection was conducted intradermally at the base of the tail. The severity of arthritis was observed for 28 days after the first injection. The severity of arthritis was monitored and scored as determined by hind paw swelling and clinical scoring: 0 = normal, 1 = slight swelling, 2 = moderate swelling, 3 = severe swelling and reversible joint immobility, and 4 = severe swelling and irreversible joint immobility (Delgado et al., 2001). To evaluate therapeutic efficacy, $1 \times 10^6$ AMMs and AMM-S were injected intraperitoneally twice a week when the arthritis score reached 3 or more.

**Flow cytometric analysis**

Th17 and Treg cell populations were examined using flow cytometry. The antibodies used were phycoerythrin-conjugated rat anti-mouse CD4 (eBioscience, USA), fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IL-17A (eBioscience), and FITC-conjugated rat anti-mouse Foxp3 (eBioscience). Analyses were conducted using CellQuest software (BD, USA).

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**Fig. 1. Generation of AMM/S cell line using TALEN gene editing.** (A) Schematic diagram of the donor vector carrying TGF-β1-bearing donor plasmid DNA. An expression cassette containing the PGK promoter-driven TGFβ1 and EF1α promoter-driven GFP-T2A-puromycin was inserted into the AAVS1 site via homology-directed repair. The locations of primers for junction detection are indicated (primers F and R). HA-L, left homology arm; HA-R, right homology arm; PGK, phosphoglycerate kinase promoter; EF1α, elongation factor-1 alpha promoter; Puro, puromycin. (B) Inserted donor plasmid was confirmed using junction PCR. (C) GFP-expressing AMM/S. Transfected cells were selected using puromycin followed by FACS. Scale bars = 500 μm. (D) Expression levels of TGF-β1 were examined using qPCR. **P < 0.01, n = 4.”
Measurement of cytokines
The concentrations of cytokines were examined using Platinum ELISA kits (eBioscience) and murine IL-17A ELISA kits (R&D Systems). IL-17A from serum was quantified according to the kit manufacturer’s instructions.

Histological analysis
To obtain cartilage and paw samples, mice were euthanized with CO₂ gas and tissues were obtained by dissection. The limbs and paws were fixed overnight in 4% paraformaldehyde and decalcified. Cartilage and paw tissues were embedded in optimal cutting temperature compound and cryosectioned to 10 μm. To analyze inflammation, sections were stained with H&E. To confirm cartilage destruction in the CIA model, the specimens were stained using Safranin O (ScienCell Research Laboratories) or Alcian blue (Newcomer Supply, USA) following the manufacturer’s instructions. Cartilage degradation was measured by using a degradation score and the following scale: from 0 to 3 was defined as either no loss or complete loss of staining for proteoglycans (Wu et al., 2016). To analyze inflammation, H&E staining was performed according to previous study (Jang et al., 2020). The degree of inflammation was scored as reported previously (Razawy et al., 2020) using the following scale: 0, no inflammation; 1, minimal inflammation; 2, mild inflammation; 3, moderate inflammation; and 4, severe inflammation.

Statistical analysis
All data are presented as mean ± SD. Statistical analyses were performed using Student’s t-test for comparisons between two groups, and ANOVA with Bonferroni’s test with multiple comparison correction using SPSS (ver. 12.0; SPSS, USA). Data with P < 0.05 were considered to be statistically significant.

RESULTS
Targeted knock-in of Sirt1 in AMMs
To produce a stem cell line overexpressing Sirt1 using gene editing, we used TALEN-mediated gene integration methodology. The targeting donor plasmid carried the phosphoglycerate kinase (PGK) promoter-driven Sirt1, and elongation factor-1 alpha (EF1α) promoter-driven green fluorescent protein (GFP)-T2A-puromycin, and was designed to be integrated into AAVS1 on chromosome 19 (Fig. 1A). AMMs were transfected with the donor plasmid and a pair of TALENs. The transfected cells (<10% of GFP-positive cells) underwent in vitro selection with puromycin and were additionally isolated using FACS (98.9% GFP-positive cells) (Fig. 1B). To confirm genomic integration of the donor plasmid into the AAVS1 site, we verified the genomic DNA using standard PCR, followed by touch-down PCR (Don et al., 1991; Korbie and Mattick, 2008). The correct insertion of the donor plasmid was detected via 5-junction fragment (960 bp) amplification.
Finally, Sirt1 expression by AMM/S was confirmed via qPCR, and Sirt1 mRNA levels were found to be significantly increased in AMM/S compared to the control AMMs (Fig. 1D). A successfully gene-edited AMM/S line was subsequently used in all experiments. In vitro chondrogenic differentiation potential of AMM/S

To investigate their chondrogenic potential, we performed qPCR analysis. Intriguingly, chondrogenically-differentiated AMM/S expressed chondrogenic-specific markers, such as type A1, 10 collagen (COL10A1), cartilage oligomeric matrix protein (COMP), and SRY-box transcription factor (SOX) 9 in AMM/S (Fig. 2A). However, undifferentiated AMM did not express these chondrogenic-specific markers. Additionally, we examined chondrogenic potential using histological experiments. Safranin O staining results revealed that AMM/S had a greater degree of staining than AMM (Fig. 2B).

Next, to evaluate the in vitro immunomodulatory effects of AMM/S on T cells, AMMs or AMM/S were treated with or without TNF-α, and then co-cultured with splenocytes. Supernatants from co-cultures were evaluated for cytokine levels after 2 days. Interestingly, ELISA results revealed that co-culture with AMM/S revealed significantly higher IL-10 and lower IL-17A levels in the culture supernatant compared with AMMs (Fig. 2C).

Therapeutic properties of AMM/S in murine CIA model

To investigate the therapeutic potential of AMM/S for restoring damaged cartilage in vivo, we induced a CIA mouse model using bovine type II collagen. The arthritis clinical score was evaluated after cell injection (Fig. 3A). Interestingly, there were significantly lower arthritis clinical scores at 15 and 21 days in the AMM/S-injected group compared with the control PBS- or AMM-injected groups (Figs. 3B and 3C).

Next, to investigate possible mechanisms underlying the favorable therapeutic effects of AMM/S, we examined the influence of T cells after cell injection. The population of Treg cells increased after AMM/S was introduced into the bloodstream of mice compared to that in the PBS- or AMM-injected cohorts (Figs. 4A and 4B). However, the Th17 cell population was significantly decreased in AMM/S-injected mice compared with PBS control or AMM-injected mouse groups (Figs. 4A and 4B). We also determined the concentration of IL-17A after injection of cells in CIA mice. IL-17A levels were significantly decreased in AMM/S-injected CIA mice (Fig. 4C).

Histological analysis in joints from CIA mice

To investigate the protection of cartilage degradation in vivo, mouse joint tissues were stained with Safranin O. Such staining can detect the expression of proteoglycans in cartilage. The AMM/S-injected group exhibited increased proteoglycan expression in articular cartilage compared with the PBS- or AMM-injected control groups, suggesting protection against cartilage damage (Figs. 5A and 5B). Next, to evaluate inflammatory responses in joint tissues, H&E staining was conducted. Histological analysis showed that AMM/S-injected joint tissues showed significantly lower inflammatory cell infiltration than PBS- or AMM-injected control joint tissues (Figs. 5C and 5D).

DISCUSSION

Using targeted gene editing, we generated Sirt1-overexpressing MSCs for enhanced cartilage protection or regeneration. In this study, we first demonstrated that genome-edited AMM/S tended to protect against arthritis progression through their therapeutic effects on chondrogenesis and T lymphocyte activation. These results indicated that Sirt1-overexpressing MSCs could be an alternative therapeutic option for the treatment of OA.

Inflammatory gene expression in joints from CIA mice

To further elucidate the therapeutic mechanisms of AMM/S, we analyzed the expression levels of pro-inflammatory factors in joint tissues after injection of cells. Interestingly, pro-inflammatory factors, such as IL-1β, IL-6, MCP-1, and TNF-α were significantly decreased in AMM/S-injected joint tissues compared with PBS- or AMM-injected joint tissues (Fig. 6).
of cytokine receptor genes in stem cells promotes cell survival and tissue deposition in inflammatory environments (Farhang et al., 2017). Erythropoietin (EPO) gene-edited MSCs successfully secrete high levels of EPO (Benabdallah et al., 2010) and HGF-overexpressing MSCs generated by gene editing exhibit improved therapeutic properties in an animal model of ischemia (Chang et al., 2016). These data indicate that genome editing can provide an ideal platform for the generation of specific, safe, and novel stem cell lines. Thus, these reports prompted us to investigate the therapeutic potential of genome-edited stem cells.

Over the past decade, our laboratory has sought to identify the best sources of stem cells. Among these, AMMs offer great benefits as a source of allogeneic stem cells, as they can be readily obtained without any ethical concerns, and express low levels of immunological responses (Alviano et al., 2007). In addition, they have high cell proliferative, survival, and trans-differentiation properties (Alviano et al., 2007; Tsuji et al., 2010). Specifically, we found that AMMs are the best MSC source for genome editing because of their high transfection efficiency compared to other stem cell sources.

Even though stem cells have become attractive tools for tissue regenerative applications, controversy regarding their low therapeutic efficacy has become a major obstacle. The aim of this study was to explore genome engineering technologies to address the challenges involved in stem cell therapy. To identify favorable factors driving anti-inflammation and regeneration, we examined one of the important factors, Sirt1, involved in cartilage repair. Recently, it has been reported that Sirt1 promotes chondrogenic differentiation and reduces MSC apoptosis (Ou et al., 2020). In addition, activation of Sirt1 inhibits inflammation and degradative processes in cartilage (Backesjo et al., 2009; Buhrmann et al., 2014). Sirt1 is an enzyme that deacetylates transcription factors that contribute to cellular regulation (Peng et al., 2011). Sirt1-deficient mice exhibit an altered cartilage phenotype (Gabay et al., 2013) and overexpression of Sirt1 inhibits osteoarthritic gene expression in human chondrocytes (Matsushita et al., 2013). In line with these reports, our results also revealed that AMM/S exhibited higher chondrocyte differentiation in vitro.

Fig. 4. AMM/S transplantation influences Treg and Th17 cell populations in CIA mice. (A) Representative figures illustrating flow cytometric data for the identification of Treg and Th17 cells. (B) Quantitative data for Treg and Th17 cells were measured using murine CIA blood samples 2 weeks after injection of cells. n = 5 each; **P < 0.01. (C) Concentration of IL-17A in serum of CIA mice 2 weeks after injection of cells. n = 5 each; **P < 0.01.
and AMM/S transplantation decreased levels of pro-inflammatory factor in joints of CIA mice. These results may indicate that Sirt1 plays important roles in the pathogenesis of OA.

For cell-based therapies, another important function of MSCs is immunomodulation. The release of immunomodulatory factors, such as hepatocyte growth factor (HGF), IL-10, and TGF-β1, protect cartilage in the synovium (Kehoe et al., 2014). In addition, it has been reported that T cells regulate arthritic pathogenesis. Immunomodulatory factors induce Treg cells and suppress inflammation by reducing proliferation of Th17 cell (Aggarwal and Pittenger, 2005). However, Th17 cells are involved in inflammatory processes, and Th17/Treg cell imbalances could present problems in arthritic therapy. Interestingly, AMM/S transplantation resulted in a significant suppression of Th17 and protection of cartilage against damage. These data indicated that therapeutic functions of AMM/S might affect reciprocal regulation of Th17/Treg cell imbalances in CIA mice.

In summary, this study revealed that Sirt1 overexpression after AMM gene editing resulted in robust therapeutic effects without changes in MSC properties in injured cartilage. Our observations indicated that transplantation of AMM/S involved in the pathogenesis of OA and Sirt1 overexpression might contribute to the prevention of OA and chondrocyte

Fig. 5. Histological staining of joints in CIA mice after injection of cells. (A) Proteoglycan expression was identified using Safranin O staining of the joints of CIA mice after injection of MSCs. Scale bars = 200 μm. (B) Quantification of cartilage degradation scores. Loss of proteoglycans was identified after staining for proteoglycans. n = 5 each: **P < 0.01. ns, not significant. (C) Representative images of H&E-stained sections of joint tissues. Scale bars = 200 μm. (D) Quantification of inflammatory response histological scores. Mononuclear cell infiltration and inflammatory pathological scores were measured after cell transplantation. AMM/S-injected Treg and Th17 cells exhibited low mononuclear cell infiltration and normal cartilage surface morphology. n = 5 each: **P < 0.01.
degradation. Further investigations are required to evaluate the efficacy and safety of AMM/S for treating joint OA in the context of clinical settings.

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AUTHOR CONTRIBUTIONS

S.W.K. conceived and designed the experiments. D.S.C. and S.H. performed the experiments. S.W.K. analyzed the data. M.K.L. contributed reagents, materials, and analysis tools. S.W.K. wrote the paper.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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