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Antisenescence activity of G9a inhibitor BIX01294 on human bone marrow mesenchymal stromal cells

조선대학교 대학원

생명과학과

안 민 지

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인간의 골수 유래 중간엽 줄기세포에서 G9a 억제제인
BIX01294를 이용한 항노화 활성 연구

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ABBREVIATIONS

ANG	angiogenin
bFGF	basic fibroblast growth factor
BM-MSCs	bone marrow-mesenchymal stromal cells
EHMT2	euchromatin histone methyltransferase2
FBS	fetal bovine serum
G9a	H3K9 methyltransferase
MSCs	mesenchymal stromal cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole
Oct-4	octamer-binding transcription factor-4
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RIPA	radio-immunoprecipitation
SA- β-gal staining	senescence-associated beta-galactosidase staining
TERT	telomerase
VEGF	vascular endothelial growth factor.

ABSTRACT

Antisenescence activity of G9a inhibitor BIX01294 on human bone marrow mesenchymal stromal cells

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Alterations in epigenomic patterns are associated with diverse physiological functions and pathological mechanisms causing aging-related diseases, such as degenerative disorders and cancers. We investigated the senescent effects of BIX01294, a G9a (histone methyltransferase) inhibitor, in human bone marrow mesenchymal stromal cells (hBM-MSCs). We determined the optimal dose and time of BIX01294 treatment in MSCs to be 1 μM and 12 h, respectively. Under these conditions, the expression of the antisenescent genes *TERT*, *bFGF*, *VEGF*, and *Oct*-4 was increased, whereas the expression of the senescent factors p16, p21, and was p53 decreased. The number of β-galactosidase-positive cells decreased significantly, and the rates of migration and cellular protection against oxidative damage increased in BIX01294-treated hBM-MSCs. These data indicate that an optimized dose of BIX01294 may improve the potency and senescence of stem cells, which may improve the efficacy of stem cell therapy.

국문 초록

인간의 골수 유래 중간엽 줄기세포에서 G9a 억제제인 BIX01294를 이용한 항노화 활성 연구

후생유전학적 패턴 변화는 퇴행성 신경질환과 암, 노화 관련 질병을 일으키는 원인이 다양한 생리학 기능과 병리학 기전과 연관되어 있다. 본 연구는 히스톤 메틸화효소 억제제로 알려진 BIX01294(G9a 억제제)의 처리를 통해 인간의 골수 유래 중간엽 줄기세포에서의 항-노화 효과를 갖는지 확인하고자 연구를 진행하였다. 먼저, Real-time PCR을 통해 노화와 관련된 유전자들인 p53과 p21의 발현을 관찰함에 따라 BIX01294 처리의 최적농도(1 μM)와 시간(12 h)을 결정하였다. 이 처리 조건에서 항-노화 관련된 유전자들인 TERT, bFGF, VEGF 그리고 Oct-4가 증가하는 것을 관찰하였고, 반면에 노화 관련된 유전자들인 p53, p21 그리고 p16은 감소하는 것을 관찰하였다. 그리고 세포 수준에서 노화의 정도를 확인할 수 있는 Senescence associated β-galactosidase staining을 통해서 세포 수준에서의 줄기세포의 다양한 passage에서의 항-노화 효과를 관찰하였고 , BIX01294에 대한 항-노화에 대한 효과가 세포의 기능적인 개선에 영향을 미치는지 확인하고자 Wound healing assay, MTT assay를 실험하여 적절한 조건의 시간과 농도에서 BIX01294 처리하여 세포 이동 능력의 향상과 산화스트레스로부터 보호 효과가 있는 것을 확인 하였다. 따라서 BIX01294은 적정한 조건에서의 인간 유래 골수 중간엽 줄기세포에서 항-노화 효과를 나타냈고 이는 줄기세포 치료의 효율을 증가시키는 데 개선될 수 있음을 시사 하였다.

1. INTRODUCTION

Stem cells are often expanded under in vitro culture conditions to obtain sufficient quantities of cells for cell therapy. However, the cellular protection [1, 2] and differentiation [3, 4] abilities of stem cells decline during long-term expansion [5, 6]. Thus, the risk of cellular senescence increases in stem cell expansions [7], resulting in reduced efficacy of cell therapies. This limitation suggests that stem cells need to be maintained under antisenescent conditions during long-term expansion.

Epigenetic modifications in DNA and nucleus histones play a crucial role in regulating chromosomal functions and gene expression. Recent studies have shown that the senescent phenotype is induced by increased epigenetic modifications [8], resulting in the formation of senescence-associated heterochromatin foci [9, 10, 11]. The replicative senescence in long-term in vitro cultures of mesenchymal stromal cells (MSCs) is also caused by increased DNA methylation in the genome [12]. Thus, there is growing evidence that deviant alterations in the epigenomic patterns are associated with diverse physiological functions and pathological mechanisms causing aging-related diseases, such as degenerative disorders and cancers [13, 14].

G9a is known as an H3K9 methyltransferase (also called EHMT2) and it transfers the methyl group to the 9th lysine residue on histone 3 and plays a role in catalyzing monomethylation and dimethylation (H3K9me1 and H3K9me2) in euchromatin [15, 16]. G9a also transfers the methyl group to nonhistone substrates [9]. Methylation by G9a is mostly associated with transcriptional repression, but in some cases activates gene transcription depending on the recruited proteins [17]. G9a is highly expressed in many cancer tissues compared with normal tissues [18, 19] and thus it has been regarded as a therapeutic target for cancer and cellular senescence.

BIX01294, a selective inhibitor of G9a and the G9a-like histone methyltransferase [20, 21] has been developed as an antitumor drug [22]. It induces transcriptional stimulation by removing H3K9me2 at the promoters of several tumor suppressor

genes [23]. However, BIX01294 enhances the cardiomyogenic potential of bone marrow mesenchymal stromal cells (BM-MSCs) by inhibiting chromatin methylation [24]. Moreover, treatment with BIX01294 in embryonic fibroblasts of Oct-4/Klf4-transduced mice improves the reprogramming efficiency of induced pluripotent stem cells (iPSCs) [25], and epigenetic modifications by BIX01294 can increase the ability of adipose-derived mesenchymal stromal cells to differentiate into endothelial cells [26], suggesting that the effects of epigenetic regulation by BIX01294 may depend upon the state of the cells.

We investigated the effects of BIX01294 on cellular senescence in human BM-MSCs (hBM-MSCs), and identified that an optimal treatment of BIX01294 leads to attenuated cellular senescence and improves the stem cell potency in hBM-MSCs.

2. Materials and Methods

ll-1. Characteristics of primary human BM-MSCs and cell culture

The hBM-MSCs were purchased from CEFO (Cell Engineering For Origin; Seoul, Korea). The cells were examined for viral infection and mycoplasma contamination, and all were presented as negative. Flow cytometric analysis of the cells revealed $CD73^{+}$, $CD105^{+}$, and $CD31^{-}$ phenotypes. The hBM-MSCs were cultured in T75 flasks (Becton Dickinson; San Jose, CA, USA) according to the supplier's recommendations. Cells were cultivated in hBM-MSCs growth medium (DMEM; Gibco; Grand Island, NY, USA), containing 10% FBS, L-glutamine, penicillin, and streptomycin, without any stimulatory supplements or vitamins, and maintained in a humidified incubator at 37 °C, using a standard mixture of 95% air and 5% CO_2. Cells were subcultured every 5 days, with medium replacement every 3 days. The hBM-MSCs at passage 7, 12, or 17 (P-7, P-12, or P-17) were used for these experiments.

II-2. Real-time PCR

hBM-MSCs were harvested and total RNA were extracted using RNAiso reagent (TAKARA; Shiga, Japan) according to the manufacturer's instructions. The Primescript II 1st strand cDNA synthesis kit (TAKARA; Shiga, Japan) was used to reverse transcribe 3-5 μg of total RNA with 5 μM Oligo(dT) primers (TAKARA; Shiga, Japan), 1 mM dNTP, and the supplied buffer. First-strand cDNAs were amplified using the Power SYBR Green PCR master mix (Applied Biosystems Inc., USA) with gene-specific primers for human *bFGF*, *Oct-4*, *p21*, *p53*, *TERT*, *VEGF*, or *β-actin*. The real-time PCR cycling parameters were as follows: 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. The primers were synthesized by GenoTech (GenoTech Corp.; Daejeon, South Korea) and IDT (Integrated DNA Technologies Inc.; Coralville, IA, USA) and are summarized in the Table 1.

Table 1. Oligonucleotides used for real-time PCR.

Gene	Forward primer (5'→ 3')	Reverse primer (3'→ 5')	Acc. No.
bFGF	AAAAACGGGGGCTTCTTCCT	ACGGTTAGCACACACTCCTT	NM_002006
TERT	ATCGCCAGCATCATCAAACC	GGTAGAGACGTGGCTCTTGA	NM_198253
Oct4	GCCCGAAAGAGAAAGCGAAC	AACCACACTCGGACCACATC	NM_002701
VEGF	AGAAAATCCCTGTGGGCCTT	GTCACATCTGCAAGTACGTTCG	NM_001025368
p21	GTCTTGTACCCTTGTGCCTC	GGCGTTTGGAGTGGTAGAAA	NM_000389.4
p53	AGGAAATTTGCGTGTGGAGT	AGTGGATGGTTGTACAGTCA	NM_000546
β-actin	ATCCGCAAAGACCTGTACGC	TCTTCATTGTGCTGGGTGCC	NM_001101

Acc. No. indicates gene access number.

II-3. MTT assay

The protective effects against H_2O_2-induced oxidative stress were measured by MTT assay (Sigma; St. Louis, MO, USA) according to the manufacturer's instructions. Briefly, 2.5 × 10^3 hBM-MSCs were seeded into each well of a 96-well plate. The next day, the cells were incubated with or without 1 μM BIX01294 for 12 h, treated with 0-1.5 mM H_2O_2 for 1 h, and then subjected to MTT assay. To examine the cell toxicity effects of BIX01294, hBM-MSCs were incubated with several concentrations (0-5 μM) of BIX01294 for 12 h and then measured by MTT assay.

II-4. Immunoblot analysis

Cells were lysed by treating them with 40 μL of RIPA buffer containing protease and dephosphatase inhibitors (Santa Cruz Biotechnology; Dallas, TX, USA) for 30 min at 4 ℃, and then centrifuged at 16,000 × g for 20 min. The total proteins were then subjected to immunoblotting with antibodies specific for TERT (1:500; Santa Cruz Biotechnology), p53 (1:500; Santa Cruz Biotechnology), p16 (1:500; Santa Cruz Biotechnology), p21 (1:200; Santa Cruz Biotechnology), angiogenin (ANG, 1:500; Santa Cruz Biotechnology), cleaved-caspase-3 (1:500; Santa Cruz Biotechnology), H3k9me2 (1:1000; Merck Millipore; Darmstadt, Germany), histone 3 (1:2000; Cell Signaling Technology; Danvers, MA, USA), or β-actin (1:5000; Sigma; St Louis, MI, USA), and subsequently to the appropriate horseradish-peroxidase-conjugated secondary antibodies (1:10,000; Jackson Immuno Research Laboratories; West Grove, PA, USA). The western blots were quantified with ImageJ (Softonic International, S.A.; Barcelona, Spain)

II-5. Senescence-associated beta-galactosidase (SA-β-gal)

SA-β-gal staining was carried out using the Senescence β-Galactosidase Staining kit (Cell Signaling Technology Inc.) according to the manufacturer's instructions. The hBM-MSCs were seeded into 6-well plates at a density of 1×10^3 cells/well and incubated until the appropriate confluence was reached. The cells were washed with PBS and fixed with 2% formaldehyde and 0.2% glutaraldehyde in distilled water for 15 min at room temperature. The cells were then washed twice with PBS containing 1 mM $MgCl_2$ (pH 7.2) and stained overnight in β-galactosidase staining solution (1 mg/mL X-gal, 5 mM $K_3Fe[CN]_6$ (potassium ferricyanide), 5 mM $K_4Fe[CN]_6$ (potassium ferrocyanide), 2 mM $MgCl_2$, 40 mM citric acid/sodium phosphate (pH 6.0), and 150 mM NaCl in distilled water) at 37 °C without CO_2. Images were captured with a microscope (Canon i-Solution IMTcam3; Tokyo, Japan). The results are presented as the means of four independent experiments.

II-6. Wound healing test

The hBM-MSCs were seeded into 6-well plates and incubated overnight in standard growth medium at 37℃ and 5% CO_2. A uniform scratch was made in the 100% confluent monolayer culture. The wound was introduced by scraping the monolayer with a sterile 200-μL pipette tip and then washing the monolayer with growth medium to remove cell debris. The cells were then replenished with fresh growth medium, and wound closure was documented by photography of the same region at different times (0-18 h). Migrating cells were counted at each time point (0-18 h). The results are presented as the means of four independent experiments.

II-7. Statistical analysis

The data are represented as mean ± standard deviation (SD) of three or more independent experiments. Statistical comparisons between groups were made using an independent t-test. A P-value of < 0.05 was considered statistically significant.

III. Results

III-1. BIX01294 suppressed the expressions of p53 and p21 in human BM-MSCs

To determine the optimal concentration of BIX01294, hBM-MSCs were incubated with 0, 0.5, 1, or 1.5 μM BIX01294 for 12 h. The expressions of senescence-related genes *p53* and *p21* were examined by real-time PCR. Figure 1A shows that the transcripts of the tested genes were minimal in hBM-MSCs treated with 1 μM BIX01294 (Figure 1A; $^{\#}P < 0.01$, mean ± SD, n = 4). To evaluate the optimal treatment time of BIX01294 in hBM-MSCs, the cells were incubated with 1 μM BIX01294 for several incubation times (0-24 h). Decreasing gene expressions were identified at 12 h of incubation (Figure 1B; $^{*}P < 0.05$, $^{\#}P < 0.01$, mean ± SD, n = 5). Cellular toxicity was not observed at BIX01294 concentrations of up to 1.5 μM for 12 h, as determined by the MTT assay (Figure 1C; $^{\#}P < 0.01$, mean ± SD, n = 5). From these data, we determined the optimal treatment conditions in hBM-MSCs to be 1 μM BIX01294 for 12 h.

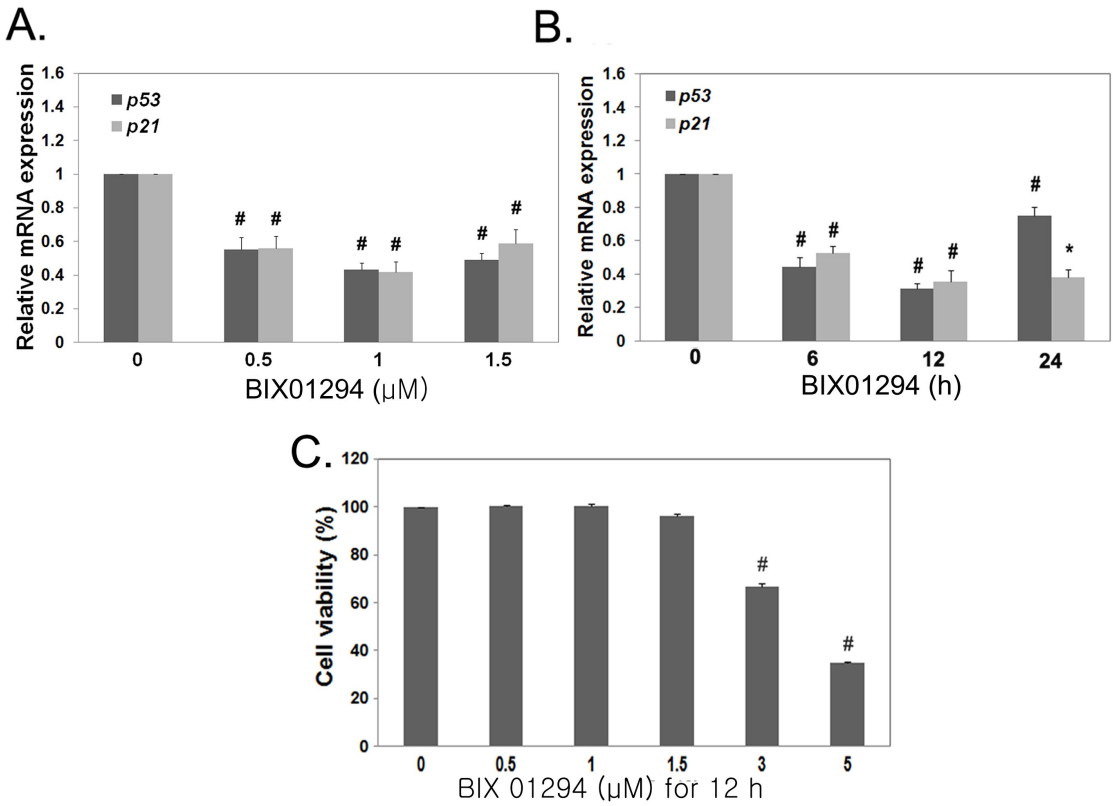

Figure 1. BIX01294 reduces the expression of the *p53* and *p21* genes in hBM-MSCs.

(A) The expressions of *p53* and *p21* in hBM-MSCs treated with BIX01294 (0-1.5 μM) for 12 h were measured by real-time PCR. (B) The suppressed expressions were maximal at 12 h of incubation with 1 μM BIX01294 treatment, as measured by real-time PCR. (C) No cytotoxicity was observed in hBM-MSCs treated with up to 1.5 μM BIX01294, as tested by MTT assay.

lll-2. The expression of senescence-related factors were regulated by BIX01294 treatment

I have shown that BIX01294 suppressed the expression of antisenescent factors at the determined conditions (Figure 1). To further investigate the role of BIX01294, various senescent or antisenescent genes were examined using real-time PCR. Transcripts of the antisenescent genes *TERT*, *Oct-4*, *bFGF*, and *VEGF* were significantly increased in BIX01294-treated hBM-MSCs (BIX01294 hBM-MSCs) [27, 28, 29], whereas the senescent genes p53 and p21 were decreased compared with nontreated hBM-MSCs (Control hBM-MSCs) (Figure 2A; *t*-test, $^{*}P < 0.05$, $^{\#}P < 0.01$, mean ± SD, n = 4). Consistent results were obtained from immunoblot analysis with specific antibodies against TERT, p53, p16, p21, and H3K9me2 (Figure 2B).

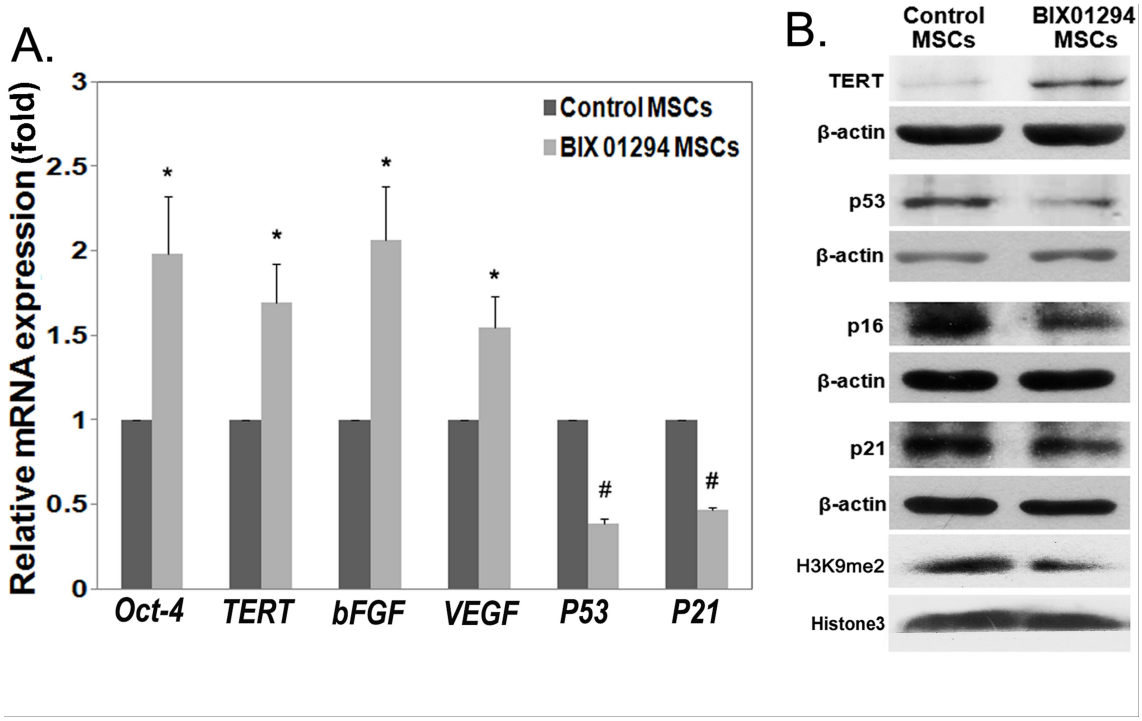

Figure 2. Transcripts of the senescence-related factors were regulated by BIX01294-treated hBM-MSCs.

(A) The expressions of the antisenescent factors *TERT*, *bFGF*, *VEGF*, and *Oct-4* increased significantly, whereas those of the senescent factors *p21* and *p53* decreased significantly in BIX01294 hBM-MSCs (B) The expressions were confirmed by immunoblot analysis with antibodies specific for TERT, p53, p16, p21, and H3K9me2.

III-3. BIX01294 prevented cellular senescence in hBM-MSCs

I have shown that senescence-related factors were modulated following BIX01294 treatment. To examine this effect in replicative senescent hBM-MSCs, hBM-MSCs were cultured up to passage 17 (P-17) according to the procedures described in the Materials and methods. Cells at P-7, P-12, and P-17 were incubated with or without BIX01294 (1 μM BIX01294 for 12 h) and subjected to SA-β-gal assays (Figure 3A). The number of SA-β-gal-stained cells increased with increasing passage number. Meanwhile, the number of SA-β-gal-stained cells decreased at BIX01294-treated hBM-MSCs in comparison with nontreated hBM-MSCs (Figure 3B; *t*-test, $^{\#}P < 0.01$, mean ± SD, n = 4). Immunoblot analyses with senescent proteins showed decreasing expressions of p53 and p16, and an increasing expression of TERT in BIX01294-treated hBM-MSCs (P-12; Figure 3C).

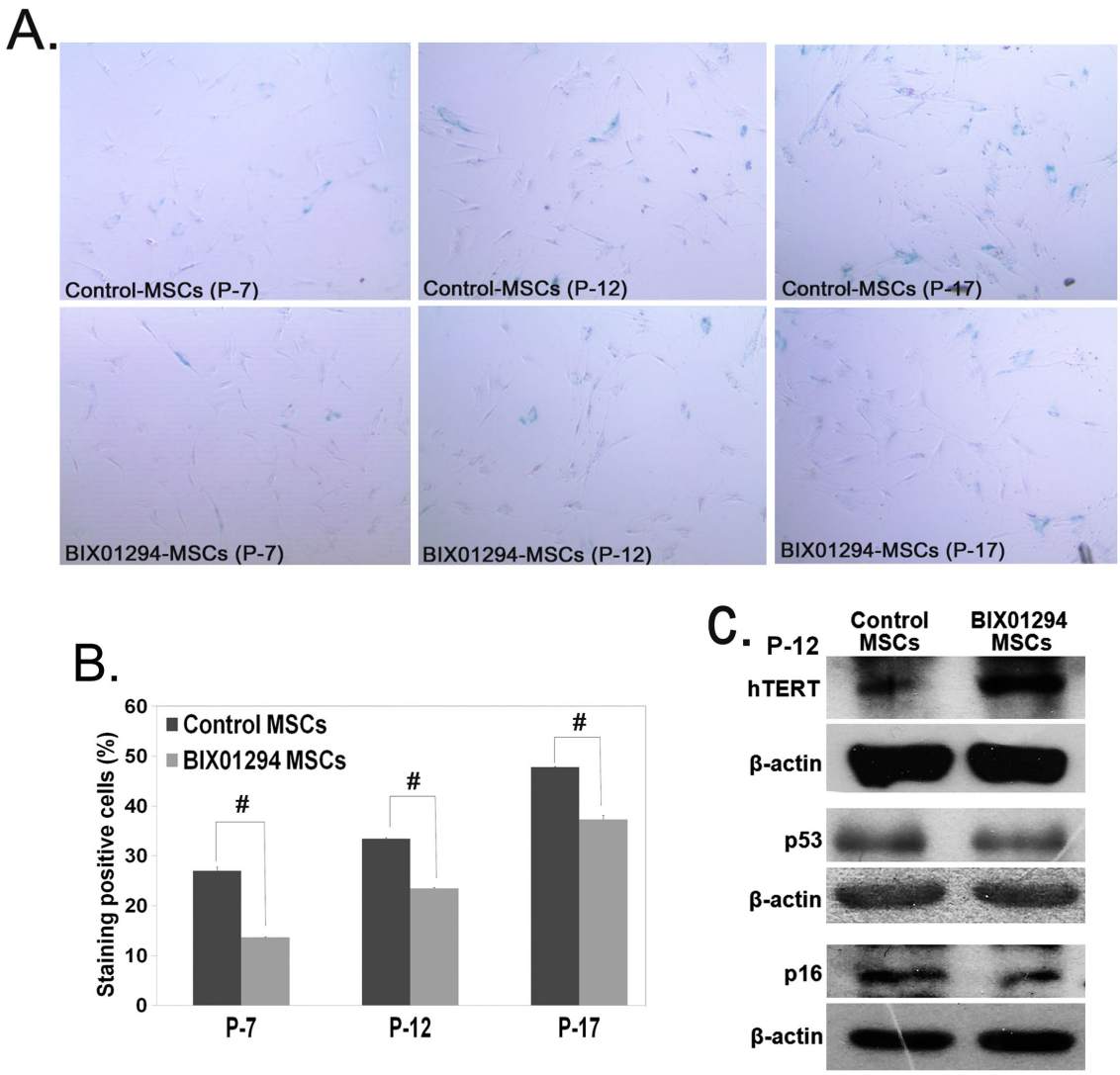

Figure 3. BIX01294 prevents cellular senescence in hBM-MSCs.

(A) Senescence-associated beta-galactosidase (SA-β-gal) assays were performed in BIX01294-treated hBM-MSCs (BIX01294 hBM-MSCs; 1 μM, 12 h) or nontreated hBM-MSCs (Control hBM-MSCs) at P-7, P-12, and P-17. P-7, P-12, and P-17

indicate passages 7, 12, and 17. The senescent cells are indicated by the blue staining. (B) The number of total cells and senescent cells (blue) were counted and the results are presented graphically. (C) Immunoblot analysis was performed with the senescence-related proteins p16, p53, and TERT in BIX01294-treated or nontreated P-12 hBM-MSCs.

III-4. BIX01294 improved cellular migration in hBM-MSCs

The migratory ability of stem cells is associated with stem cell potency [30]. To examine the migratory effects of BIX01294, hBM-MSCs were incubated with or without 1 μM BIX01294 for 12 h. Cells were then tested with a wound healing assay (Figure 4A). Increased migration rates were observed in BIX01294-treated hBM-MSCs (Figure 4B; *t*-test, $^{\#}P < 0.01$, mean ± SD, n = 3). To confirm these results at the protein level [31], immunoblot analysis was performed with antibodies against ANG and β-actin, and increasing expressions were identified in BIX01294 hBM-MSCs (Figure 4C).

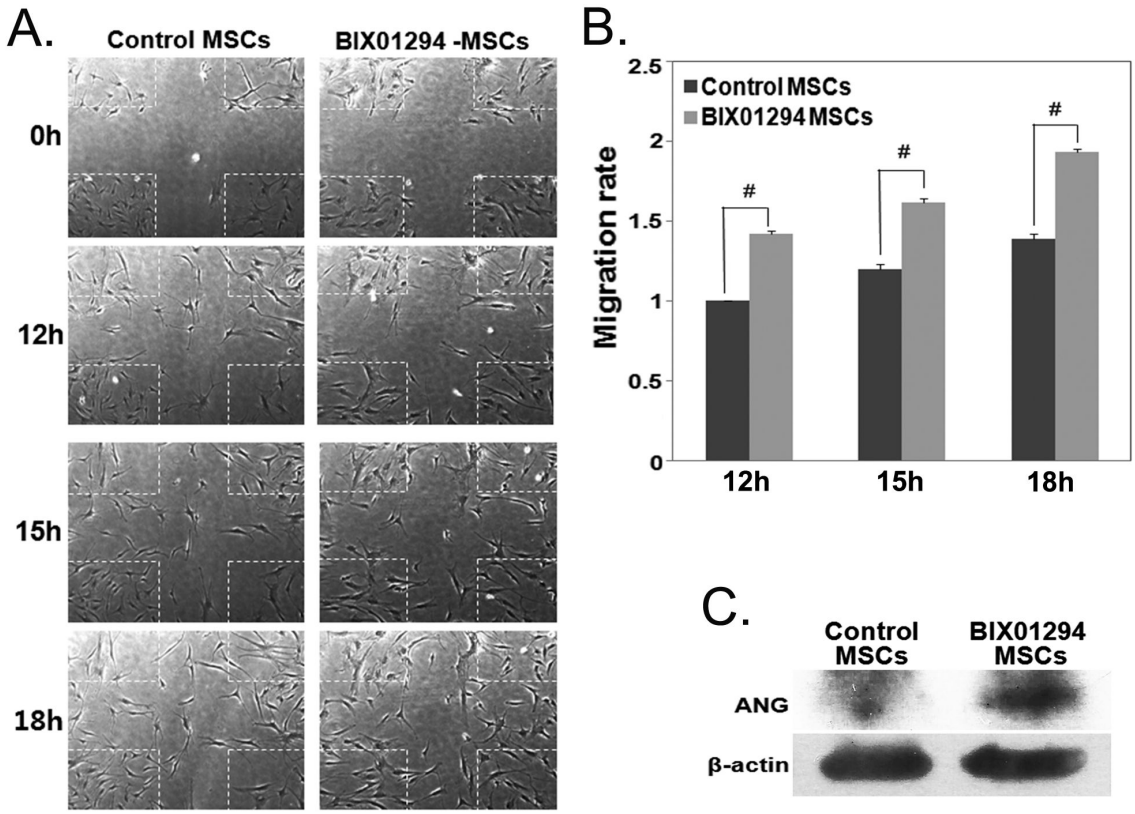

Figure 4. BIX01294 improves the cellular migration of hBM-MSCs.

(A) The migration of BIX01294-treated hBM-MSCs was observed for 18 h after being scratched (inside dotted line). (B) Migrated hBM-MSCs inside the dotted lines were counted. (C) Immunoblot analysis was performed with the migration marker protein, ANG.

lll-5. Protective effects of BIX01294 in hBM-MSCs

Since BIX01294 increased the expression of antisenescent factors in hBM-MSCs, we examined the cell-protective effects in BIX01294-treated hBM-MSCs. BIX01294 hBM-MSCs were exposed to H_2O_2 (0-1.5 mM) for 1 h and then subjected to a cell viability assay. The viability of BIX01294 hBM-MSCs was significantly greater than that of nontreated hBM-MSCs (Control hBM-MSCs) (Figure 5A; *t-test,* $^{*}P < 0.05$, $^{\#}P < 0.01$, mean ± SD, n = 5). To confirm the protective effects at the protein level, immunoblot analysis was performed with antibodies against cleaved caspase-3 and β-actin, and consistent results were obtained (Figure 5B).

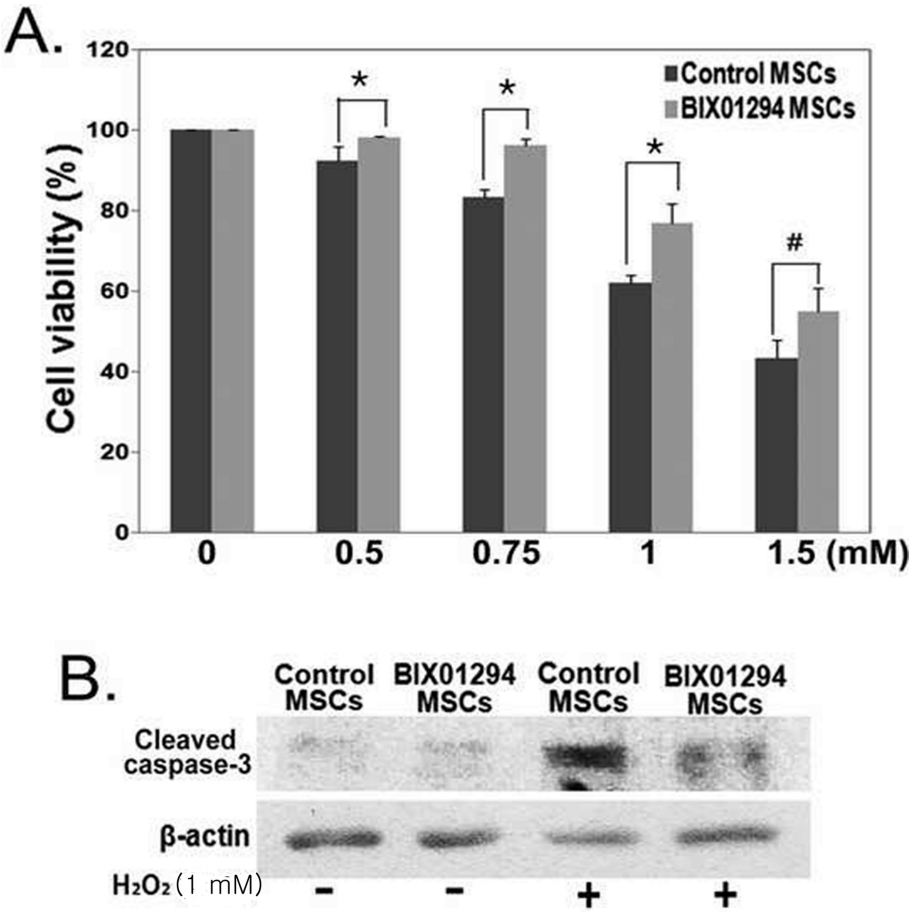

Figure 5. Protective effects of BIX01294 in hBM-MSCs.

(A) BIX01294-pretreated hBM-MSCs were exposed to 0-1.5 mM H_2O_2 and the cell viabilities were examined by MTT assay. (B) Immunoblot analysis was performed with antibodies specific for cleaved caspase-3 or β-actin.

IV. Discussion

G9a has been studied as a therapeutic target for cancers [18, 19]. However, the G9a inhibitor BIX01294 improves stem cell potency [24, 26] and reprogramming efficiency [25], suggesting that the effects of BIX01294 may vary depending on cell type, treatment dose, and exposure time. In this study, we assessed the effects of BIX01294 under various doses and times using human BM-MSCs (Figure 1). We identified that a 12-h treatment at a low micromolar concentration of BIX01294 (1 with or without 1 μM) decreased the expression of the senescent genes p53 and p21, whereas high doses of BIX01294 and longer treatment times did not (Figure 1). With further investigation under the determined conditions (1 with or without 1 μM BIX01294 for 12 h), we observed increasing expressions of the antisenescent factors bFGF, TERT, Oct-4, and VEGF, and decreasing expressions of the senescent factors p21, p16, and p53 in BIX01294-treated hBM-MSCs (Figure 2). These data suggest the possibility of antisenescent effects in BIX01294-treated hBM-MSCs. It is well known that increasing replicative senescence caused by continuing subcultivation can induce the expression of the senescence-associated serial proteins p53, p16, and p21 [31]. Since we have shown that low doses of BIX01294 modulated the expression of senescence-associated factors in hBM-MSCs (Figure 2), BIX01294 hBM-MSCs may attenuate cellular senescence in hBM-MSCs. To examine this, SA-β-gal assays were performed on BIX01294-treated hBM-MSCs. The number of senescent cells was lower in BIX01294-treated hBM-MSCs than in the nontreated hBM-MSCs (Control hBM-MSCs; Figures 3A and 3B). Similar results were obtained in long-term cultures of hBM-MSCs (P-12, P-17; Figures 3C and 3D).

Migration and protective ability are also important factors for stem cell potency. In cell therapy, injected stem cells migrate toward the site of injured cells [32] and protect themselves from inflammatory attacks [33]. I showed that BIX01294 induced the expression of antisenescent proteins and decreased prosenescent proteins, suggesting that BIX01294 treatment may improve the migration and protection of

hBM-MSCs. As shown in Figures 4 and 5, BIX01294 treatment increased the migration ability (Figure 4) and protective effects against damage induced by oxidative stress in the hBM-MSCs (Figures 5). Thus, BIX01294 induces antisenescent effects by transcriptional modulations of senescence associated genes. We have described the antisenescent effects in BIX01294-treated hBM-MSCs. However, there are limitations to this study; we were unable to confirm whether or not this modulation is direct. Based on the functional mechanism of BIX01294 as a histone methyltransferase inhibitor, it is possible that BIX01294 induction acts to decrease the number of dimethylated histones on their chromatin, thereby activating many gene loci and regulating the expression of various genes. Therefore, global genomic analysis of BIX01294 hBM-MSCs may provide a better picture of how the global genes are modulated by BIX01294, which may offer clues to understanding the overall mechanisms of BIX01294.

Conclusion

I have investigated the antisenescent effects of BIX01294 in hBM-MSCs and confirmed its attenuation of cellular senescence. The antisenescent effects of BIX01294 were achieved through the expression of senescence-related genes. An optimized dose of BIX01294 may improve stem cell potency and cellular senescence, which may provide better efficacy in stem cell therapy.

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감 사 의 글

꿈과 열정적인 마음을 가지고 실험실 문을 열고 들어 온지 벌써 3년 5개월의 시간이 지났습니다. 마무리하는 시간이 가까워졌다는 사실이 믿겨지지 않았지만 저에게는 꿈을 향해 달려가는 잊지 못할 시간들 이였습니다. 꿈을 위한 시간들을 잘 보내게 해주신 소중한 분들에게 이 자리를 들어 감사 인사를 드리려 합니다.

먼저, 전과하여 전공적인 지식도 많이 부족하였지만 많은 부분을 채워주시고, 항상 자신감을 주셨던 지도교수님, 조광원 교수님께 감사의 인사드립니다.

학부 생 때 실험에 흥미를 갖게 해주시고, 좋은 말씀 많이 해주신 이준식 교수님, 생물학의 필수적인 지식을 많이 가르쳐주신 전택중 교수님께 감사 인사드립니다.

학문에 대한 넓은 시야를 갖게 해주신 늘 인자하신 윤성명 교수님, 박현용 교수님, 송상기 교수님, 이현화 교수님, 정민주 교수님께도 감사 인사드립니다.

오랜 기간 동안 함께 해준 소중한 실험실 식구들 부족한 저를 많이 도와주신 신구 오빠, 친구처럼 이야기도 잘 들어주고, 착한 호태 오빠. 오빠와 함께 실험실 생활을 하면서 많은 도움도 받고 너무 고마웠어. 앞으로도 하는 일 모두 잘 되길 기도할게! 동생이지만 언니 같고, 말도 잘 통하는 우리 다희야 언니가 진짜 너 많이 좋아하는 거 알지? 더 가까워질 테니 자주 만나자. 공부도 잘하고 똑똑한 영찬이, 실험실 귀염둥이 막내 향이야 만난 지 얼마 안 되서 떠나지만 너와 함께 지내서 좋았어! 실험실 생활 절반 이상을 함께한 윤서오빠, 항상 힘나는 말 많이 해주고, 고민도 많이 잘 들어줘서 감사했어요. 우리 이슬언니! 언니가 있어서 실험실 생활하는 동안 정말 행복했어요. 지금도 저를 위한 충고와 위로 감사해유 진짜 최고 사랑해요 저두 언니 응원 많이 하고 있어요! 그리고 대학원 동기인 혜선아 너는 모든 일에 최선을 다하는 친구니깐 박사학위도 잘 해낼 거야 멀리서도 응원할게! 학부생 때부터 이야기도 잘 들어주고, 힘이 되어준 푸름아 고마워, 승미야 나도 너에게 도움만 받았던 거 같은데 너가 졸업하고 난 뒤엔 허전 하더라 우리 자주 하는 말처럼 파이팅 하자 우리!

언제나 내 편에 서서 힘이 되어주고 함께 해준 내 소중한 사람들 달해 언니, 지은이, 수민이, 양유정, 박유정, 연송이, 승하, 하경, 세나, 경연, 설아, 미혜똥, 애라똥, 네 명 민뚜루미 그리고 여섯 명 보름달 친구들에게 정말 내 곁에 있어줘서 고맙고 사랑한

다는 말 전하고 싶습니다. 좋은 말로 용기와 힘을 주고 힘들 때 든든하게 내 옆에 있어줘서 고맙고, 다정한 대영아. 각자 하는 일 최선을 다해 열심히 하고, 지금처럼 사이좋게 오래 오래 잘 지내자 ♡

마지막으로, 지금 이 순간에도 항상 사랑해주시고, 아낌없는 지원 해주시는 사랑하는 우리 부모님, 앞으로도 노력해서 좋은 모습만 보여 드릴게요! 정말 사랑합니다. 지금 군대에서 고생하고 있는 멋진 내 동생, 떨어져 있지만 너의 존재만으로도 누나에겐 큰 힘이 되었어. 너무 고맙고 사랑한다.

여기에 다 적진 못했지만 공부하는 동안 많은 분들의 도움으로 힘을 얻어 열심히 하여 석사학위까지 마무리를 잘 할 수 있었던 것 같습니다.

모두 정말 정말 감사하고 사랑합니다.