이학석사 학위논문

간엽줄기세포 증식 조절에서 CIP2A의 역할

Role of CIP2A in regulation of mesenchymal stromal cells proliferation

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간엽줄기세포 증식 조절에서

CIP2A의 역할

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2018 년 8 월

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Abstract

Mesenchymal stromal cells (MSCs) are multipotent and are widely used as cell therapy agents for the treatment of many diseases such as neurodegenerative diseases, cartilage damage, Parkinson's disease. However, mesenchymal stromal cells are difficult to expand in large amounts of healthy cells because aging progresses rapidly and cells are sensitive to the environment when the subculture is repeated several times. We could solve this problem by introducing the CIP2A gene into MSCs.

CIP2A is an endogenous inhibitor of protein phosphatase 2 (PP2A), a highly expressed protein in cancer cells, but also has recently been shown to be highly expressed in precursor cells and related to cell proliferation. Based on the findings that CIP2A in MSCs is more abundant than normal cells and decreased by subculture, we hypothesize that CIP2A is involved in the proliferation of MSCs.

When CIP2A inhibitor celastrol was treated, the decrease of mTOR –related proteins and proliferation were observed when the expression level of CIP2A was declined. We found reduced proliferation when we knocked out CIP2A. Conversely, overexpressed CIP2A at the gene level increased proliferation, confirming that CIP2A is involved in the proliferation of MSCs. Anti-inflammatory and angiogenic genes of MSC and migration were enhanced by overexpression of CIP2A. Through in vivo wound healing assay, we confirmed that CIP2A is involved in the therapeutic effect of MSCs.

In conclusion, through these changes caused by expression of CIP2A we identified that CIP2A could affect proliferation and therapeutic effect.

the oncoprotein CIP2A, which is not well known as a target gene to increase the proliferation and therapeutic effect of MSC, will be an important research that can overcome the limitation of MSC as a cell therapy agent.

Key words : mesenchymal stromal cells, proliferation , CIP2A

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Introduction

Mesenchymal stromal cells (MSCs) have multipotent stem cells and exist umbilical cord, bone marrow, and fat tissues.[1] MSCs play a role in tissue maintenance and refresh themselves process also called self-renewal.[2] And also though division, they do self-regeneration and differentiate to bone, cartilage, muscle and fat tissues.[3] As a cell therapy agent, MSCs functions as an immunomodulator of immune cell, effectors of tissue repair and homing to injury or inflammation sites make to try many clinical trials.[4,5] In addition, MSCs produce various cytokine, growth factors that can get better for proliferation and treatment of damaged cells.[6] Also MSCs avoid the ethical issues of embryonic stem cells, as they can be derived from sources that include adult bone marrow and adipose tissue.[7,8] Advanced knowledge exists for how to grow MSCs in culture, including protocols for isolation, expansion.[9] For these reasons, MSCs have been used cell therapy than other stem cells.

Cell therapy has been studying because of treatment of fundamental treatment of disease using live cells.[10] But new mechanism different from existing agents bring some problems. Major problems are decreasing proliferations of MSCs processing subculture and sensitivity about culture environment.[11] Replicative senescence, MSCs gradual decreasing that the capacity of proliferation after repeated replication is irreversible cell cycle arrest that reduces the proliferation of damaged cell.[12] These issues make commercialization of MSCs difficult. Recently advanced culture skills and gene editing are presented as solution. However, there is still insufficient research on how to maintain mesenchymal stem cell characteristics and increase cell proliferation rate. Much research is needed, as not only the

efficacy of new functional groups but also the unexpected variety of problems in existing therapeutic agents.

Cancerous Inhibitor of Protein phosphatase 2A (CIP2A) is a 90 kDa protein that found as an inhibitor of protein phosphatase 2A (PP2A), Ser/Thr phosphatase that involved in negative control of cell growth and division.[13] Consistent with the important role of PP2A in regulating various phosphorylation-dependent pathways. CIP2A inhibits PP2A activity towards transcription factor Myc and thereby prevent Myc proteolytic degradation. Also CIP2A regulates phosphorylation and activity of many critical signaling proteins in cancer, including Myc, E2F1 and mTORC1-dependent growth signaling. Many cancer cells including head and neck squamous cell carcinoma, colon cancer and breast cancer express higher CIP2A mRNA and protein than healthy cells.[14] More recently CIP2A has been shown to regulate phosphorylation and activity of many other oncoproteins and to drive malignant cell growth and tumorigenesis in various human cancer types.[15] And surprisingly, Studies have shown that CIP2A is also expressed at high concentrations in progenitor cells. Both Myc and CIP2A enhance each other' s expression and increase self-renewal and proliferation thus indicating a functional connection between CIP2A and Myc in neural progenitor cells.[16] Also in spermatogonial progenitor cells, CIP2A is co-expressed with ki67 and with self-renewal protein PLZF.[17]

Through these studies, we verified CIP2A was involved in the proliferation of cancer cells as well as progenitor cells. We hypothesized that CIP2A also plays a role in MSCs. But Whether CIP2A is involved in the spread of MSC is little known.

There is no specific study yet on the MSCs, we conducted this study to investigate the role of CIP2A in the MSCs. We identified the expression of CIP2A in MSC and found changes in CIP2A in various situations. We then examined the changes in proliferation and CIP2A-related proteins and proliferation-related proteins. MSCs Also we confirmed migration and major gene expression related to therapeutic effect of MSC.

Materials and Methods

Reagent

Celastrol was purchased from Sigma-Aldrich (C0869-10mg). A stock solution made in 5mM is prepared using DMSO and aliquoted at -80℃.

Cell culture

The mesenchymal stem cells from umbilical cord blood derived MSCs by Medi-post(Korea). Culture condition was maintained at 37℃ in a humidified atmosphere containing 5% CO_2 incubator. The cells were plated with culture medium α-MEM (Gibco) supplemented 10% FBS(Gibco) and 100μg penicillin and streptomycin(welgene) in T175 flask. After 3~4days, subculture performed when the cell reached 80% confluence.

Cell proliferation

The proliferation of cells is determined by colorimetric assay using the CCK-8 kit (dojindo). Cells were seeded in 96 well plates for 1000 cells per well. When confluence reached 80%, CIP2A inhibitor was treated. After one day, media removed, the CCK-8 solution is treated with new culture media. After 4h incubation, the absorbance is measured at 450nm using microplate reader.

Knock-out of CIP2A

Designed gRNA with PAM sequence was liagated with CRIPSPR vector. Vector was packaged in lenti virus. Transfection was conducted with 293T. After 2 day, supernatants containing lentvirus was harvested and infected

with the prepared MSC. The stable cell lines were selected in the presence of 2.5 μg/ml puromycin .

T7E1 assay

The target locus where nuclease cleavage was expected was amplified via PCR. Annealing was performed with 20 μl of purified PCR product and 3 μl of NEB2 buffer (New England Biolabs) using the thermocycler program. After oligo formation, 2 μl of T7E1 (New England Biolabs) was added and the reaction was incubated for 1hr at 37° C. Product was mixed with loading dye and loaded on a 2% agarose gel. Band strengths were measured with the Bio Rad Gel Doc EZ Imager and Image Lab(Bio Rad).

Overexpression of CIP2A

The plenti-su-CMV-CIP2A expression plasmid was made. Transfection of the plenti-su-CMV-CIP2A plasmid into 293 cells were carried out using neon electroporation system. The stable cell lines were selected in the presence of 2.5 μg/ml puromycin .

RNA isolation and cDNA synthesis

Total RNA was isolated from MSCs using Trizol reagent (Invitrogen) according to the manufacturer's protocols and quantified using take3. 1000 ng of total RNA was reverse-transcripted to cDNA using oligo dT primers using TOPscript cDNA synthesis lit(enzynomics).

Quantitative Real-time PCR

Using TOPreal qPCR premix(enzynomics), qRT-PCR was performed on CFX Connect real-time PCR detection system (Bio-Rad). Cycle was 45 cycles. Quantification was carried out by correcting for amplication efficiency of the primer using a standard curve, followed by nomalizing gene levels to the amount of expressed GAPDH gene. All used primers are listed in table1 .

Western blot

the sample cells were lysed into RIPA buffer. Lysates were sonicated and incubated on ice for 10 min, spinned at 12,000 g for 10 min and the protein content was quantified by using the BCA Protein Assay Kit (PIERCE). Laemli sample buffer was added and the samples were boiled for 3 min prior to loading of the samples to SDS-PAGE gels (10 μg per lane). Proteins were transferred to a nitrocellulose blotting membrane(GE Healthcare). The membrane was blocked in TBST with 5% bovine serum.

Migration assay

Using Scarblock (SPL), 100ul of cell is seeded each block.

When the confluence reaches 90 to 100%, treat the inhibitor and incubate one more day. Subsequently, the block is removed, replaced with cerum free media, cultured for one day, and cells counted and captured by camera.

in vivo wound-healing assay

Remove hair from the mice the day before surgery. On the day of surgery, anesthetize mice. punch through the folded skin (both layers) with a 5-mm-diameter sterile biopsy punch to create two symmetrical full-thickness

excisional wounds besides the midline. We MSCs into the dermis at four sites around the wound. We also topically apply 0.3 × 106 MSCs in Matrigel onto the wound. Secure the splint to the skin with four interrupted sutures. Completely cover the wounds and splints with Tegaderm (3M) sterile transparent dressing. Dress the wounds with self-adhering elastic bandage. Uncover wound bandages and take photographs of individual wounds on days 3, 7, 10 and 14.

Table 1 . quantitative RT-PCR primer sequences of MSCs stemness, proliferation and angiogenesis

GENE		PRIMER SEQUENCE
OCT4	F	TCAGGTTGGACTGGGCCTAGT
	R	GGAGGTTCCCTCTGAGTTGCTT
NANOG	F	GAAATCCCTTCCCTCGCCATC
	R	CTCAGTAGCAGACCCTTGTAAGC
ANG1	F	GCCTACACTTTCATTCTTCCAGA
	R	TCTTCCTTGTGTTTTCCTTCCAT
VEGF	F	ATGAACTTTCTGCTGTCTTGGGT
	R	TGGCCTTGGTGAGGTTTGATCC
TSG-6	F	ACGATGTCCACGGCTTTGTAGG
	R	GACGCATCACAAACTTCAAGG
IDO	F	AGGATCCTTGAAGACCACCA
	R	CCAATAGAGAGACGAGGAAG
GAPDH	F	GCCAAGGTCATCCATGACAACTTTGG
	R	GCCTGCTTCACCACCTTCTTGATGTC

Results

CIP2A is expressed in human mesenchymal stromal cells

To provide an understanding of the biological function of CIP2A in MSCs, the endogenous expression of CIP2A and association with cell proliferation in MSCs are analyzed. Expression of CIP2A was increased in MSCs isolated from different donors than in fibroblast cells (MRC5, HS68), which is a common negative control for stem cell differentiation and stemness analysis (Fig1 A). Next, to confirm its association with cell proliferation, I observed the expression of CIP2A in the cellular senescence stage, where cell proliferation is reduced. CIP2A expression was gradually decreased in replicative senescent MSCs by continuous subculture (Fig 1B). CIP2A expression was dose-dependently reduced by treatment of doxorubicin, which induces premature senescence due to DNA damage (Fig 1C). p21 was used as cellular senescence markers. These results suggest that CIP2A is predominantly expressed in MSCs and is also associated with MSC proliferation.

Pharmacological and genetic inhibition of CIP2A reduce MSC proliferation

Celastrol, the natural inhibitor of CIP2A, inhibits cell proliferation and migration in various cells such as non-small-cell lung cancer cells (NSCLC), chondrosarcoma[18,19]. It binds directly to CIP2A protein and induces CIP2A proteasomal degradation by promoting its interaction with the ubiquitin E3 ligase CHIP[19]. To examine the significance of CIP2A in MSC proliferation, Celastrol (0.6 ug/ml) was treated for 24 hours. Celastrol reduced CIP2A

protein level as expected (Fig 2C). Consistent with reduced of CIP2A protein, visual cell imaging with microscopes showed decrease in cell confluence (Fig 2A). Cell proliferation measured by CCk-8 significantly decreased after treatment with celastrol (Fig 2B). CIP2A has been reported to regulate phosphorylation and activity of c-MYC, E2F1 and mTORC[20]. degradation of CIP2A protein by celastrol treatment inhibited phosphorylation of E2F, mTOR target proteins (p70S6K, 4EBP1) and E2F1, which are CIP2A activity related proteins. In addition, survivin and Cyclin D1 expression, which are proteins involved in regulation of proliferation and function in MSCs, were reduced (Fig 2C). To confirm efficacy of MSC proliferation by inhibition of CIP2A, I generated a CIP2A knockout cell in which CIP2A was genetically removed using the CRISPR/Cas9 system. I performed the T7E1 assay to measure insertion / deletion efficacy at target site of CIP2A (Fig 3A). Inhibition of endogenous CIP2A expression was also confirmed in CIP2A knockout MSCs using western blot (Fig 3C). Consistent with pharmacological inhibition by celastrol, proliferation of CIP2A knockout MSCs was reduced than nontarget MSCs (Fig 3 B). CIP2A-regulated proteins (p-4EBP1, p-p70S6K) and regulatory proteins of MSC proliferation (survivin, CyclinD1) were reduced in CIP2A knockout MSCs (Fig 3C). These data indicate that inhibition of CIP2A reduced MSC proliferation.

CIP2A overexpression in MSCs increase MSC proliferation and stemness

Inhibition of CIP2A reduces MSC proliferation. Conversely, I tried to investigate the effect of CIP2A on MSC proliferation when CIP2A was

overexpressed. To generate CIP2A overexpressed MSCs using the lentivirus system, plenti-suCMV /hu CIP2A plasmid was constructed by inserting CIP2A cDNA into plenti-suCMV vector. It was confirmed that the plenti-suCMV/hu CIP2A plasmid was well constructed through restriction enzyme digestion analysis (NheI, ClaI) (Fig 4A). The expression of CIP2A was significantly increased in CIP2A overexpressed MSCs. In addition, the expression of CIP2A-regulated proteins (p-c-Myc, p-4EBP1, p-p70S6K) and regulatory proteins of MSC proliferation (survivin, CyclinD1) were increased (Fig 4C). As expected, proliferation of CIP2A overexpressed MSCs was increased than vector control MSCs (Fig 4B). To investigate the effect of CIP2A on maintaining the stemness of MSCs, I quantitatively analyzed the relative mRNA expression of representative pluripotency-associated transcription factors such as Nanog and OCT4. mRNA Expression levels of all stemness markers in CIP2A overexpressed MSCs were upregulated (Fig 4D). These results suggest that CIP2A regulates the proliferation of MSCs and is involved in stemness regulation.

CIP2A regulates MSC migration

MSCs are associated with tissue regeneration and repair and have been reported to improve wound healing in preclinical studies[21]. Migration of the MSCs to the damaged site is important for tissue repair. To test whether CIP2A regulates MSC movement to the wound, I measured MSC migration after celastrol treatment. In vitro migration assay, treatment with celastrol for 24 hours reduced the number of MSCs that migrated into the wound wounded regions. (Fig 5A). Conversely, migration of CIP2A overexpressed

MSCs was increased to the wound site than vector control MSCs (Fig 5B). These results suggest that CIP2A regulates the migration of MSCs.

CIP2A overexpressed MSCs promote Wound healing in the mouse excisional wound splinting model

MSCs promotes wound closure, increase angiogenesis, resolution of wound inflammation, favorably regulate extracellular matrix remodeling, and encourage regeneration of skin with normal architecture and function. The mouse excisional wound healing model is widespread in studying wound healing and skin regeneration but fails to reflect the wound healing process occurring in humans. In rodents where skin can move, contraction is a major part of the wound closure. In humans, where skin is tethered to subcutaneous tissue, the generation of a new tissue heals the wound. In the mouse excisional wound splinting model, the splinting ring tightly attaches to the skin around the wound to prevent localized skin contraction. Hence, wounds are treated through child-rearing and re-epithelization, a process similar to that occurring in humans[22]. To observe wound healing efficacy of CIP2A overexpressed MSCs in the mouse excisional wound splinting model, I implanted equal number of CIP2A overexpressed MSCs or vector control MSCs into excisional wounds in BALB/c mice. CIP2A overexpressed MSC-treated wounds exhibited a slight effect on improving wound closure compared to vector control MSC-treated wounds at 14 days (Fig 6A, 6B).

CIP2A upregulates expression of genes related to wound healing in MSCs

MSCs secrete a wide range of factors with therapeutic potential including growth factors, cytokines and chemokines. Paracrine factors are secreted by MSCs play a vital role in each of the phases of wound healing process including homeostasis, inflammation, proliferation, contraction and remodeling[21]. To understand the wound healing mechanism of CIP2A, I analyzed growth factors and cytokines related with wound healing secreted by MSCs. Angiopoietin-1 (ANG-1) and Vascular endothelial growth factor (VEGF) have been reported to promote angiogenesis and wound closure[23]. mRNA Expression levels of ANG-1, VEGF in CIP2A overexpressed MSCs were upregulated (Fig 7A). Tumor necrosis factor-α (TNF-α) stimulated protein-6 (TSG-6) and Indoleamine 2, 3-dioxygenase 1 (IDO-1), which are anti-inflammatory cytokines released when MSCs are exposed to an inflammatory environment, are known to improve wound healing by limiting activation of immune cells (macrophages, T cells), inflammation and fibrosis[24]. The mRNA expression of TSG-6 and IDO-1, which were increased by poly IC, was upregulated in CIP2A overexpressed cells. Increased expression of therapeutic factors in CIP2A overexpressed cells suggests that CIP2A is involved in the wound healing mechanism of MSCs.

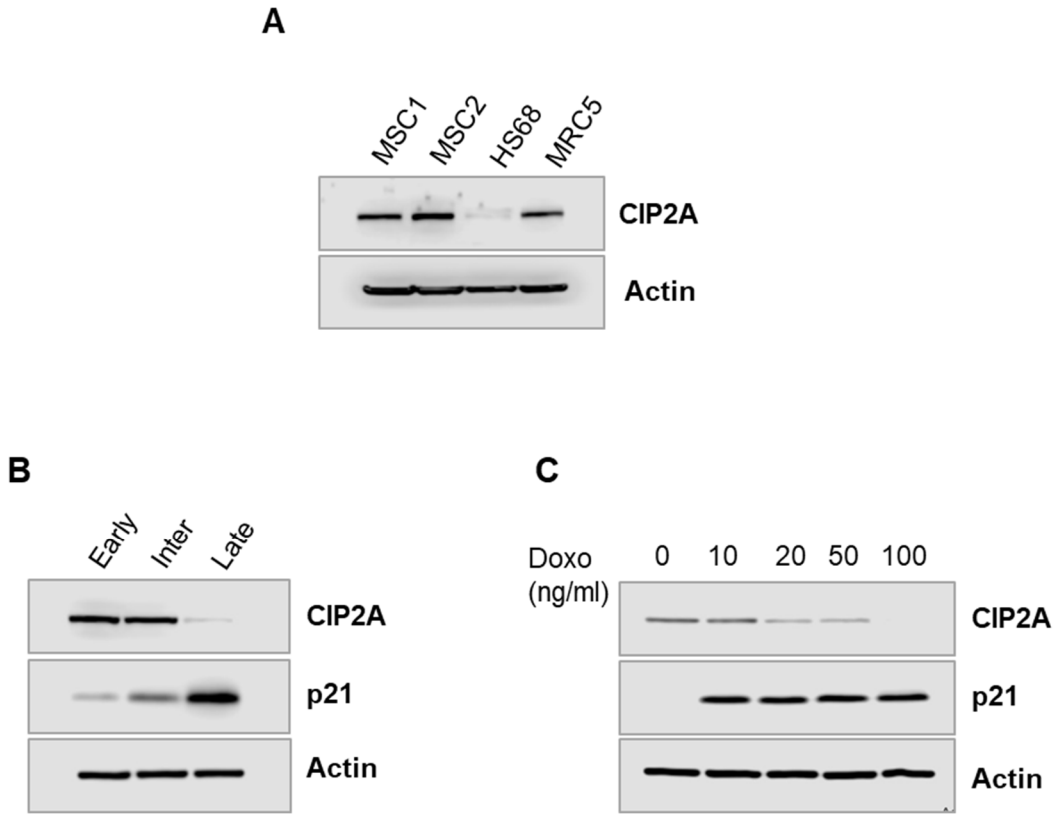

Figure 1. CIP2A is expressed in human mesenchymal stromal cells and related to proliferation of MSCs.

(A) Western blot analysis of CIP2A expression in human fore skin fibroblast HS68, human lung fibroblast MRC5 and MSCs from different donors. (B) Western blot analysis of CIP2A expression at each replicative senescence stage of MSCs. (C) Western blot analysis of CIP2A expression in MSCs treated with doxorubicin at indicated concentrations for 24h.

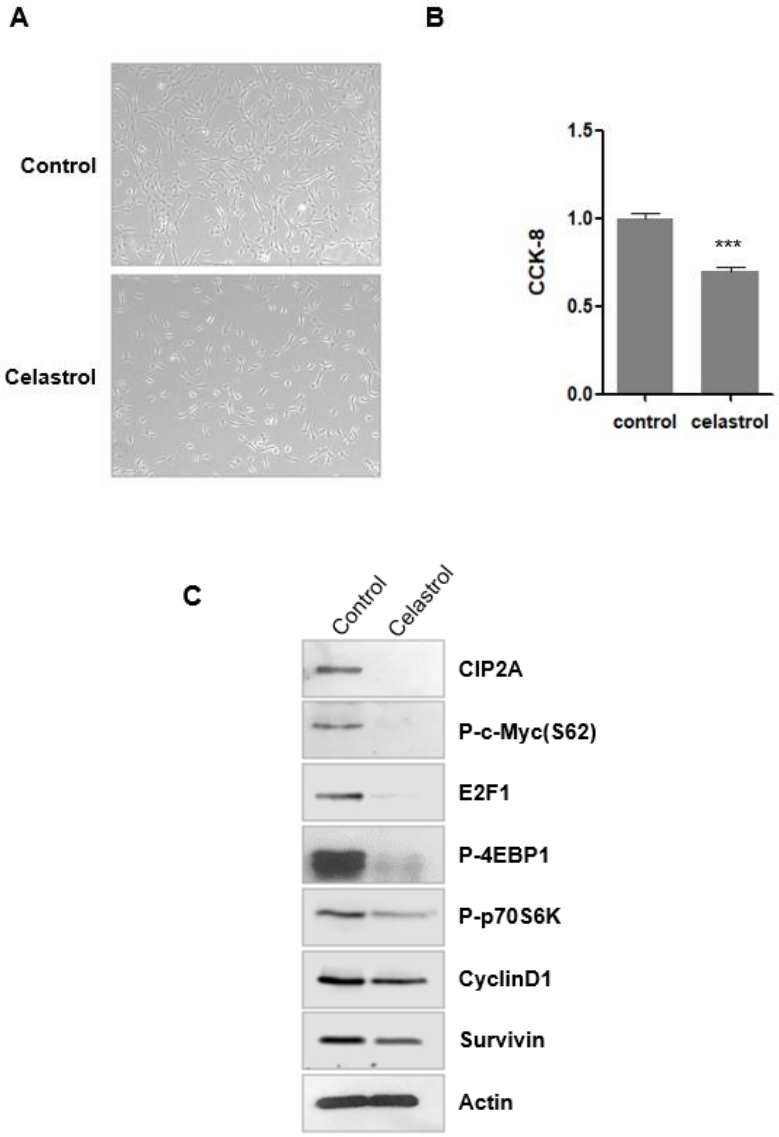

Figure2. CIP2A inhibitor reduced MSCs proliferation.

(A-C) MSCs were treated with celastrol 0.6ug/ml for 24h. (A) Light microscopic images (magnification: X50). (B) CCK-8 proliferation assay. (C) Western blot analysis using indicated antibodies. Results are representative of the mean of three independent experiments (mean±SD, n=3, ***p<0.001).

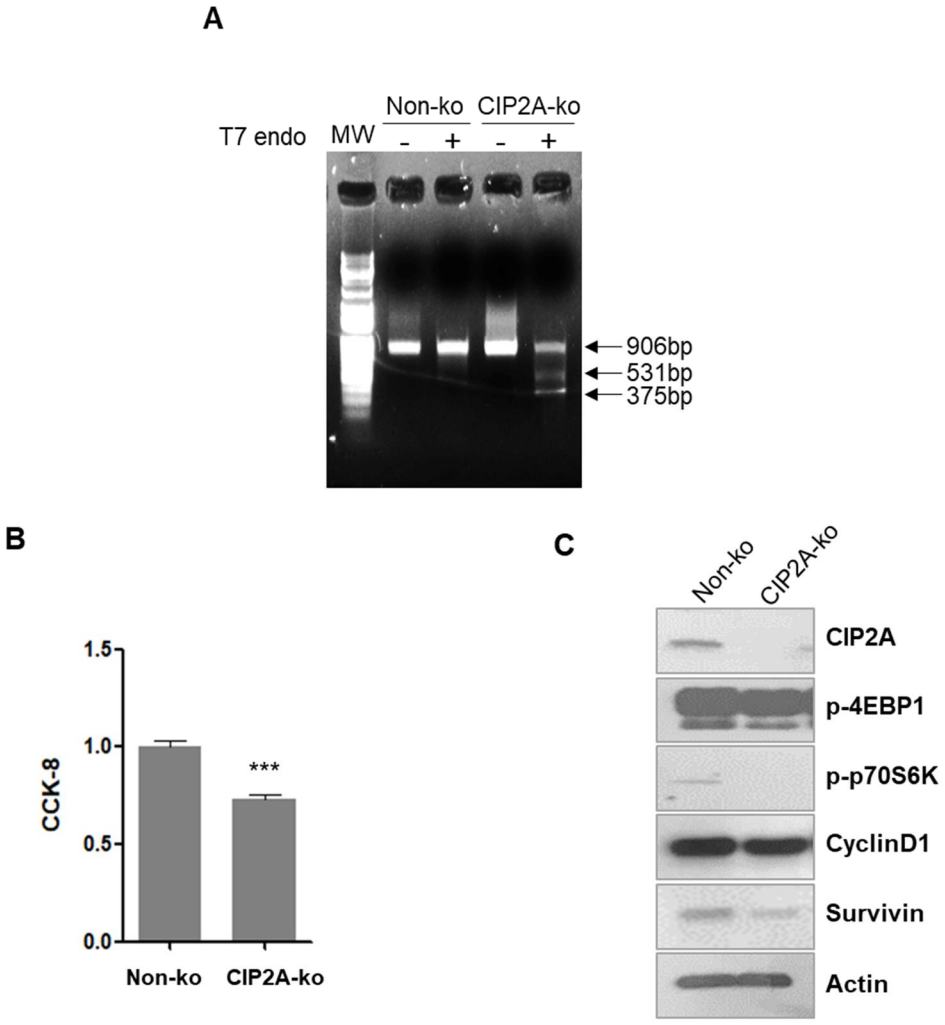

Figure 3. CIP2A knockout reduce MSC proliferation

(A) T7E1 assay of CIP2A knockout MSCs. (B) CCK-8 proliferation assay. (C) Western blot analysis using indicated antibodies. Error bars represent the mean±SD, n=3, ***p<0.001. Abbreviations: Non-ko, nontarget MSCs; CIP2A-ko, CIP2A knock-out MSCs.

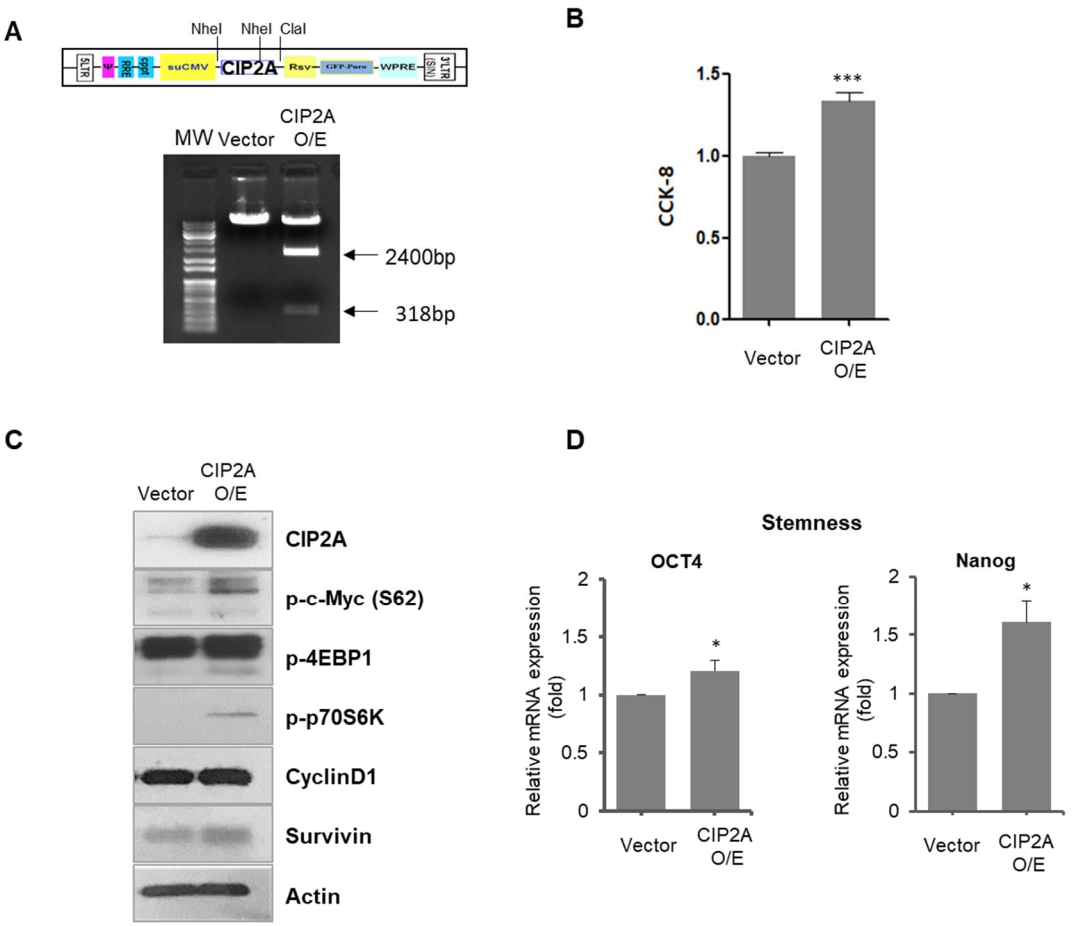

Figure 4. CIP2A overexpression increase MSC proliferation and stemness (A) Structure (top) and restriction enzyme digestion analysis (bottom) of plenti-suCMV/hu CIP2A plasmid. (B) CCK-8 proliferation assay. (C) Western blot analysis using indicated antibodies. (D) Quantitative real time PCR analysis for stemness genes expression. The expression level of each gene was normalized to the of GAPDH. Error bars represent the mean±SD, n=3, ***p<0.001. Abbreviations: Vector, vector control MSCs; CIP2A O/E, CIP2A overexpressed MSCs.

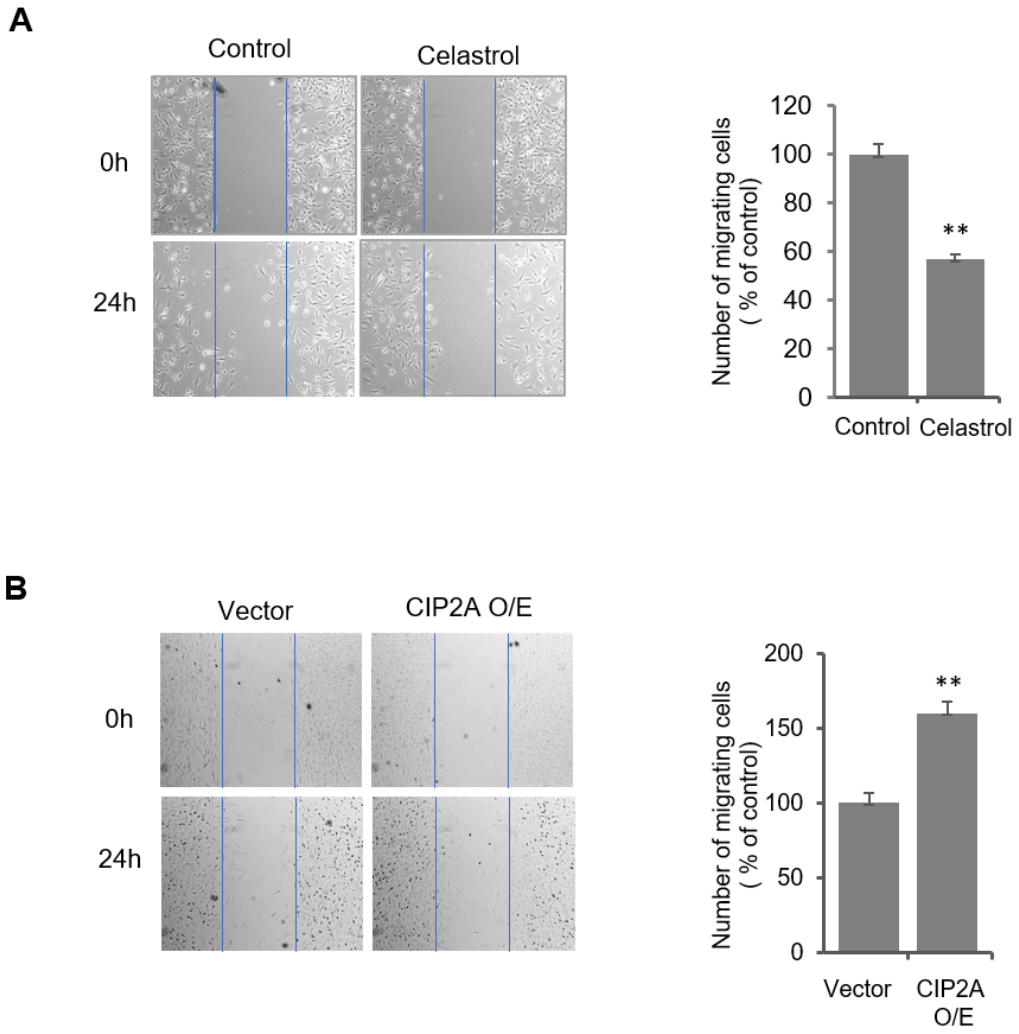

Figure 5. CIP2A regulates MSC migration.

(A-B) In vitro migration assay of celastrol-treated MSCs (A) and CIP2A overexpressed MSCs (B). Images of the migration area were captured 0 h and 24 h after removal of the Silicon block. The number of cells that moved beyond the baseline was counted based on 0h. Error bars represent the mean±SD, n= 5 to 8, ***p<0.001

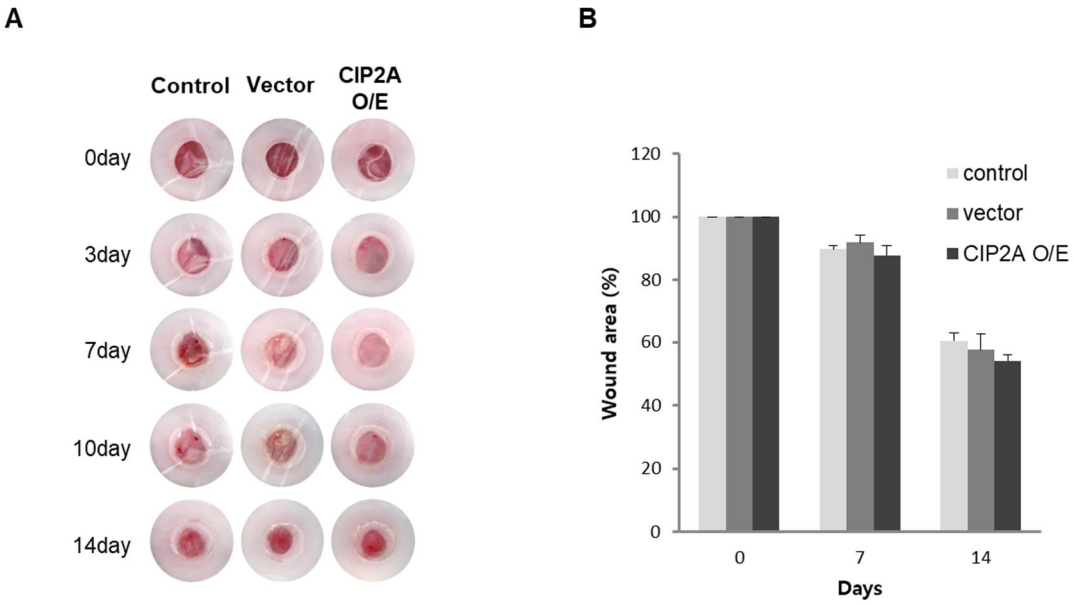

Figure 6. CIP2A overexpressed MSCs promote wound healing in the mouse excisional wound splinting model

(A) Representative images of the excisional wound splinting mouse model after transplantations of vehicle medium (control), vector control MSCs (Vector) and CIP2A overexpressed MSCs (CIP2A O/E) at 0, 3, 7, 10 and 14 days. (B) Wound measurements of each group in mice. Error bars represent the mean±SD, n=4 to 8 per group.

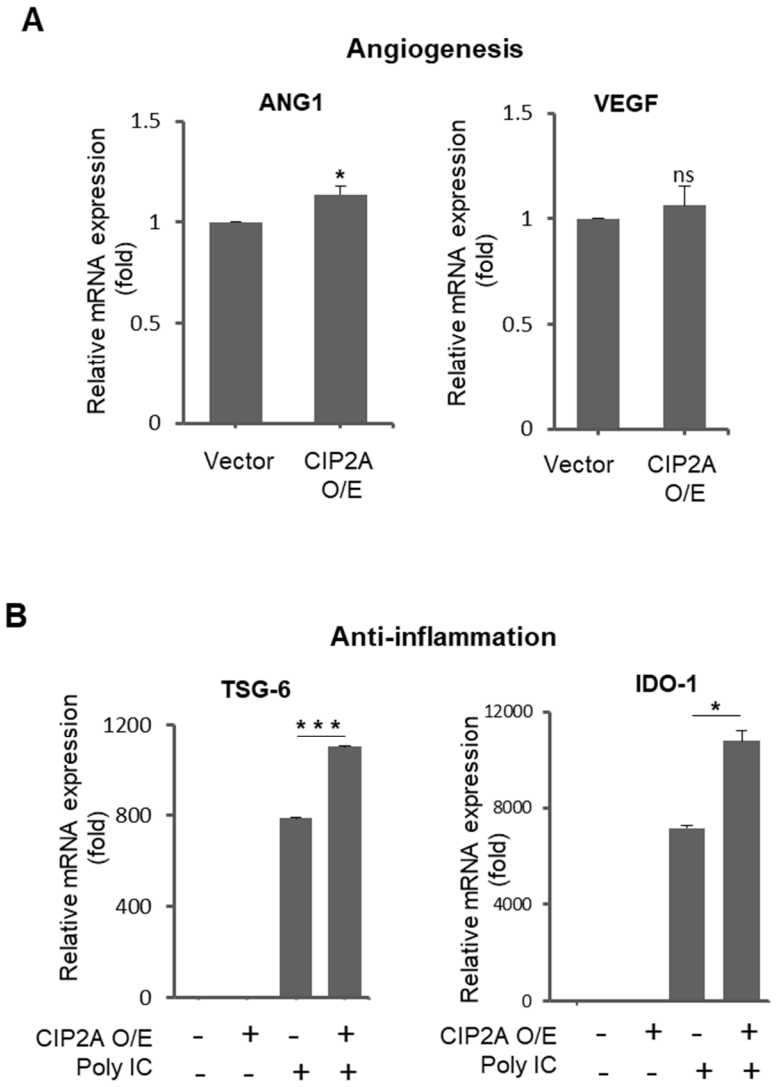

Figure 7. CIP2A upregulates expression of genes *related* with wound healing

(A) Quantitative real time PCR analysis for angiogenesis associated genes expression in CIP2A overexpressed MSCs. (B) Quantitative real time PCR analysis for anti-inflammation associated genes expression in CIP2A overexpressed MSCs. The expression level of each gene was normalized to the of GAPDH. Error bars represent the mean±SD, n=3, *p<0.05, ***p<0.001.

Discussion

This study suggested that CIP2A is involved in the proliferation of MSCs. CIP2A is also involved in various pathways such as cell cycle, CREB, mTOR, and myc in cancer.[25] For example, CIP2A modulates the cell cycle by regulating the expression and migration of p27Kip1 in breast cancer[26], and also p27Kip1 is involved in quiescence in stem cells[27]. Myc, which has been described in this study, is involved in malignant cellular growth in cancer cells, but maintains pluripotency and self-renewal in embryonic stem cell[28]. These results suggest that CIP2A is involved in proliferation because MSC is also involved in the pathway known only to cancer cells in MSC.

CIP2A has both effects that brought out by PP2A-dependent and PP2A-independent. CIP2A interacts with Myc, inhibits PP2A activity toward Myc S62, and stabilizes Myc S62. Transcription factors including Oct4 ,E2F1 and enzymes like AKT, mTOR are PP2A-dependent CIP2A targets. There are also PP2A-independent effects of CIP2A ; changing the stability, localization and activity of PLK1, enhancing NEK2 kinase activity to facilitate centrosome separation and increasing self-renewal of neural progenitor cells[29,30,31]. In this study, Whether the results about CIP2A in this study were PP2A-dependent or independent is unknown. Further studies will help identify whether the effect of CIP2A on MSC proliferation is PP2A dependent or not.

The findings of this study that CIP2A overexpression increases MSCs' proliferative potential, stemness, and induces the expression of genes including survivin, TSG-6, IDO-1 and Ang1 in MSCs will be an important beginning in the future study of CIP2A.

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국문 요약

중간엽 줄기세포는 다능성이며 신경 퇴행성 질환, 연골 손상, 파킨슨 병과 같은 많은 질병의 근본적인 치료를 위한 세포 치료제로 널리 연구되고 있다. 하지만 중간엽의 줄기세포는 계대배양으로 인한 스트레스와 배양환경에 쉽게 영향을 받기때문에 치료제로 개발될 수 있을 만큼의 건강한 세포를 다량 확보하기 어렵다. 이 연구에서는 MSC 에 CIP2A 유전자를 도입함으로써 이러한 문제점의 개선가능성을 살펴보고자 한다.

CIP2A 는 암세포에서 높게 발현되는 PP2A 의 내인성 저해제로 암세포에서 종양의 성장이나 악성에 연관이 있다고 알려져 있지만, 최근 전구세포에서도 발현되고 proliferation 과도 관련이 있는 것으로 연구 결과 밝혀졌다. MSC 에서 CIP2A 가 정상 세포보다 높게 발현하고, 계대 배양에 의해 감소한다는 결과를 바탕으로 CIP2A 가 MSC 의 proliferation 에 관련이 있다고 가정하고 실험을 진행하였다.

CIP2A 저해제인 celastrol 을 처리했을때 CIP2A 의 발현이 감소하면서 proliferation 의 감소와 함께 mTOR 관련 단백질과 CIP2A 의 target gene 의 감소를 확인할수 있었다. 반대로, 과다 발현 된 CIP2A 는 proliferation 을 증가시켜 CIP2A 가 MSC 의 proliferation 에 관여한다는 것을 확인했다. 또한 in vivo 와 in vitro migration assay 를 통해 migration 에도 관련이 있으며, 항염증반응과 혈관생성에 대한 관련성을 확인하였다.

최종적으로, CIP2A 가 증가하면 proliferation 뿐만 아니라 migration 을 포함한 다양한 MSC 의 양상에도 긍정적인 효과를 줄 수 있다는 것을 확인할수 있었다.

중심단어 : mesenchymal stromal cells, CIP2A, proliferation, therapeutic effect