Preconditioning of SIRT1 activator improves chondrogenic differentiation potential of mesenchymal stem cell

Seong Mi Choi

Department of Medical Science

The Graduate School, Yonsei University

Preconditioning of SIRT1 activator improves chondrogenic differentiation potential of mesenchymal stem cell

Directed by Professor Jin Woo Lee

The Master's Thesis

submitted to the Department of Medical Science,

the Graduate School of Yonsei University

in partial fulfilment of the requirements for the degree of

Master of Medical Science

Seong Mi Choi

December 2017

연세대학교
YONSEI UNIVERSITY

This certifies that the Master's Thesis

of Seong Mi Choi is approved.

Thesis Supervisor: Jin Woo Lee

Thesis Committee Member#1: Yong-Min Chun

Thesis Committee Member#2: Sung-Rae Cho

The Graduate School

Yonsei University

December 2017

ACKNOWLEDEGMENTS

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Seong Mi Choi

Department of Medical Science

The Graduate School, Yonsei University

(Directed by Professor Jin Woo Lee)

Osteoarthritis (OA) is the most common degenerative disease of joints affecting more than 70% of the aged population and can gradually lead to the deterioration of extensive areas of cartilage due to the lack of regeneration capacity. The major manifestations of OA are damage to cartilage, malfunction of chondrocyte proliferation and hypertrophic maturation. There are currently several therapies for cartilage regeneration, among them cell therapy is the most frequently used, particularly with mesenchymal stem cells (MSCs). However, cell therapy necessitates long-term expansion of MSCs, *in vitro,* and during this process, MSCs lose their self-renewal and multipotential capacity as well as undergoing hypertrophic maturation following chondrogenic differentiation. Therefore, a new strategy to enhance chondrogenic differentiation potential and regenerate hyaline cartilage is essential.

Resveratrol (RSV), a strong SIRT1 activator, is known to play critical roles in cell survival, proliferation, and multipotency of MSCs. Our previous study confirmed that when RSV is continuously delivered to MSCs from early passage with the expression of SIRT1, MSCs maintain their self-renewal, osteogenic and adipogenic differentiation potential. However, chondrogenic differentiation potential was not confirmed.

In the present study, we investigated and confirmed the chondrogenic differentiation potential of MSCs which are continuously treated with RSV. Chondrogenic markers were upregulated, when RSV was continuously delivered to MSCs compared to MSCs that were not treated with RSV. In addition, we confirmed the cartilage regeneration potential of RSV treated MSCs *in vivo.* A rabbit osteochondral defect model was used to evaluate the hyaline cartilage formation by MSCs treated with RSV. MSCs treated with RSV had improved delivery of RSV to MSCs maintained stemness similar to P1-MSCs as well as enhanced their multipotential differentiation capacity resulting in increased cartilage regeneration, *in vivo.*

Key words: chondrogenic differentiation, cartilage regeneration, resveratrol, SIRT1

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I. INTRODUCTION

Osteoarthritis (OA) is the most prevalent age-related or posttraumatic degenerative disease of joints, affecting more than 70% of the aged population, that can gradually deterioration of extensive areas of cartilage a due to the lack of regeneration capacity.[1-4] The major incidence of OA is damage joint. The major indicators of OA are damage to cartilage, malfunction of chondrocyte proliferation and hypertrophic maturation, thus the cartilage regeneration capacity is severely impaired.[5] There are currently, several types of therapy for cartilage regeneration such as techniques for bone marrow stimulation, mosaicplasty and cell based therapies.[6] Recently, cell based therapies have become regard as the most promising prospective treatment among the various strategies. Autologous chondrocyte implantation (ACI) is the most generally used approach for cartilage regeneration

that requires the *in vitro* expansion of autologous chondrocytes.[7,8] There are, however, several drawbacks to implementing this technique i.e., overall complexity, cost and loss of cartilage regeneration capacity.[8,9] Hence, a cell-based therapeutic approach using mesenchymal stem cells (MSCs) has emerged most recently as a new approach to cartilage regeneration. [10-13]

MSCs from adult tissues, with their multipotency capabilities, including chondrogenic differentiation, have been identified as a promising cell source.[14-16] MSCs also possess anti-inflammatory and immunosuppressive properties and it has been reported that the use of MSCs in clinical trials was highly successful in promoting cartilage regeneration without severe side effects.[17] Despite the proven efficacy of MSCs in several clinical trials,[17-19] there are several problems affecting their use in clinical trials. As discussed above, these include loss of self-renewal and multi-lineage differentiation potential during *in vitro* expansion, [20-23] and hypertrophic maturation following chondrogenic differentiation.[24-26] Therefore, the identification of new strategies that sustain stemness of MSCs during *in vitro* long-term expansion and preserve chondrogenic differentiation potential and regulation of hypertrophic maturation is vitally important.

Accordingly, I investigated the critical environments which can sustain the self-renewal and multi-lineage differentiation capacities of the cells. There are several possible strategies to enhance stemness of MSCs such as genetic modification,[27] scaffolds as a carrier[27] and growth factor treatment.[28] However, these methods had some disadvantages including safety issues and poor mechanical strength of scaffolds.[27,28] Therefore, our strategy to overcome these limitations was to provide a stable environment for the MSCs to preserve stemness in MSCs. Thus, I considered the use of resveratrol (RSV; 3,5,4′-hydroxystilbene), a phytoalexin made from plants damaged by environmental stress and strong activator of SIRT1, a class III histone

deacetylase protein.[29-31] RSV is also known to strongly influence cell survival and proliferation[32-34] enhancing the osteogenic and adipogenic differentiation potential of MSCs.[34-36] However, there are some negative effects of RSV on self-renewal and differentiation capacity of MSCs.[32] In our previous study, we have demonstrated that the appropriate application of RSV to MSCs could enhance self-renewal as well as the osteogenic and adipogenic potential of MSCs during long-term *in vitro* expansion.[37]

In the present study, I have elucidated the improvement of chondrogenic differentiation potential of MSCs treated with RSV during the long-term *in vitro* expansion. Moreover, RSV treatment inhibited hypertrophic maturation leading to the regeneration of hyaline cartilage, *in vivo.*

Ⅱ. MATERIALS AND METHODS

1. Isolation of MSCs from human bone marrow aspirates

Bone marrow aspirates obtained from the posterior iliac crests of ten adult donors, with approval from the Institutional Review Board of Yonsei University College of Medicine. MSCs were selected and cultured for seven days in Dulbecco's modified Eagle medium-low glucose (DMEM-LG; Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Gibco) and 1% antibiotic antimycotic solution (Gibco) and incubated at 37℃ in 5% CO_2 humidity. MSCs were subcultured at a 1:3 ratio when they were 80% confluent.

2. Chemical treatment of MSCs

Resveratrol (RSV; Sigma, St. Louis, MO) was dissolved in ethanol (EtOH) which has concentration of 1uM. The RSV is continuously treated from passage (P) 1 to P5 MSCs (P5-RMSC) and subcultured as previously described.[21,38] Since MSCs lower than P5 are considered as optimal passage for clinical application, they were cultured up to P5.

3. *In vitro* chondrogenic differentiation of MSCs via micromass culture method

Micromass culture method was used for *in vitro* chondrogenic differentiation of MSCs. MSCs that are 80% confluent was harvested using 0.05% trypsin-EDTA (Gibco). Cells were washed, centrifuged and resuspended at density of 1 x 10^7 cells/mL, and 10 ul of the resuspended cells was dotted on the center of individual

wells of 24-well plates (1 x 10^5 cells/well). The cells were allowed to adhere at 37ºC for 2h, and then chondrogenic medium, consisting of Dulbecco's modified Eagle medium-high glucose (DMEM-HG; Gibco) supplemented with 1% antibiotic-antimycotic solution, 1% Insulin Transferrin Selenium-A (ITS; Invitrogen, Carlsbad, CA), 50 mg/mL ascorbic acid (Invitrogen), and 10ng/mL TGF-β3 (R&D System, Minneapolis, MN), was gently added. The chondrogenic medium was changed every 2 days during *in vitro* differentiation periods.

4. Quantitative real-time polymerase chain reaction

Total RNAs from MSCs were isolated using Trizol (Invitrogen) following the manufacturer's instructions. For cDNA reverse transcription, RNAs were reverse transcribed using an Omniscript Reverse-Transcription Kit (Qiagen, Hilden, Germany). The cDNA was used in real-time polymerase chain reaction (PCR) with an SYBR Green PCR Master Mix (Applied Biosystems, London, UK). Real-time PCR was performed using an ABI7500 real-time machine by Applied Biosystems. All primers were purchased from Bioneer. The primers that have no validation were designed as following Table 1. The validated primer, SOX9 (P232240), IHH (P101104) and ALP (P324388), was purchased from Bioneer. The PCR procedure was initiated for 30 s at 95ºC, followed by 40 thermal cycles of 5 s at 95ºC and 20 s at 60ºC. SYBR fluorescence was detected during the annealing/extension phase and all real-time PCR products had a final size of 100 base pair. Values from each samples were normalized to β-Actin as an internal control.

Table 1. A list of primers used for real-time PCR

Gene symbol		Sequence (5′ → 3′)
β-ACTIN	Forward	GTCCTCTCCCAAGTCCACACAG
	Reverse	GGGCACGAAGGCTCATCATTC
SOX5	Forward	AGCCCCACATAAAGCGTCCAAT
	Reverse	GGTCCTCCTCCTCCTCATCGTA
SOX6	Forward	AGCAGAGCCTGTGAAGTCC
	Reverse	GGTCCTCCTCCTCCTCATCGTA
COL2A1	Forward	GGCAATAGCAGGTTCACGTACA
	Reverse	CGATAACAGTCTTGCCCCACTT
AGGRECAN	Forward	CCTGGCCTGACATGGAGCTG
	Reverse	GGACTGGGGGAGACCTCGAA
RUNX2	Forward	CCCAGTATGAGAGTAGGTGTCC
	Reverse	GGGTAAGACTGGTCATAGGACC
OSTEOCALCIN	Forward	AGCAAAGGTGCAGCCTTTGT
	Reverse	CTTCACTACCTCGCTGCCCT
MMP13	Forward	GACGGGGTTTTGCCACACTG
	Reverse	ATTGGGTGTGGTGGCTCACG
COL1A1	Forward	GCCCTGCTGGAGAGGAAGGA
	Reverse	ATTGGGTGTGGTGGCTCACG
COL10A1	Forward	CCAGGACAGCCAGGCATCAA
	Reverse	ATTGGGTGTGGTGGCTCACG

5. Western blotting

For protein extraction, cell pellets were suspended in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, and 0.1% sodium dodecyl sulfate (SDS), followed by gentle pipetting and heating at 100ºC for 10 min with vortex mixing every 3 min. Lysates were centrifuged at 13,000 rpm for 10 min and supernatants were collected into new tube. To measure the concentration of proteins, we used the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Prior to western blotting, 30ug protein was mixed with 5× loading dye (Pierce) and heated at 10ºC for 3 min. The protein samples were run on 10% SDS polyacrylamide gel electrophoresis (PAGE) gels. Then proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Hybond, Escondido, CA) for 90 min. Membranes were blocked within 5% skim milk (BD Biosciences, San Jose, CA) for 1h, following the incubation of primary antibodies at 4ºC overnight. Antibodies used were anti-SOX9 (Millipore, Billerica, MA, 1:1,000 in 1% skim milk); anti-β-ACTIN (Santa Cruz Biotechnology, Santa Cruz, CA, 1:1,000 in 1% skim milk); and anti-COL2A1 (Santa Cruz Biotechnology, 1:500 in 1% BSA). Finally, membranes were developed using enhanced chemiluminescence (ECL) solution (Amersham, Buckinghamshire, UK).

6. Preparation of Hydrogel

Hydrogel (Hy) was prepared as previously described.[39,40] In short, the cosolvent, consists of water and dimethylformamide within the ratio of 3:2, was added with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) that activates the 3-(4-hydroxyphenyl)propionic acid (HPA) and solution was added to preheated gelatin solution. After 24h of reaction at 40 ºC, the solution was dialyzed with deionized water, filtered and lyophilized.

7. Animal experiments

Twelve New Zealand white rabbits (male, 3.5 – 4 kg; Doo Yeol Biotech, Seocho-gu, Seoul) were used for osteochondral defect model which had been previously established.[41] Briefly, the cylindrical osteochondral defect (6 mm diameter, 3 mm depth) was formed and applied with following: None (Defect), hydrogel only (Hy), Hy + P5 MSCs (Hy/MSC) and Hy+ P5 RSV-MSCs (Hy/RMSC). Each cell is applied with 2 x 10^6 cells. 8 weeks after operation, the rabbits are euthanized and defect sites are extracted for histological analysis in vitro. All animal experiments are approved by the Committee on the Ethics of Animal Experiments of Yonsei University College of Medicine (Permit No. 2016-0200).

8. Histological analysis and immunohistochemistry

8 weeks post-operation, the regenerated cartilage tissues were fixed for 7 days in 10% formalin. After fixation, the formalin-fixed specimens were embedded in paraffin and then paraffin blocks were sliced at a thickness of 4mm. The sections were deparaffinized, rehydrated and washed twice with PBS and stained with hematoxylin-eosin (HE) to observe the cell morphology and Masson's trichrome (MT) to assess total collagen synthesis and safranin O/fast green to detect glycosaminoglycans (GAGs). The stained samples were observed using VS120 virtual microscope (Olympus, Tokyo, Japan), and images were analyzed using OlyVIA 2.5 program (Olympus). We used O'Driscoll scoring system for histological examination and the regenerated cartilage was evaluated by three independent experts using grading scale. All scores were the means of the three independent evaluations.

9. Statistical analysis

Each experiment was performed in triplicate using samples more than three donors. For detection of difference between two groups are confirmed by t-test. The statistical significance of the differences among three or more groups was calculated using one-way analysis of variance (ANOVA) with Tukey's post hoc analysis. All data are presented as mean and 95% CIs of the values from different donors per group.

Ⅲ. RESULTS

1. Enhanced stemness and inhibited senescence of MSCs via continuous treatment of RSV

During long-term *in vitro* expansin of MSCs, we have continuously treated RSV from P0 to P5 MSCs (P5-RMSC) while the other cells are cultured up to P5 without RSV treatment (P5-RSV) (Figure 1).

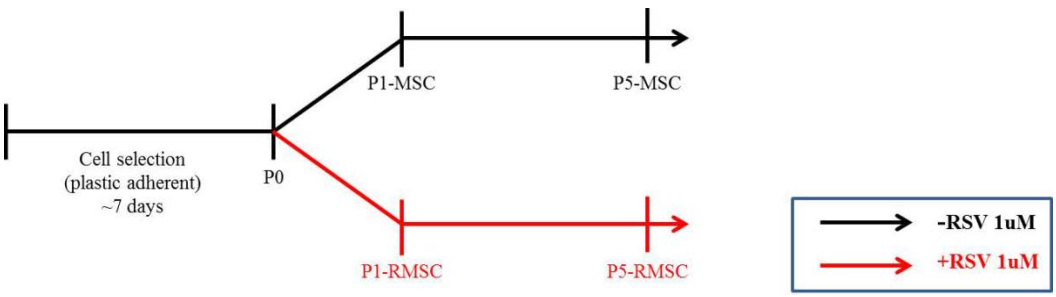


Figure 1. Long-term *in vitro* expansion of MSCs with or without treatment of RSV. MSCs were isolated from bone marrow aspirates and cultured for 7 days. From P0-MSC, the cells were treated with 1uM of resveratrol up to P5.

To identify the similar stemness with P1-MSC, we compared the morphological changes between P1- and P5-MSC with and withour RSV treatment. The P5-RMSC showed similar morphology with P1-MSC whereas P5-MSC showed opposite morphology as flat and large size (Figure 2A). Also, the proliferation assay was performed to evaluate sustained proilferative capacity of P5-RMSC. As a result, the P5-RMSC had improved proliferation capacity in comparison with P5-MSC (Figure 2B).

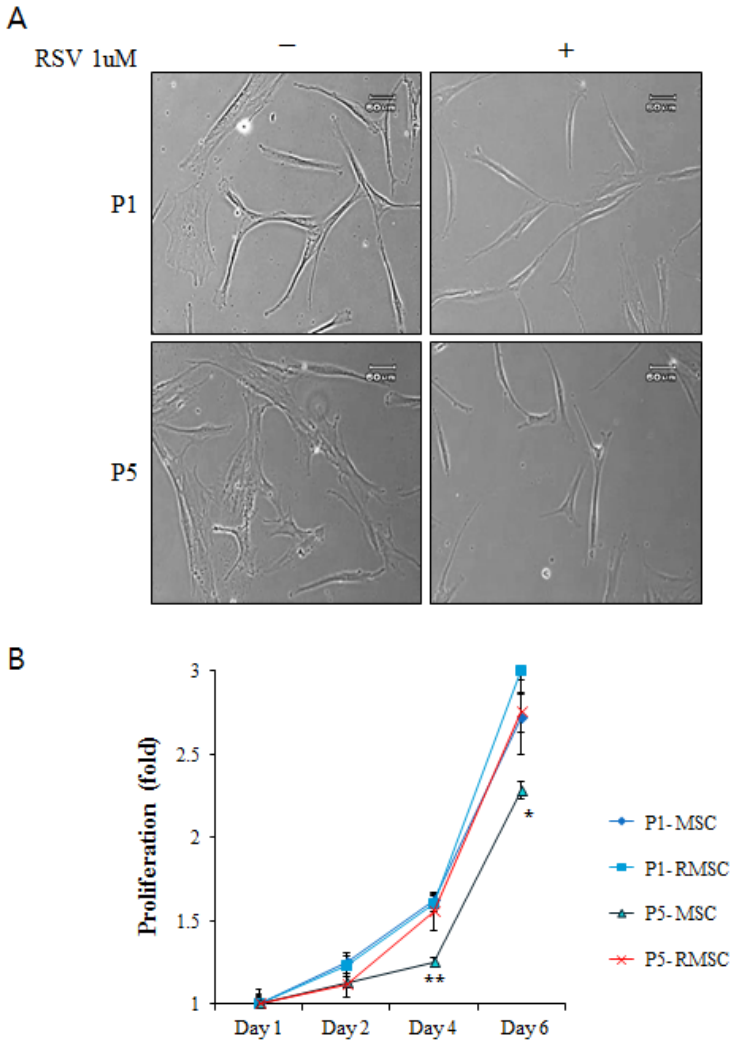


Figure 2. Comparison of morphological change and proliferation capacity between P1 and P5 MSCs. (A) Small and spindle-like morphology of P1-MSC and large and flat morphology of P5-MSC. P5-RMSC had similar morphology with P1-MSC. (B) Proliferative potential of P5-RMSC had similar potential with P1-MSC. **p*<0.05, ***p*<0.01.

Furthermore, to demonstrate the effects of RSV in cell senecence, we assessed protein level of senecence and stemness markers. When MSCs are culutred up to P5, senescence markers were upregulated whereas P5-RMSC had downregulated that had similar expression with P1-MSC (Figure 3A). The stemness markers were highly expressed in P5-RMSC which has similar expression level of P1-MSC (Figure 3B). Therefore, when resveratrol is continuously treated to MSC from P1 to P5, the stemness of MSC was upregulated while senecence was inhibited.

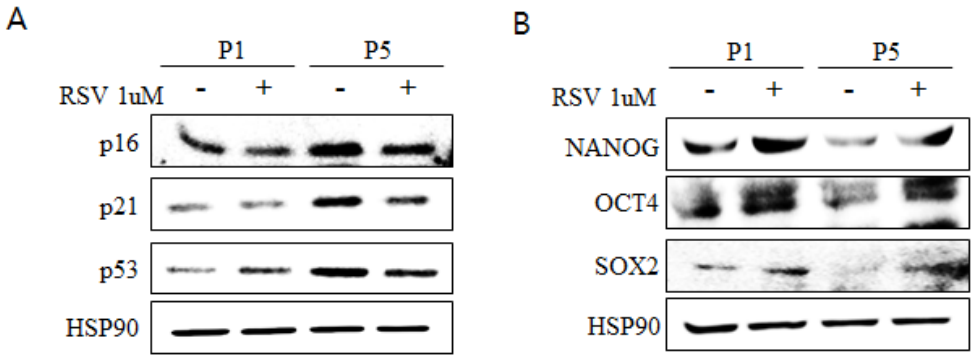


Figure 3. Sustained expression of stemness markers and inhibited expression of senescence markers. (A) P1-MSC had low expression of senescence markers, p16, p21, and p53, whereas P5-MSC had higher expression of that. However, the P5-RMSC had decreased level of senescence marker in comparison with P5-MSC. (B) Stemness markers, NANOG, OCT4, and SOX2, showed opposite result of senescence markers. When RSV is continuously treated, P5-RMSC had similar expression of stemness markers with P1-MSC.

2. Evaluation of in vitro chondrogenic differentiation of P5-RMSC

To compare enhanced chondrogenic differentiation potential of P5-RMSC, we performed micromass culture of P1-MSC, P5-MSC and P5-RMSC. The mRNA level of chondrogenic markers, SOX-5,-6,-9, COL2A1, and AGGRECAN, were up-regulated up to similar level with P1-MSC in P5-RMSC while P5-MSC was down-regulated (Figure 4). The western blot demonstrated that protein expression level of chondrogenic markers were up-regulated up to similar level with P1-MSC in P5-RMSC while P5-MSC was down-regulated (Figure 5A and B).

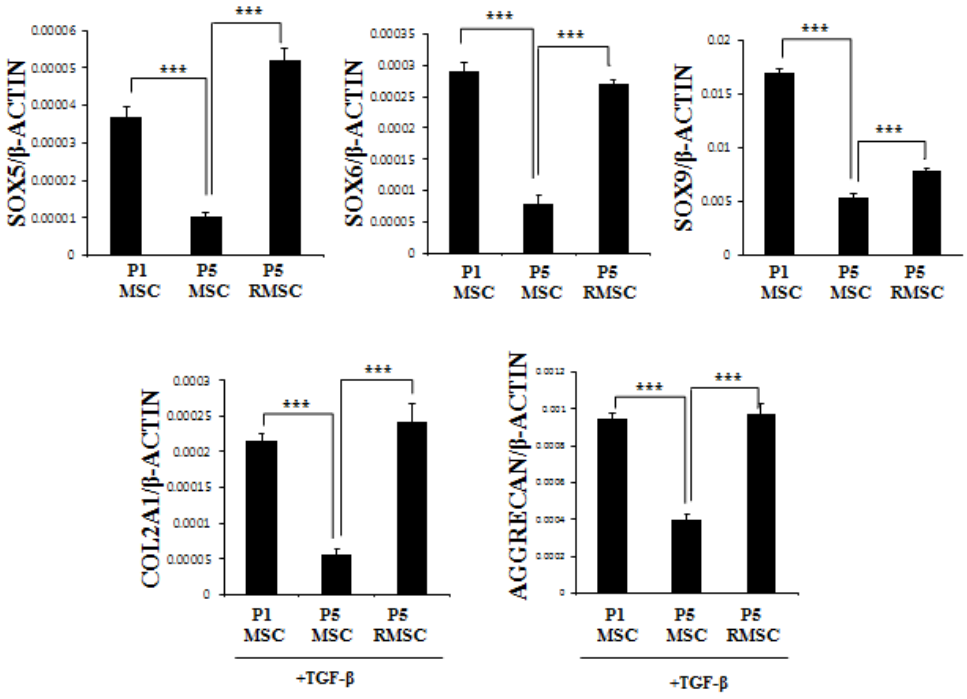


Figure 4. The mRNA expression level of chondrogenic markers in MSCs following chondrogenic differentiation. mRNA expression level of chondrogenic markers, SOX-5,-6,-9, COL2A1, and AGGRECAN, was highly up-regulated in P5-RMSC when comparison to P5-MSC, and has similar expression level with P1-MSC on day 5. ***$p < 0.001$.

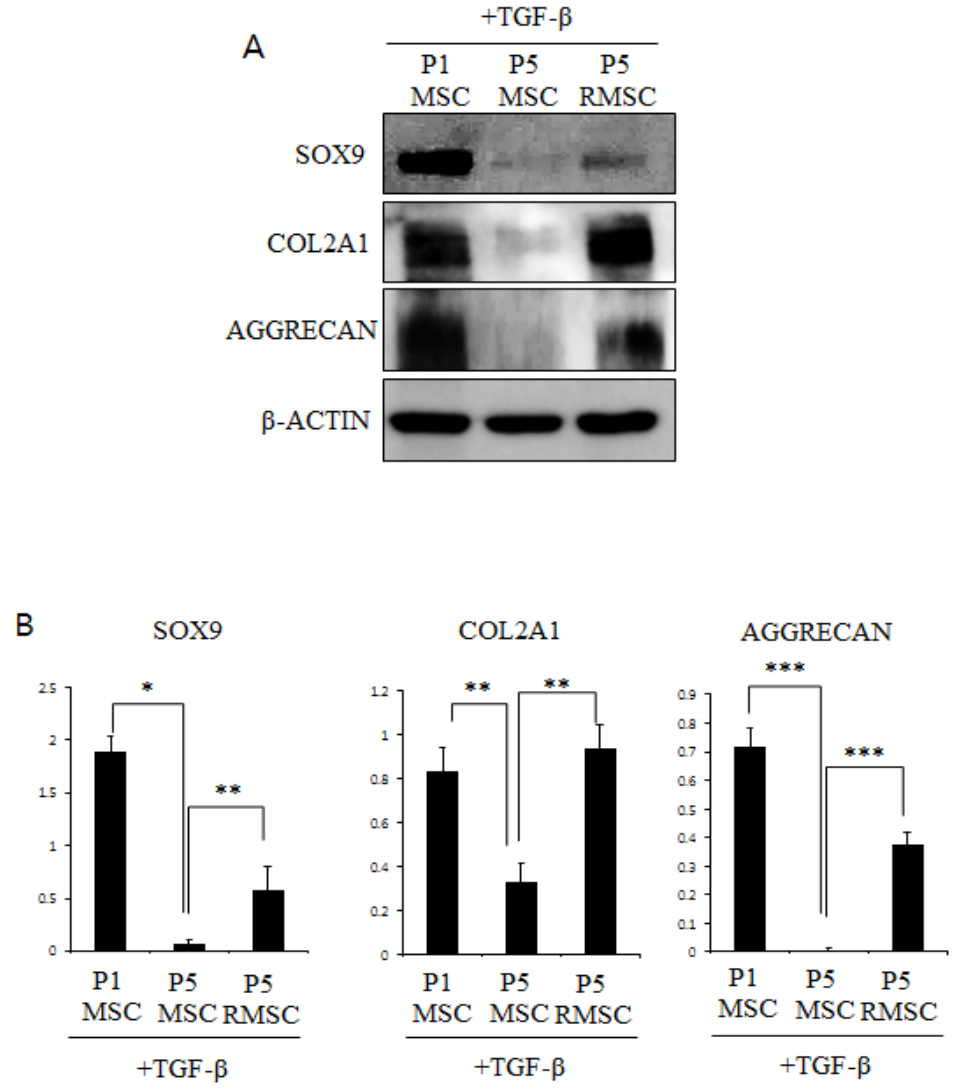
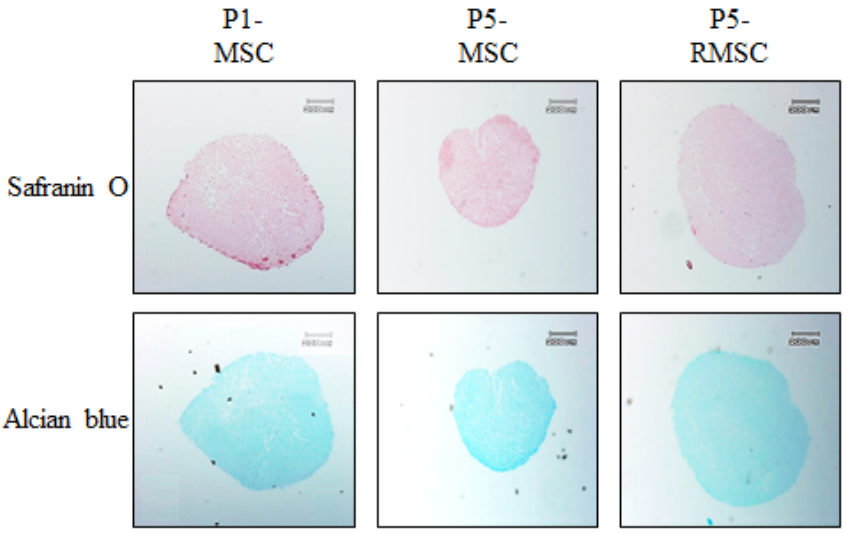
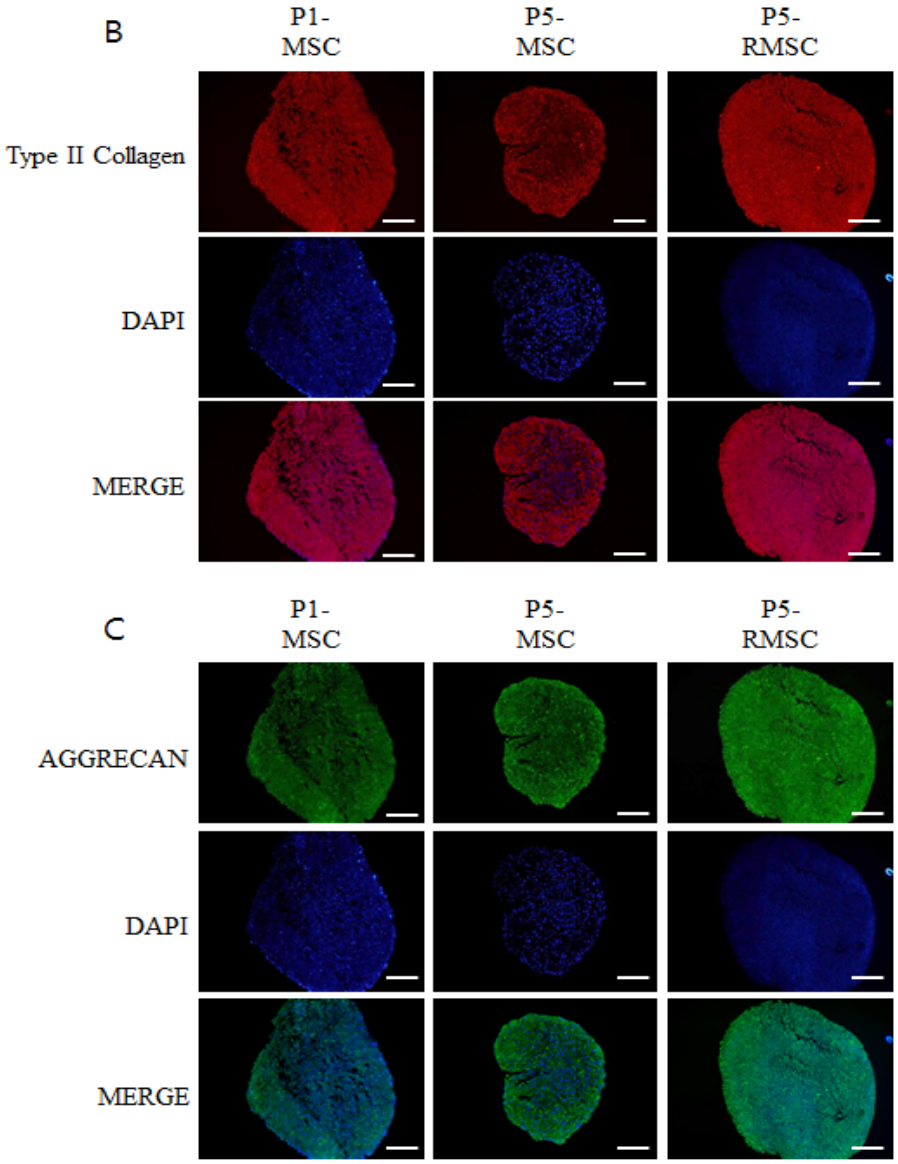


Figure 5. The protein expression of chondrogenic markers in MSCs following chondrogenic differentiation. (A) Enhanced protein expression level of P5-RMSC in chondrogenic markers, SOX-9, COL2A1 and AGGRECAN on day 10. (B) Quantitative analysis of protein expression level in each group was confirmed by Image J Software Ver. 1.48. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

To further demonstrate the enhanced capacity of chondrogenic differentiation of P5-RMSC, we performed the safranin O and alcian blue staining. The P5-MSC had smaller size of micromass in comparison with both P1-MSC and P5-RMSC which has larger size and higher contents of glycosaminoglycan (GAG) and proteoglycan (PG) (Figure 6A). We preformed immunocytochemistry to confirm the increased expression level of COL2A1 and AGGRECAN in P5-RMSC (Figure 6B) and the quantitative analysis of those was conducted (Figure 6C). Thus, the P5-RMSC had increased chondrogenic potential when compared with P5-MSC in concurrence with similar level of P1-MSC.

A





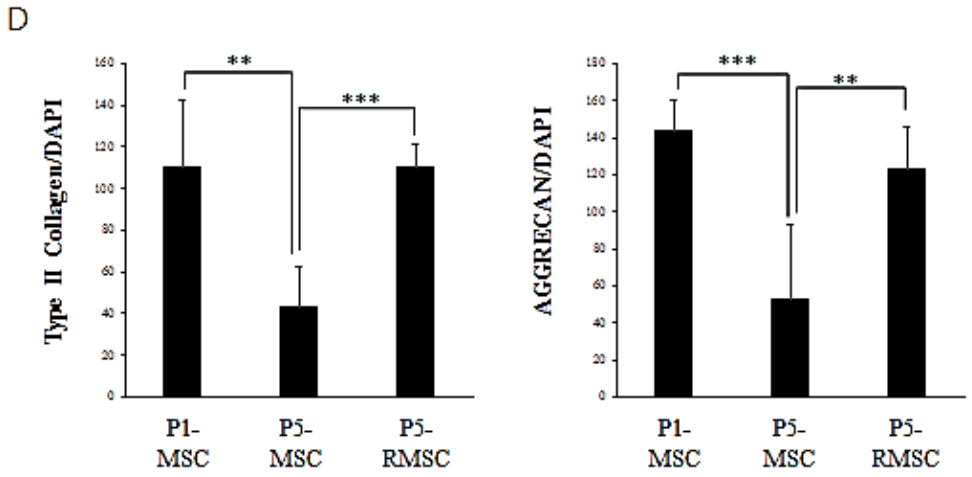


Figure 6. Histological analysis of GAGs and PGs in chondrogenic micromass and detection of chondrogenic markers by immunocytochemistry. (A) The safranin O and alcian blue staining demonstrated increased contents of GAGs and PGs, respectively, on day 14. (B) Immunocytochemistry of COL2A1 (PE; red fluorescence) and AGGRECAN (FITC; green fluorescence) showed higher expression level in P5-RMSC on day 14. DAPI is stained with nucleus (blue). (C) Quantitative analysis of COL2A1 and AGGRECAN was confirmed via Image J Software Ver. 1.48. $^{**}p < 0.01$, $^{***}p < 0.001$.

3. Inhibition of hypertrophic maturation of RSV treated MSCs

The primary limitation to use MSCs in cartilage regeneration is their tendency to become hypertrophic maturation during chondrogenic differentiation followed by increased expression of COL10A1, matrix metalloproteinase 13 (MMP13) and alkaline phosphatase (ALP).[24,42] Thus, we investigated whether the P5-RMSC could inhibit hypertrophic maturation during *in vitro* chondrogenic differentiation. In mRNA level of hypertrophic markers are down-regulated in P5-RMSC when compared to P5-MSC (Figure 7). Also, the western blot demonstrated the decreased protein level of hypertrophic markers (Figure 8A). Moreover, the immunocytochemistry showed decreased expression level of COL10A1, the major hypertrophic marker (Figure 8B and C). These results suggest that continuous treatment of RSV could inhibit the hypertrophic maturation during chondrogenic differentiation of MSC.

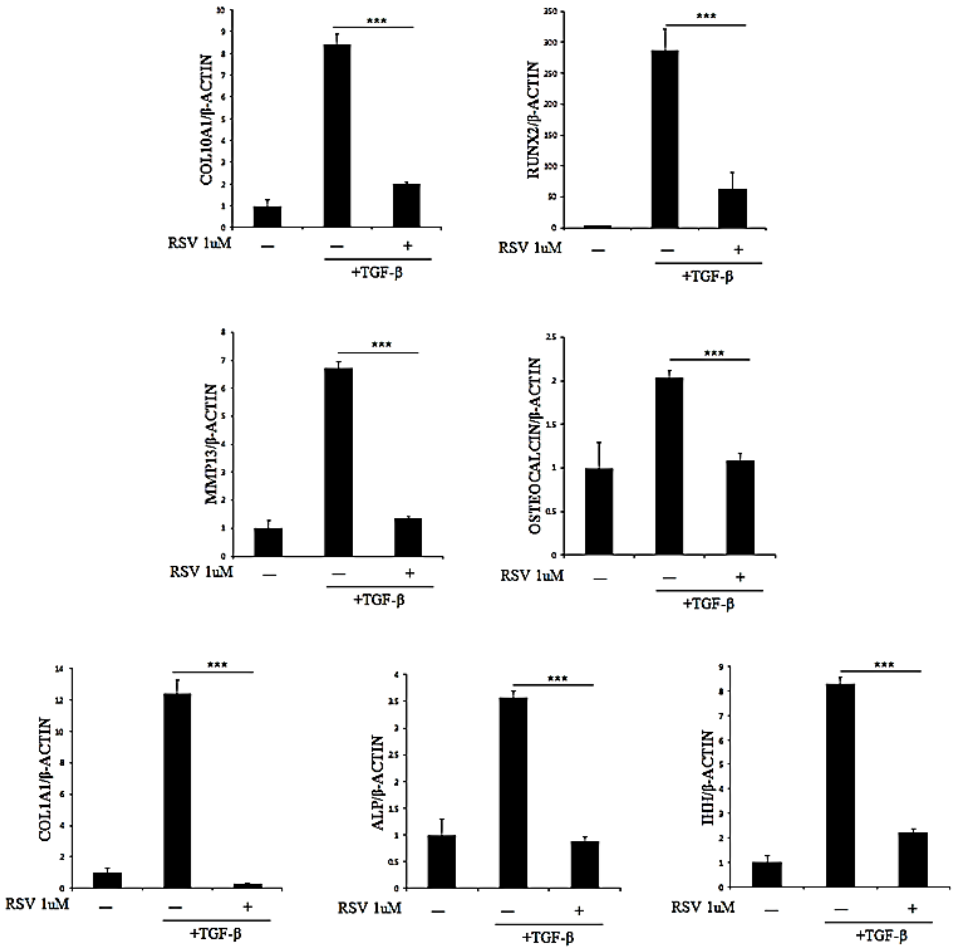


Figure 7. Effects of RSV treatment on MSCs in hypertrophic maturation during chondrogenic differentiation. Continuous treatment of RSV on MSCs had decreased expression level of hypertrophic markers following chondrogenic differentiation, on day 21. ***p<0.001.

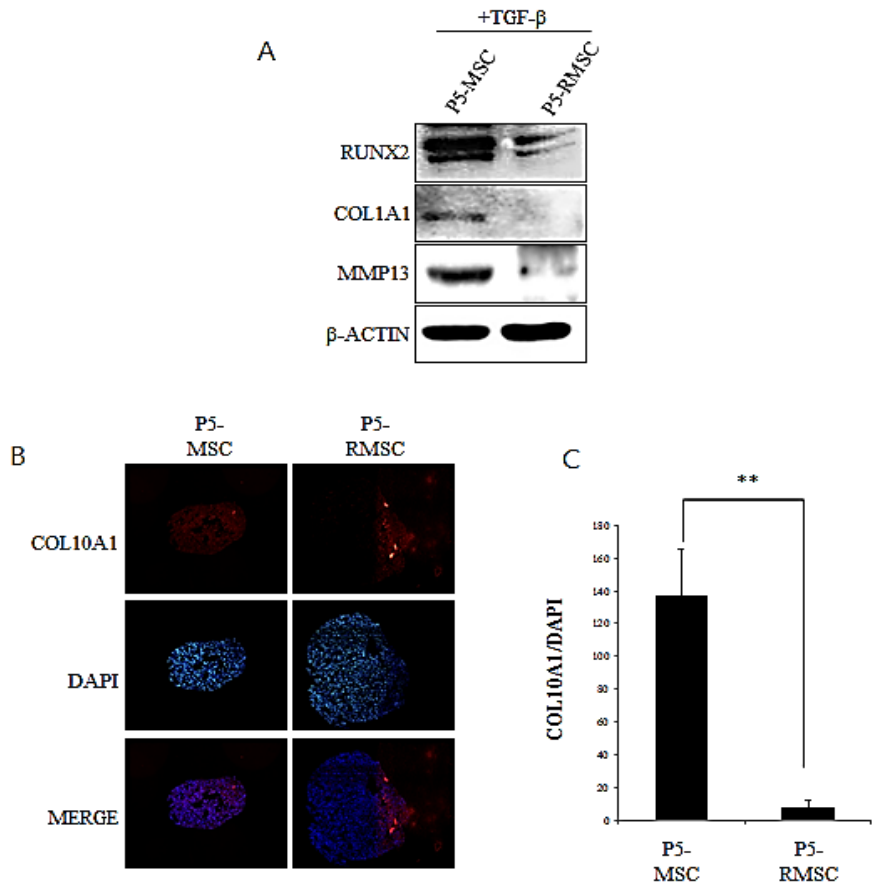


Figure 8. Expression of representative hypertrophic markers in protein level. (A) Western blot analysis of hypertrophic markers, RUNX2, COL1A1 and MMP13, on day 21. (B) On day 21, immunocytochemistry determines the expression level of COL10A1 (PE; red fluorescence) and nucleus was stained with DAPI (blue). (C) Quantitative analysis of COL10A1 expression by Image J Software Ver. 1.48. **p < 0.01.

4. Enhanced cartilage regeneration potential *in vivo*

To identify whether the continuous treatment of RSV to MSCs could acquire increased cartilage regeneration capacity *in vivo*, we have developed osteochondral defect model (Figure 9).

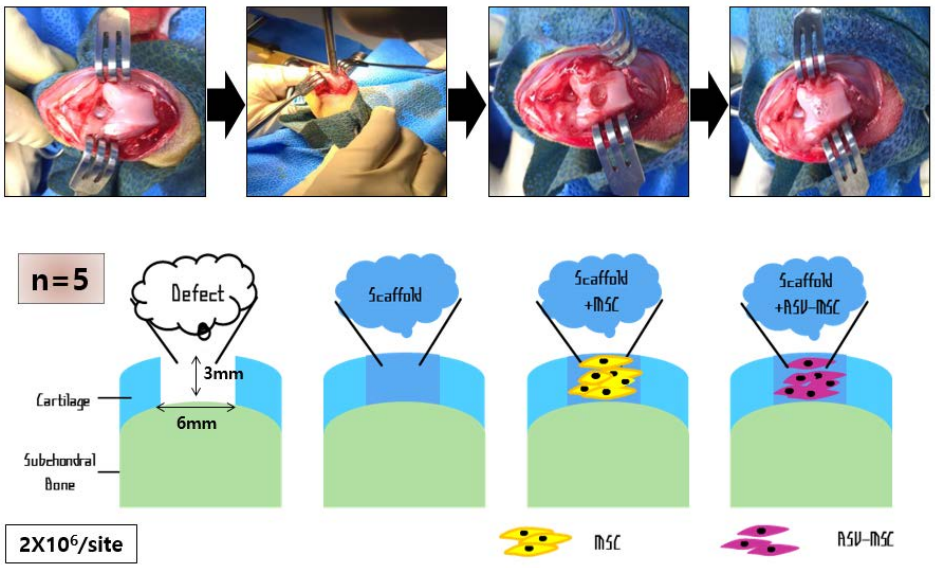
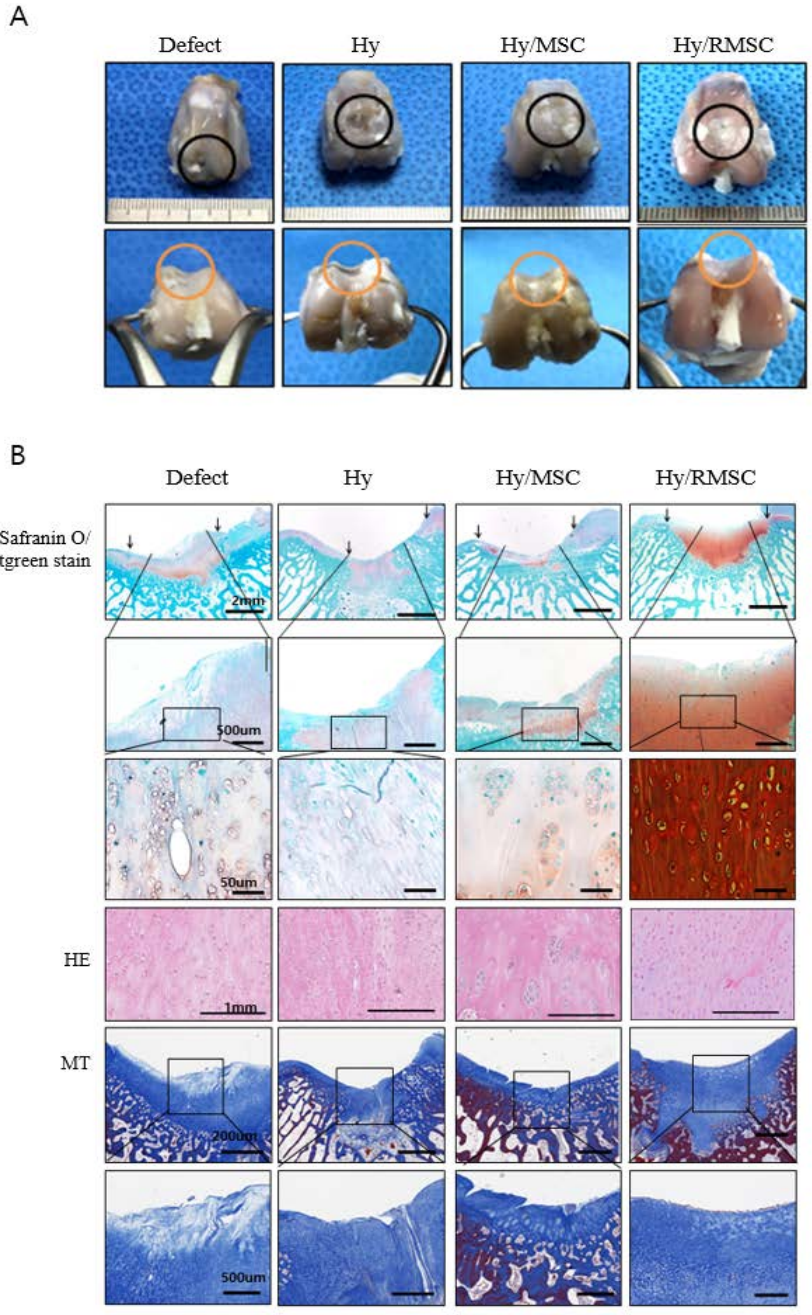


Figure 9. Establishment of rabbit osteochondral defect model. The size of defect is 6mm diameter and 3mm depth. $2x10^6$ cells are applied onto the defect site to compare the effectiveness of P5-RMSC in cartilage regeneration.

8weeks post-operation, we observed the gross morphology of regenerated cartilage. In Hy/RMSC group, the surface of defect site was almost fully filled with cartilage-like tissue as the nearby cartilage while other groups were not fully filled with cartilage like tissue and in some parts it had lamination and cysts on the surface. Moreover, the Hy/RMSC group showed more transparent cartilage-like tissues (Figure 10A). Furthermore, we observed regenerated cartilage tissues via safranin O/fastgreen staining. The results showed enhanced GAG formation in Hy/RMSC group whereas the Hy/MSC group had slightly increased synthesis of GAG when compared to other groups (Figure 10B, upper lane). Then, we analyzed HE and MT staining to confirm the histological characteristics of newly formed cartilage in osteochondral defects. In HE stain, we observed that more chondrocyte-like cells were formed and also the there was no clustering. However, the other groups had a few chondrocyte-like cells and they formed fibrous tissues on cartilage (Figure 10B, middle lane). The MT stain demonstrated higher collagen deposition and no fibrous tissue formation on the surface of the cartilage in Hy/RMSC group in comparison with other groups (Figure 10B, bottom lane). Moreover, we performed O′Driscoll scoring which shows the Hy/MSC groups had slightly higher score than defect or Hy group however the Hy/RMSC group had significantly higher score than other groups (Figure 11). These results suggest that the Hy/MSC group had slightly increased effects in cartilage regeneration while the Hy/RMSC group had significantly enhance cartilage regeneration.



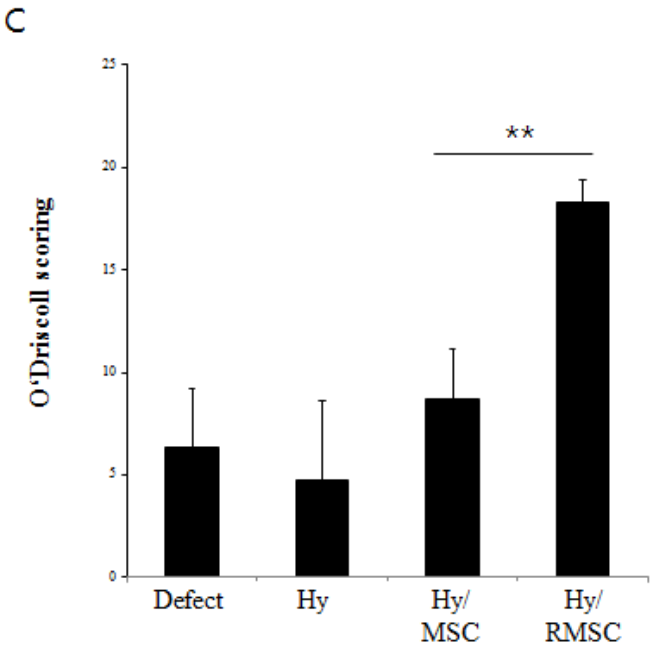
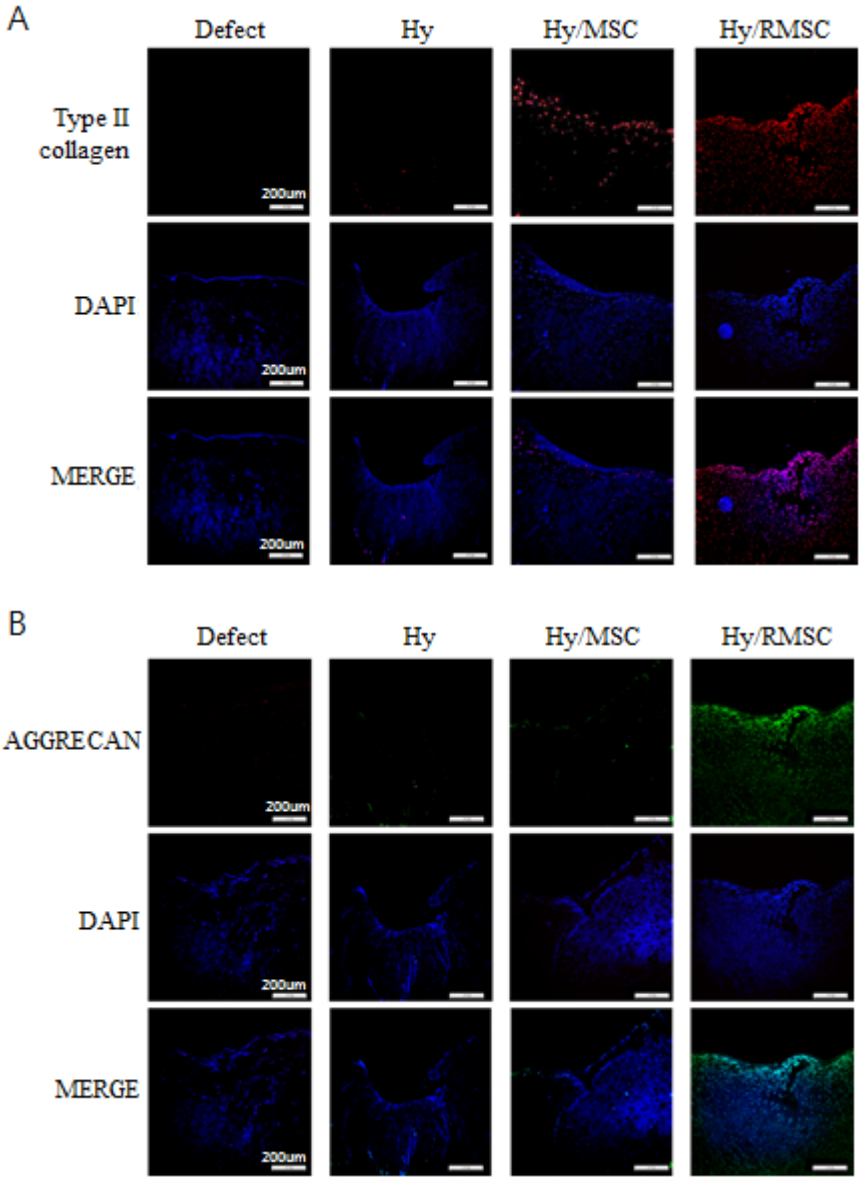


Figure 10. Gross morphology and histological analysis of rabbit osteochondral defect sites after 8 weeks of surgery. (A) The gross morphology of osteochondral defect sites was photographed. (B) Formation of GAGs at osteochondral defect sites was evaluated by safranin O/fast green staining. GAGs were stained with cartilage tissue and fast green was stained in non-collagenous proteins (upper lane). HE stain shows the chondrocyte-like cell morphology (middle lane). MT stain demonstrates the collagen fiber formation which is stained with blue (bottom lane). (C) Quantitative histological analysis of regenerated cartilage tissue was performed via O′Driscoll scoring system. Three independent experts assessed the cartilage regeneration and all scores were means of three independent assessments (n = 3). $^{**}p<0.01$.



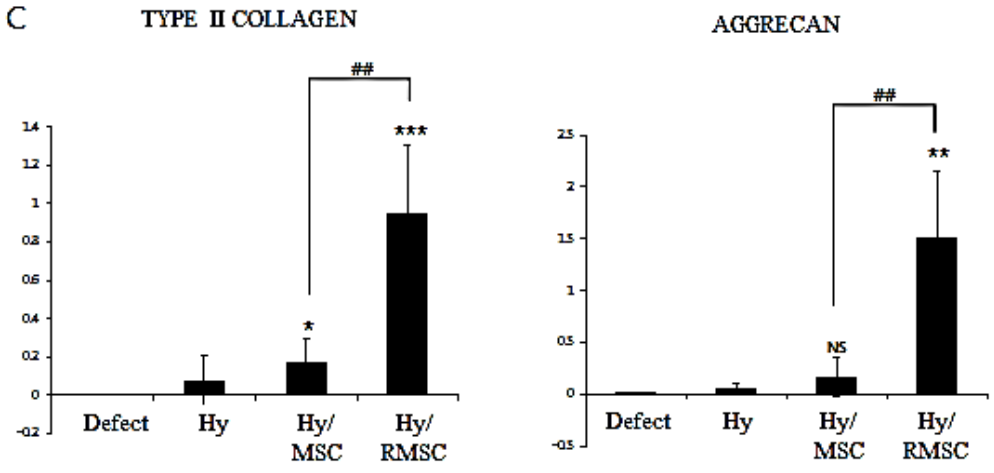


Figure 11. Evaluation of cartilage regeneration potential *in vivo* via immunohistochemical analysis. The effects of RSV treated MSCs in cartilage regeneration was confirmed by detecting (A) type II collagen (PE; red fluorescence) and (B) aggrecan (FITC; green fluorescence). (C) Quantitative analysis of type II collagen and aggrecan was confirmed via Image J Software Ver. 1.48. Hydrogel only vs. *$p<0.05$, **$p < 0.01$, ***$p < 0.001$.

5. Inhibition of hypertrophic maturation of cartilage

In order to identify the regeneration of hyaline cartilage, the immunohistochemistry was performed to detect expression of type X collagen, the hypertrophic marker. The Hy/RMSC group had scarce expression level of type X collagen while the defect, Hy, and Hy/MSC groups showed high expression level of that (Figure 12A and B). Consequentially, the P5-RMSC could inhibit the hypertrophic maturation *in vivo*, thus regenerated the hyaline cartilage.

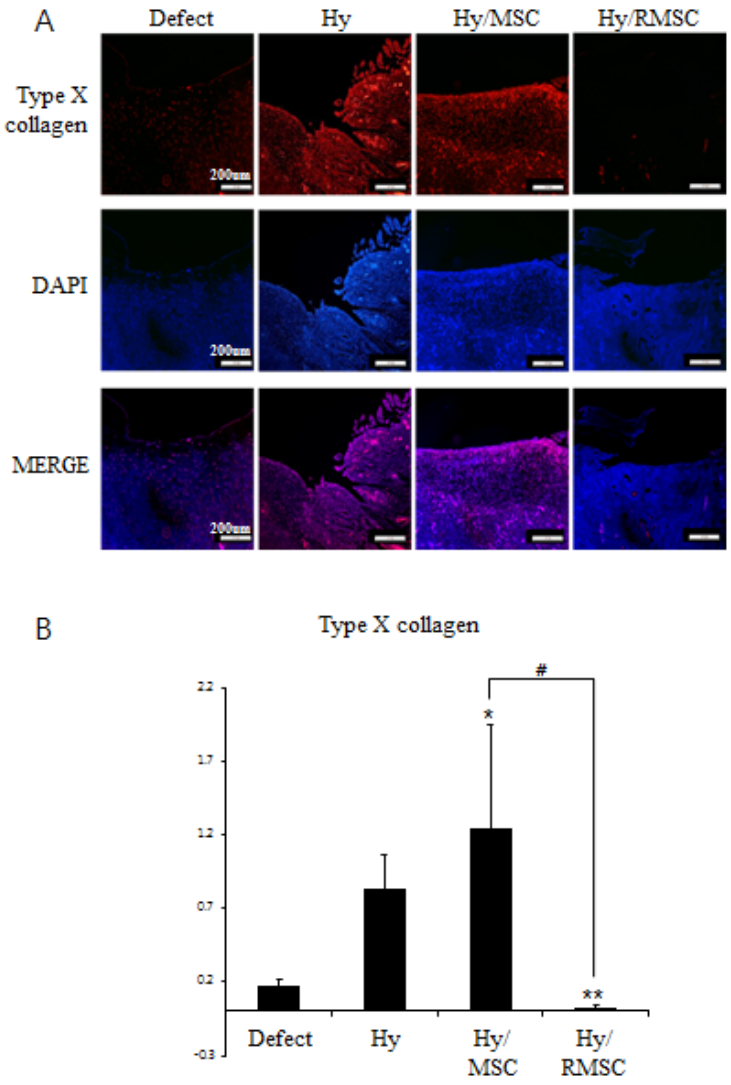


Figure 12. Inhibition of hypertrophic maturation in RSV treated MSCs. Type X collagen (PE; red fluorescence), the hypertrophic maturation marker, was detected to confirm the inhibition of hypertrophic maturation in RSV treated MSCs. (B) Quantitative analysis of type X collagen was confirmed by Image J Software ver. 1.48. Hydrogel only vs. *$p < 0.05$, **$p < 0.01$.

Ⅳ. DISCUSSION

In cartilage regeneration, the MSCs are the most commonly used cell type but they have some limitations to use them. When we use MSCs as clinical application, the long-term *in vitro* expansion is necessary which cause the cellular senescence that leads to loss of self-renewal and multipotency.[43] In present study, I found that MSCs treated with RSV from P0 to P5 had enhanced stemness and inhibited senescence (Figure 2 and 3), simultaneously. When chondrogenic differentiation was performed, I found that P5-RMSC had increased chondrogenic differentiation potential when compared with other groups (Figure 4). Also, the expression levels of hypertrophic markers were confirmed to investigate the inhibition of hypertrophic maturation. The P5-RMSC had decreased expression level of hypertrophic markers while P5-MSC had increased hypertrophic maturation (Figure 7 and 8).

After confirmation of enhanced chondrogenic differentiation of P5-RMSC *in vitro*, I investigated whether the P5-RMSC could enhance the hyaline cartilage regeneration *in vivo*. I established osteochondral defect model in rabbit and evaluated the regenerated cartilage. The histological analysis demonstrated that P5-RMSC had improved regeneration of hyaline cartilage. Typically, when the MSCs that have high potential of stemness are used in cartilage regeneration, there are several shortcomings including formation of fibrous tissue and hypertrophic maturation[44]. However, in my study, I have overcome these limitations as described in Figure 7 and 8. Consequentially, the P5-RMSC had enhanced hyaline cartilage regeneration in concurrence with inhibited hypertrophic maturation because the maintenance of stemness via treatment of RSV.

RSV is known to play critical roles in not only cell survival and proliferation[32-34] but also enhances multipotential differentiation.[34-36] However, the RSV had contradictory effects when it is treated to MSCs.[32] Several studies have

demonstrated that RSV treatment could enhance multipotential differentiation[34,35] while others demonstrated that successive treatment of RSV to MSCs could increase cellular senescence.[34] Thus, in our previous study, we confirmed that when RSV is treated to MSCs at appropriate time point, they can enhance the stemness and multipotency.[37] Yoon et al. demonstrated that early passage MSCs which has high expression of SIRT1 were treated with RSV and these early passage MSCs treated with RSV could sustain the stemness. However, the late passage MSCs which has low expression of SIRT1 were treated by RSV could induce the cellular senescence. Also, other studies have treated RSV in high dose while yoon et al. confirmed the optimal concentration of RSV with consistent results of MSCs which have enhanced cartilage regeneration. Therefore, we treated 1uM of RSV from P0 to P5-MSCs.

In cellular therapy, the recommendable passages for MSCs are between 3 and 5.[45,46] Generally, the MSCs at passage 1~2 have high multipotency but MSCs at passage 4~5 start to lose their multipotency.[22,47] To utilize MSCs in cellular therapy, the large numbers of cells are required for the treatment.[48] To obtain large number of cells, maintaining the stemness of MSCs is essential. Bonab et al. demonstrated that bone marrow derived MSCs lose the number of population doubling and also possess decreased telomere length as cells are subcultured.[21] Thus, I cultured MSCs up to passage 5 and obtained higher number of cells when RSV is treated (data not shown).

In my study, I have used human bone marrow derived MSCs not rabbit MSCs. Since the MSCs have anti-inflammatory and immunosuppressive effects,[17] the utilization of human MSCs on rabbit osteochondral defct model did not cause any

side effects. Additionally, there are several studies using human MSCs in animal model experiments.[49,50]

In my *in vivo* study, I have made critical size (diameter 6mm, depth 3mm) of osteochondral defects on rabbit, which is the size that was not able to self-heal. During establishing rabbit osteochondral defect model, the bone marrow from rabbit was emerged. In previous study, Gobbi et al, have demonstrated that the usage of bone marrow concentrate for cartilage regeneration was effective.[51] However, in our previous study, we have proven that the bone marrow concentrates did not have significant effects in cartilage regeneration.[41,52] Despite the presence of bone marrow concentrates, the defect and Hy groups did not regenerate cartilage. Thus, the evaluation of effectiveness of RSV treated MSCs is sufficient to compare each other without any other disturbance.

Taken together, the continuous treatment of RSV on MSCs had sustained stemness which is similar to P1-MSCs. Consequently, the RSV treated MSCs had not only enhanced the chondrogenic differentiation potential but also promoted the regeneration of hyaline cartilage via maintenance of stemness.

Ⅴ. CONCLUSION

In summary, the treatment of RSV from P0 to P5 MSCs could enhance the stemness of MSCs and inhibited senescence of cells. Since the differentiation potential is up-regulated via continuous treatment of RSV to MSCs, they had enhanced chondrogenic differentiation potential and also inhibited hypertrophic maturation to synthesize hyaline cartilage. Furthermore, the MSCs that are continuously treated with RSV were applied onto the rabbit osteochondral defect sites. The osteochondral defect sites that had P5-RMSC transplantation showed enhanced cartilage regeneration in concurrence with formation of hyaline cartilage

which inhibited the hypertrophic maturation. In conclusion, the continuous treatment of RSV from P0 to P5 MSCs could support the environment for maintaining stemness result in enhanced chondrogenic differentiation. Thus, the methods which continuously treat RSV to MSCs can be a promising method of MSCs for cellular therapy.

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ABSTRACT (in korean)

SIRT1 활성제 전처리에 의해 증진된 중간엽 줄기세포의 연골 분화능

<지도교수 이 진 우>

연세대학교 대학원 의과학과

최 성 미

골 관절염은 퇴행성 관절 관련 질병으로, 이는 연골 재생 능력이 부족하여 점진적으로 광범위한 연골 손상이 발생하게 된다. 골 관절염의 주요한 세포 병리학적 현상은 연골의 손상, 연골세포 증식 문제, 그리고 연골의 비대화이다. 현재, 이를 치료하기 위한 여러 가지 연골 재생 치료 방법이 진행되고 있는데, 여러 가지 방법 중 세포 치료제가 최근 주목 받고 있다. 세포 치료제 중 특히 중간엽 줄기 세포를 이용한 방법이 가장

널리 이용되고 있다. 중간엽 줄기 세포를 세포 치료제로 이용하기 위해서는 부족한 세포를 증식하기 위한 체외 배양이 필수적이다. 또한 세포 치료제로써의 유효량을 얻기 위해서는 체외에서 장기간 배양이 불가피하다. 체외에서 장기 배양 시, 중간엽 줄기 세포는 자가 증식력과 다분화능을 잃게 되며 또한 연골로 분화 시 연골의 비대화가 유발되게 된다. 이와 같은 문제점을 극복하여 중간엽 줄기 세포의 줄기 세포능과 연골 분화능을 향상시키기 위한 새로운 전략이 필요하다.

Resveratrol 은 강력한 SIRT1 활성제로, 중간엽 줄기 세포에서 세포의 생존, 증식력 그리고 다분화능에서 주요한 역할을 한다고 알려져 있다. 이전 연구에 따르면, SIRT1 의 발현이 유지되고 있는 초기 계대의 중간엽 줄기 세포에 resveratrol 을 처리하였을 경우, 중간엽 줄기 세포의 자가 증식력, 골 분화능 그리고 지방세포 분화능이 후기 계대가 되어도 초기 계대와 유사하게 유지 되는 것을 알 수 있었다. 하지만 이 때, resveratrol 을 초기 계대부터 지속적으로 처리하였을 경우 연골세포로의 분화능은 검증 되지 않았다.

본 연구에서는 resveratrol 을 1 계대의 중간엽 줄기 세포부터 지속적으로 처리하면서 배양하였을 경우 중간엽 줄기 세포의 증진된 연골 세포 분화능을 검증하였다. 따라서, 중간엽 줄기 세포를 micromass culture 방법을 이용하여 연골 분화를 유도 하여 real-time PCR 과 western blot 을 통하여 연골 분화 마커의 발현을 비교하였다. 그 결과,

resveratrol 을 처리해 주었을 경우에 더 높은 연골 분화능을 유지하고 있는 것을 확인 할 수 있었다. 또한 염색 및 면역 염색법을 이용하여 분화된 세포의 GAGs 와 PGs 가 형성된 정도를 확인하고 연골 분화 마커의 발현을 면역 염색법을 이용하여 확인하였다. 또한 연골 세포로 분화되었는지 확인하기 위하여 연골 비대화 관련 마커를 real-time PCR 과 western blot 을 이용하여 확인한 결과, resveratrol 을 처리한 중간엽 줄기 세포에서는 연골 비대화가 감소되어 있는 것을 알 수 있었다.

이어서 체내 연골 재생능을 검증하기 위하여 토끼의 골연골 결손 모델을 확립하여, 결손 부위에 세포를 이식하였다. 그 결과를 조직학적 분석을 통하여 확인을 하였다. Resveratrol 을 처리한 중간엽 줄기 세포는 Safranin O/ Fast green stain 을 통하여 형성된 GAG 를 확인할 수 있었고, HE stain 을 통해서 온전한 연골 세포가 형성 된 것을 확인하였으며, MT stain 을 통하여 교원질 증착을 확인 할 수 있었다. 그리고 연골이 재생되었는지 확인하기 위해, 연골 분화 마커와 비대화 마커를 면역 염색법을 이용하여 발현 정도를 확인 결과 resveratrol 을 처리한 중간엽 줄기 세포가 연골 분화 마커는 증가 되었지만 반대로 비대화 마커는 감소된 것을 알 수 있었다.

요약하면, resveratrol 을 SIRT1 이 발현되고 있는 1 계대의 중간엽 줄기 세포부터 지속적으로 처리를 하게 되면, 세포 치료제로 주로 이용하는 5 계대인 중간엽 줄기 세포의 줄기 세포능과 분화능이 passage

1 과 유사하게 유지하게 됨으로써, 연골 분화능이 증진됨과 동시에 비대화를 억제하는 것을 체외와 체내 실험을 통해 알 수 있었다. 이와 같이 resveratrol 을 지속적으로 처리해 함으로써 세포의 치료적 유효량을 더욱 빨리 획득이 가능하며 더불어 더욱 효율적인 연골 분화가 가능한 점에서 골관절염의 효과적인 세포 치료제로써의 역할을 할 것으로 사료된다.

핵심되는 말: 연골분화, 연골 재생, 레스베라트롤, SIRT1