공학석사학위논문

Inducing Osteogenic Differentiation of Human Mesenchymal Stem Cell Using 30Kc19α-Runx2 Protein

30Kc19α-Runx2 단백질을 이용한
인간 중간엽 줄기세포의 골 분화 유도

2019년 2월

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ABSTRACT

Inducing Osteogenic Differentiation of Human Mesenchymal Stem Cell Using 30Kc19α-Runx2 Protein

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Recently, there have been many studies on bone regeneration by differentiating human mesenchymal stem cells (hMSCs) into osteoblasts. Runt-related transcription factor 2 (Runx2) is known as a key transcription factor directing hMSCs to osteoblastic lineage. However, there have been difficulties in utilizing recombinant Runx2 protein due to its low solubility and poor cell-penetration ability. In this study, we fused Runx2 with 30Kc19α, which is a cell-penetrating protein, for soluble expression and intracellular delivery of Runx2 to induce osteogenesis of hMSCs. After construction of pET-23a/*30Kc19α-Runx2* plasmid, the recombinant protein was expressed in *E. coli* then purified by nickel affinity chromatography. The soluble expression of Runx2 protein was enhanced through fusion with 30Kc19α. The

fusion protein presented low cytotoxicity regardless of the protein concentration after 72 h treatment. Also, we observed that 30Kc19α could drag Runx2 into cells by confocal laser scanning microscopy. The effect of 30Kc19α-Runx2 on osteogenesis of hMSCs was confirmed by osteogenic differentiation assays (ALP staining and ARS staining). Therefore, we suggest that 30Kc19α-Runx2 protein would be used as a new osteogenic material for tissue engineering and regenerative medicine.

Keywords: stem cell differentiation, mesenchymal stem cell, osteogenesis, transcription factor, Runx2, cell penetrating protein, 30Kc19α

Student number: 2017-25452

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1. Introduction

Many researches have been focused on not only development of therapeutic molecules, but also their delivery into cells. One of the tools for facilitating cellular uptake of therapeutic molecules is cell-penetrating peptide (CPP), a short peptides that can penetrate the cell membrane. CPPs have been utilized as 'vehicles' for intracellular delivery of various cargos (e.g. plasmids, siRNAs, proteins, nanoparticles) [1, 2]. And many cell-penetrating peptides have been discovered and developed. Although cell penetrating mechanism of CPP is not fully understood, it is presumed that CPP drags cargos into cells through direct translocation or endocytosis [3]. The first discovered CPP is trans-activator of transcription (TAT) protein from human immunodeficiency virus 1 (HIV-1) [4].

30Kc19 is a member of 30K family, which originates from hemolymph of *Bombyx mori* [5, 6]. Previous studies have discovered that 30Kc19 has cell-penetrating ability due to its intrinsic CPP, Pep-c19 [6, 7]. It is meaningful in that pep-c19 is first CPP derived from insect hemolymph.

Although 30Kc19 first attracted attention as a cell-penetrating protein, further studies on 30Kc19 discovered that it also has remarkable functions additional to cell-penetrating ability: protein stabilization and enhancement of soluble expression. Supplementation of 30Kc19 increased the stability of enzymes, such as alkaline phosphatase (ALP), horseradish peroxidase (HRP) and sialyltransferase (ST), and isolated mitochondrial complex I/III [8, 9]. Furthermore, 30Kc19 improved the stability and soluble expression of transcription factors through conjugation [10].

30Kc19 is composed of two domains: 30Kc19α and 30Kc19β. 30Kc19α is N-terminal domain with α-helix structure and 30Kc19β is C-terminal domain with β-trefoil structure. Recently, it was observed that 30Kc19α showed cell-penetration and protein stabilization functions of 30Kc19. Rather, the delivery efficiency of 30Kc19α was higher than that of 30Kc19 whole protein [11].

Stem cell is defined as a cell which self-renews and has a potency to differentiate into other cells. Many attempts are tried to use stem cells for therapeutic purposes [12]. Embryonic stem cell (ESC) is pluripotent but has an ethical issue in using it [13, 14]. Therefore, many researches on stem cell therapy have been focused on multipotent adult stem cells.

Mesenchymal stem cell (MSC) is a kind of adult stem cell, which can be differentiated into various mesodermal lineage cells [15]. Lineage specification of MSC is determined by combined effects of biochemical and physicochemical factors [16]. Especially, inducing differentiation of MSC into osteoblast is focused on by many researches because of the importance of osteoblasts in the initial formation of bone, maintaining bone ossification and fracture repair [17].

Bone morphogenetic protein 2 (BMP-2) was firstly characterized by its effects on bone formation [18]. It has been discovered that BMP-2 plays important roles in almost all organ systems besides bone, therefore may cause side effects in using it [19]. Biological factors which have more specific effects on bone formation are targeted for the replacement of BMP-2. Located in the downstream of BMP-2 signaling, Runt-related transcription factor 2 (Runx2) is a key transcription factor of bone, which is essential for directing mesenchymal stem cells to the osteoblastic lineage [20]. Runx2 binds to the promoter of osteoblast-specific genes (e.g. osterix, collagen type 1, bone sialoprotein), then upregulates gene expression of these genes

[21, 22]. Due to its crucial role in osteogenesis, some researches utilized Runx2 by direct Runx2 gene delivery or stimulation of Runx2 gene expression to induce osteogenic differentiation of MSC [23].

However, there have been difficulties in utilizing recombinant Runx2 protein due to its two defects: its low solubility and poor cell-penetration ability. Runx2 was expressed in insoluble form in *E. coli*. And it was unable to penetrate in cells therefore couldn't role as a transcription factor [24]. Herein, we conjugated Runx2 to 30Kc19α so that 30Kc19α-Runx2 could induce osteogenic differentiation.

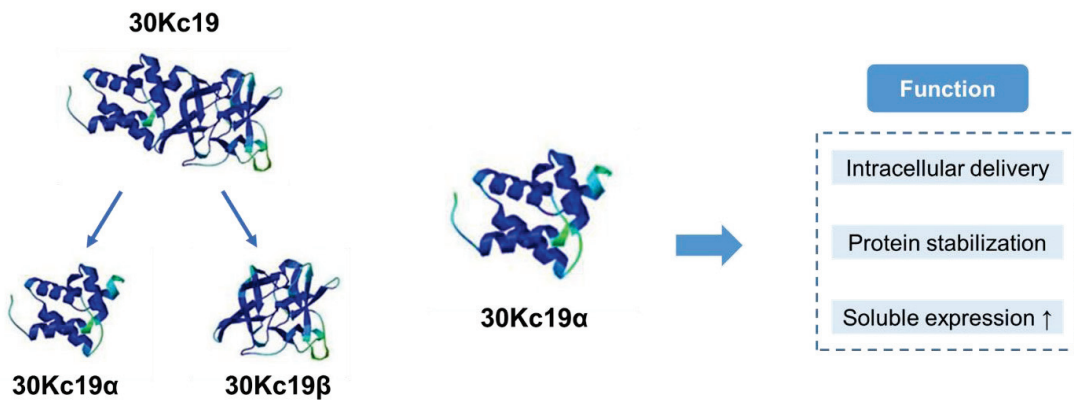


Figure 1. Structure of 30Kc19 and its functional subunit, 30Kc19α

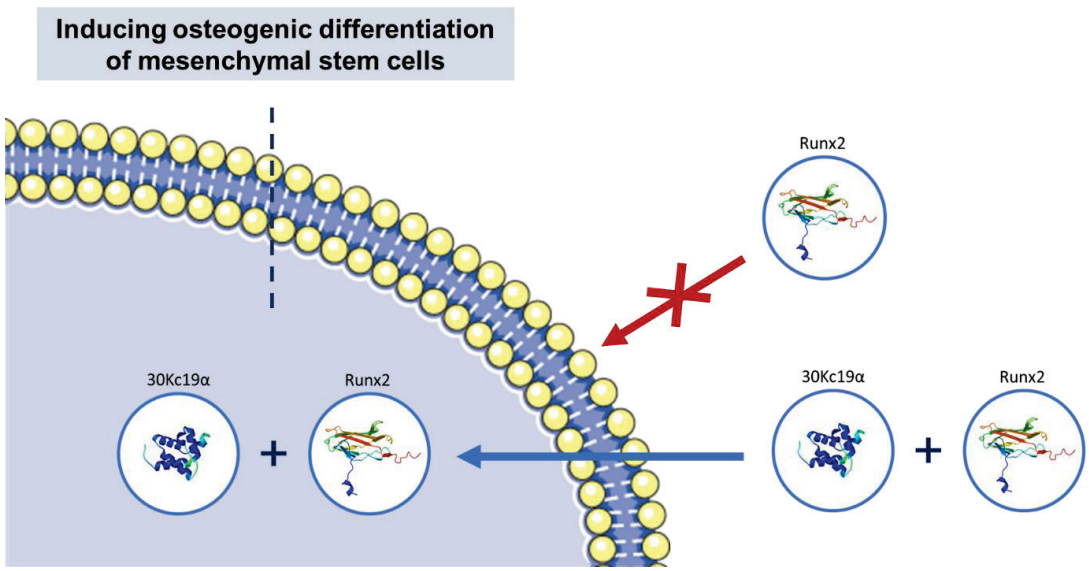


Figure 2. Schematic diagram of the research

2. Materials and methods

2.1 Plasmid construction of pET-23a/*Runx2* and pET-23a/*30Kc19α-Runx2*

Runx2 gene and 30Kc19α gene were amplified by polymerase chain reaction (PCR). Primer designs for PCR are as follows: *Runx2* forward primer: 5'-AGT GCT AGC ATG GCG TCA AAC AGC CTC T-3'; *Runx2* reverse primer: 5'-AGT CTC GAG ATA TGG CCG CCA AAC AGA C-3'; *30Kc19α* forward primer: 5'-AGT CAT ATG GAT TCC GAC GTC CCT AAC G-3'; *30Kc19α* reverse primer: 5'-AGT GCT AGC TTT CGG CGA AGA TAA GTC T-3'.

Runx2 and *30Kc19α-Runx2* were constructed into pET-23a expression vector (Novagen, USA). *Nhe*I and *Xho*I were used as restriction sites for *Runx2* in pET-23a/*Runx2*. *Nde*I and *Nhe*I were used as restriction sites for *30Kc19α*, while *Nhe*I and *Xho*I for *Runx2* in pET-23a/*30Kc19α-Runx2.* Both vectors were designed to contain His-tag at the C-terminus in order to identify and purify the recombinant protein.

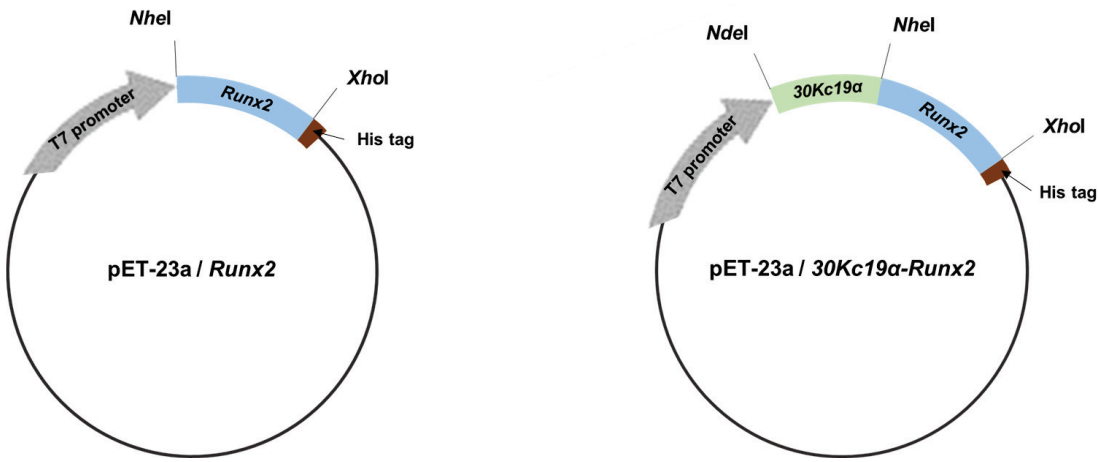


Figure 3. Plasmid construction of pET-23a/*Runx2* and pET-23a/*30Kc19α-Runx2*

2.2 Protein expression and purification of 30Kc19α-Runx2

Protein expression

pET-23a/*30Kc19α-Runx2* was transformed into BL21 (DE3) competent *E. coli* (Novagen) to express protein. Then, the transformed *E. coli* was precultured at 37°C in LB broth (Miller, USA) with 100 μg/ml of ampicillin (Sigma-Aldrich, USA) overnight. Next day, 30 ml of the overnight-cultured *E. coli* was transferred to 1L of LB broth and incubated at 37°C for cell growth. 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG; Calbiochem, USA) was added for induction once OD_{600} value reached 0.4. After IPTG induction, *E. coli* was further incubated for 6 hours at 25°C or 37°C. The cells were collected by centrifuging at 12000 rpm for 10 min at 4°C. After removing the supernatant, remained pellet was frozen at -20°C for later processing.

To compare the solubility of Runx2 and 30Kc19α-Runx2, recombinant Runx2 protein was also expressed in the same manner as 30Kc19α-Runx2.

Protein purification

The pellet collected from 1L of LB broth was resuspended with 20 ml of His-binding buffer (20 mM imidazole (Sigma-Aldrich), 20 mM Tris (Sigma, USA), 500 mM NaCl (Junsei, Japan), pH 8.0). Then, resuspended pellet was poured into an ice bucket and sonicated for 10 minutes (25% amplitude, pulse on 5s, pulse off 5s). The cell lysates were centrifuged at 12,000 rpm for 30 minutes at 4°C. The supernatants were filtered with 0.22 μm bottle top filter (Jetbiofil, Korea). 30Kc19α-Runx2 protein was purified using fast protein liquid chromatography (FPLC; GE Healthcare, Sweden). Filtered soluble proteins were loaded onto

Histrap HP (GE Healthcare) filled with His-binding buffer. To washout unbound or weakly-bound proteins, His-washing buffer (50 mM imidazole, 20 mM Tris-HCl, 0.5 M NaCl, pH 8.0) flowed through the column. Remained proteins were eluted with His-elution buffer (350 mM imidazole, 20 mM Tris-HCl, 0.5 M NaCl, pH 8.0) After elution, we changed a buffer to Dulbecco's Modified Eagle's medium (DMEM; Biowest, France) by using a desalting column (GE Healthcare) for further cellular use.

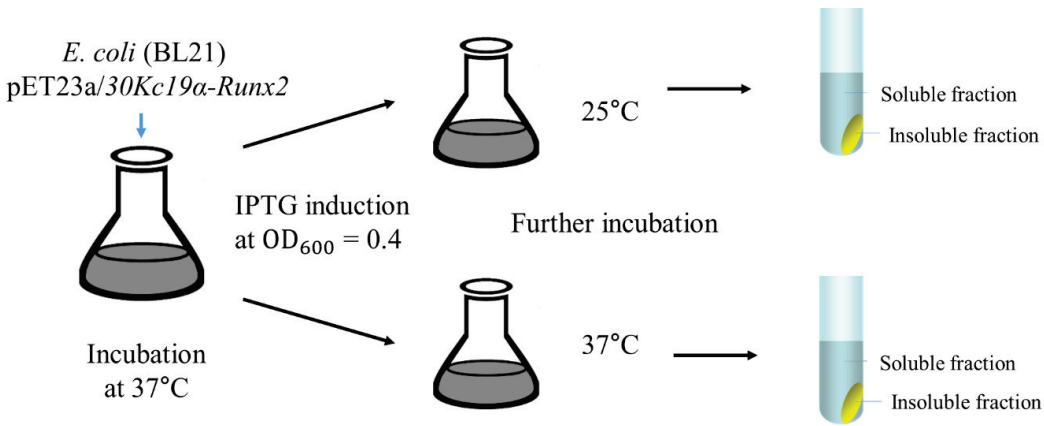


Figure 4. Recombinant protein expression in *E. coli*

2.3 Cytotoxicity assay

The viability of hMSCs exposed to different concentrations of fusion proteins for 24, 48 and 72 hours was determined by cell counting kit-8 (CCK-8; Dojindo, Japan). hMSCs were seeded into 48-well (Eppendorf, Germany) with MSCGM (Lonza, USA) one day before the experiments and preincubated for 24 hours. Then 5, 10, 20 and 40 μg/ml of proteins were treated to hMSCs and the cells were further incubated for 24 hours. After that, 10 vol% of CCK-8 solution was added to each well of the plate and the plate was incubated for 2 hours. The absorbance at 450 nm was measured by the microplate reader (Tecan, Switzerland). The cytotoxicity tests for 48 and 72 hour-protein treatment were also performed in the same way.

2.4 Cell penetrating assay

Cell penetration of 30Kc19α-Runx2 was observed with immunocytochemistry (ICC). hMSCs were seeded into 8-well confocal plate (Thermo Fisher Scientific, USA) with MSCGM. When it reached 50% confluent, 10 μg/ml of the proteins were treated for 4 hours. Following the fixation and permeation steps, proteins were labeled with 30Kc19 antibody as the primary antibody and Alexa Fluor® 488 (Invitrogen, USA) as the secondary antibody. And nucleus was stained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). Confocal laser scanning microscopy (Olympus, USA) was used for observing various focal planes of the cells.

2.5 hMSC culture and differentiation

Bone marrow derived hMSCs (Lonza) were cultured in 24-well plate (Eppendorf) with MSCGM until the cells reached 80% confluency. Then, cells were transferred to osteogenic medium (OM). Two kinds of OM were used: conventionally used OM (DMEM supplemented with 10% fetal bovine serum (FBS; Biowest), 1% penicillin streptomycin (PS; Sigma), 50 μM ascorbic acid (Sigma) and 10 mM β-glycerophosphate (Sigma) and 100nM dexamethasone (DEX; Sigma)) and OM without DEX (DMEM supplemented with 10% FBS, 1% PS, 50 μM ascorbic acid, 10 mM β-glycerophosphate). When OM was used, equimolar proteins were once treated in the cells on day 0. When OM without DEX was used, cells were treated twice with equimolar proteins on day 0 and day 2. The medium was replaced to fresh medium every 2 days.

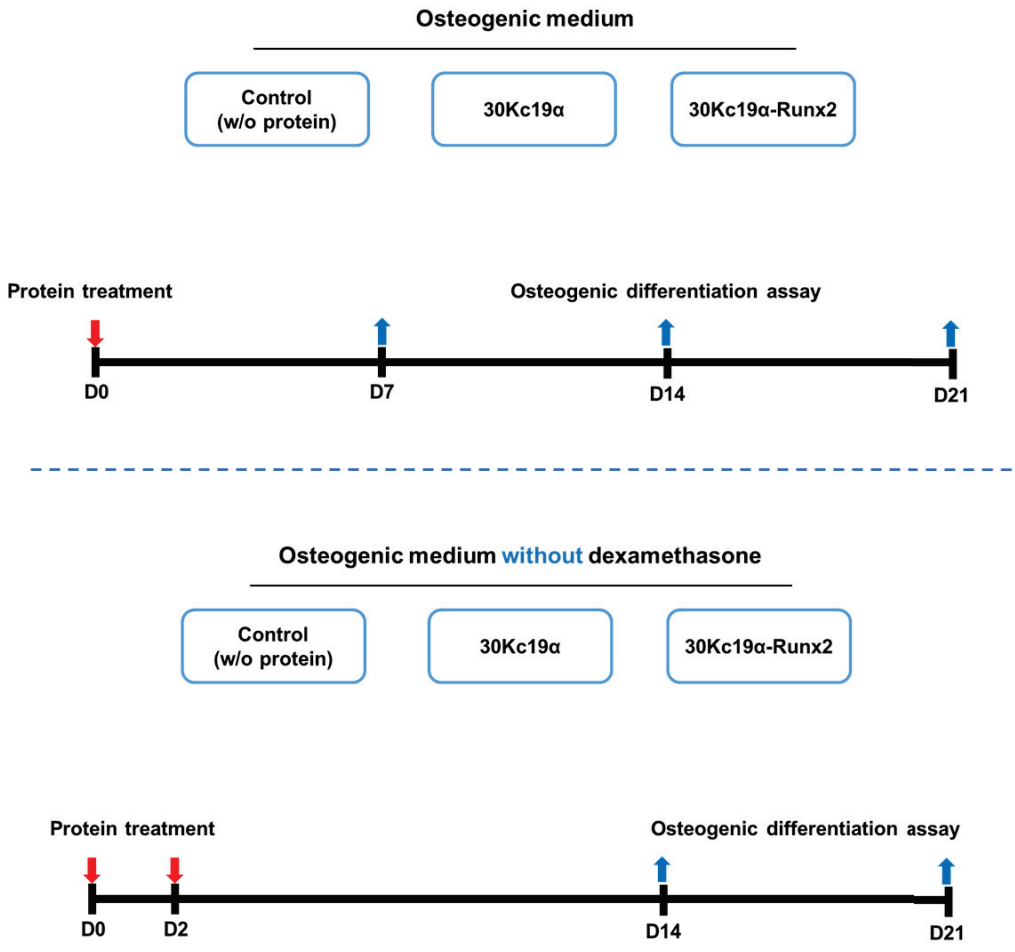


Figure 5. Experimental design for osteogenic differentiation of hMSCs

2.6 ALP and ARS staining

Alkaline phosphatase (ALP) staining and alizarin red S (ARS) staining were conducted on hMSCs differentiated in OM (with or without DEX) for 7, 14 days and 14, 21 days respectively to visualize the extent of osteogenic differentiation of hMSCs. The medium was aspirated from the wells and then each well was washed with phosphate buffered saline (PBS; Welgene, Korea). In the case of ALP staining, fixative solution, mixture of acetone (Aldrich, USA) and citrate solution (Sigma-Aldrich) (3:2, v/v) was used for fixation. After 1 minute of fixation, wells were washed with PBS and added with ALP staining solution (0.1% naphthol AS-MX phosphate (Sigma-Aldrich) and 0.1% fast blue BB salt (Sigma)) for 30 minutes. Then, wells were washed with PBS for 1 minute. In the case of ARS staining, 4% paraformaldehyde (PFA; Biosesang, Korea) in PBS was used for fixation. After 5 minutes of fixation, wells were washed with DDW and added with 2% w/v ARS (Sigma) in DDW for 10 minutes. Then, wells were washed with DDW for 10 minutes. Finally, optical microscope (Olympus) images of staining results were obtained.

3. Results and discussion

3.1. Plasmid construction and protein expression of 30Kc19α-Runx2

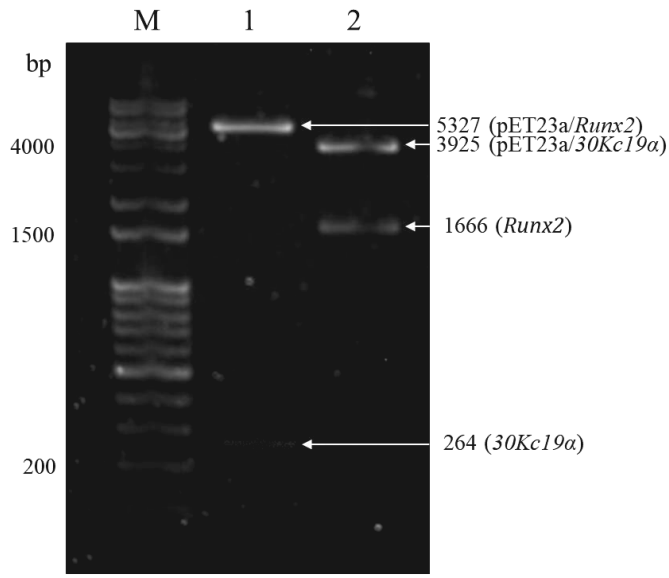
To confirm that *30Kc19α* and *Runx2* were successfully inserted to the pET-23a vector, the plasmid was double-digested using *Nde*I and *Nhe*I for *30Kc19α,* and *Nhe*I and *Xho*I for *Runx2*. Consequently, the digested plasmids were loaded onto agar gels to conduct electrophoresis and verify their respective sizes. As demonstrated in Figure 6, lane 1 contains two bands with theoretical base pairs of pET-23a/*Runx2* and *30Kc19α,* indicating the successful insertion of *30Kc19* at *Nde*I/*Nhe*I restriction enzyme sites. In the case of *Runx2*, lane 2 contains two bands with theoretical base pairs of pET-23a/*30Kc19α* and *Runx2* (Fig. 6).

Some recombinant proteins expressed in *E. coli* can be obtained as inclusion bodies due to aggregation. Inactive inclusion bodies could regain their functions via solubilization and refolding process. However, these processes require extensive amount of time and manual effort and do not always lead to high production yields and restoration of their native activities [25]. Therefore, soluble-expressed 30Kc19α-Runx2 was used for further experiments.

Lowering expression temperature is one method of providing the appropriate amount of stress to *E. coli* to obtain soluble recombinant protein. By decelerating the process of protein synthesis, protein aggregation and degradation of recombinant protein can be abated [26]. Therefore, IPTG induction temperature was decreased from the conventional expression temperature, 37°C, to 25°C to augment the soluble expression of the protein of interest. Western blot analysis was conducted to compare the amount of 30Kc19α-Runx2 expressed in

soluble form in two conditions, 25°C versus 37°C. When the western blot bands of soluble fractions (Sol) of cell lysates were compared between the two conditions, the 25°C band was thicker than that of 37°C condition (Fig. 7). This indicated that soluble expression of 30Kc19α-Runx2 increased when *E. coli* was incubated at 25°C after IPTG induction. Therefore, we produced 30Kc19α-Runx2 at 25°C expression condition.

Protein expressed under this condition was identified as 30Kc19α-Runx2, as the band was detected on the expected size; 71.2 kDa in coomassie blue staining and western blot analysis (Fig. 8). The concentration of purified protein was 200 μg/ml.



M: 1 kb DNA marker

1: *Nde*I/*Nhe*I digestion of pET23a/*30Kc19α-Runx2*
2: *Nhe*I/*Xho*I digestion of pET23a/*30Kc19α-Runx2*

Figure 6. Restriction digestion of 30Kc19α-Runx2

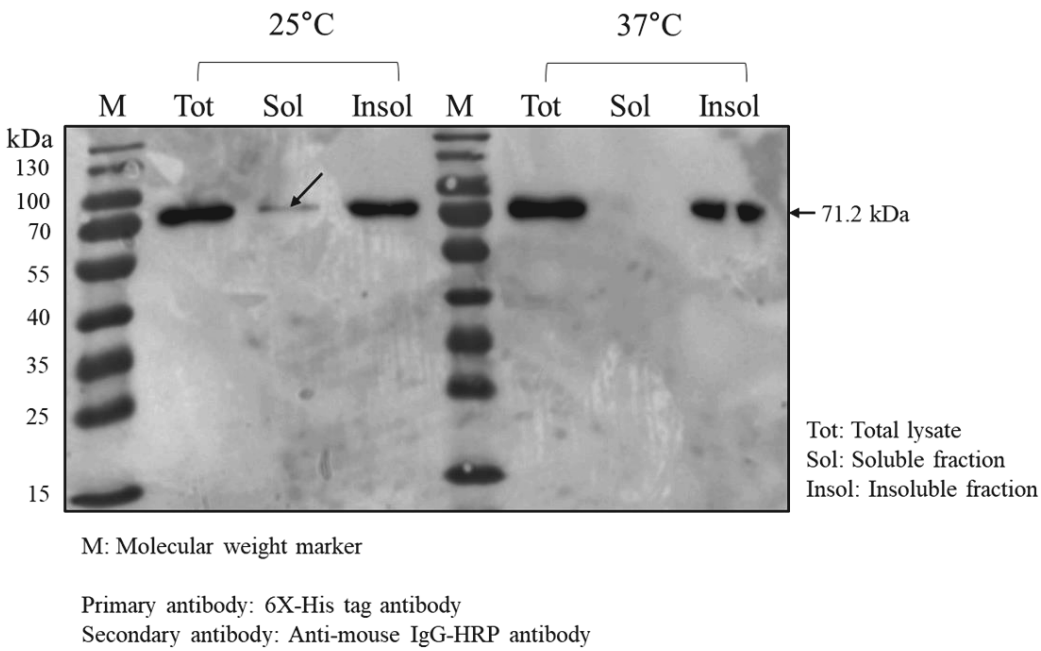


Figure 7. Effect of expression temperature on solubility of 30Kc19α-Runx2

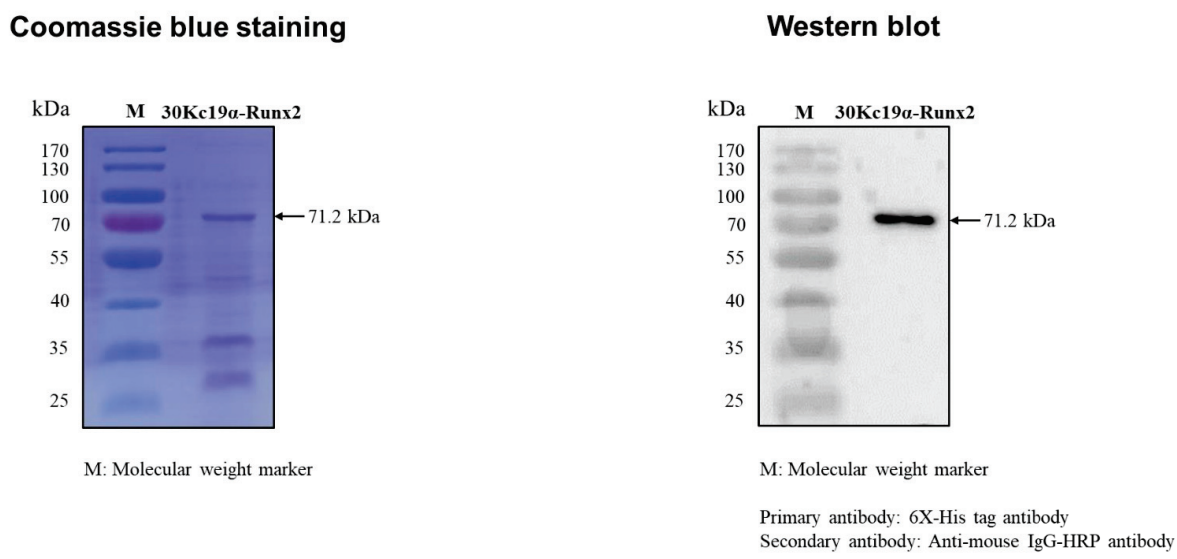


Figure 8. Coomassie blue staining and western blot analysis of 30Kc19α-Runx2

3.2. Enhanced soluble expression of Runx2 by fusion with 30Kc19α

30Kc19 has ‘soluble expression enhancement’ function in addition to cellular penetration and protein stabilization functions [10]. As 30Kc19α showed cellular penetration and protein stabilization functions, we expected 30Kc19α could improve solubility of fusion protein like 30Kc19. So, we compared the extent of soluble expression between Runx2 and 30Kc19α-Runx2 expressed in the same condition by western blot analysis.

On Fig. 9, each band was consistent with the theoretical size of Runx2 (61.1 kDa) and 30Kc19α-Runx2 (71.2 kDa). While there was no visible band in soluble fraction of cell lysates in the case of Runx2, the band was detected in soluble fraction of cell lysates in the case of 30Kc19α-Runx2. The solubility of Runx2 was improved by fusion with 30Kc19α. Therefore, we could confirm that 30Kc19α also enhances soluble expression of fusion protein as expected.

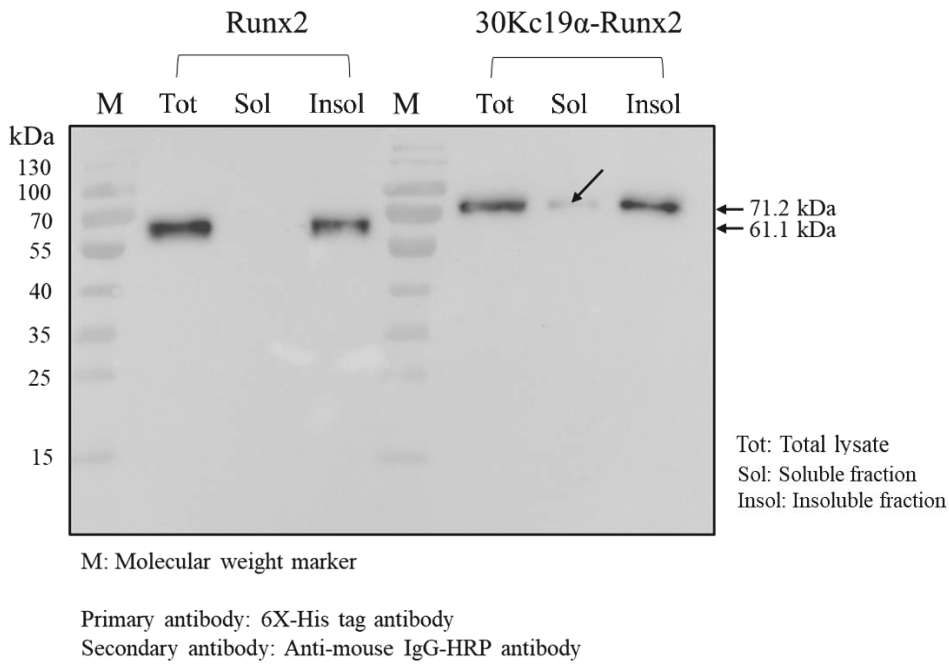


Figure 9. Western blot analysis of the soluble and insoluble fractions of Runx2 and 30Kc19α-Runx2

3.3. Low cytotoxicity of 30Kc19α-Runx2

To test whether there is cytotoxic effect of 30Kc19α-Runx2 on hMSCs, CCK-8 assay was conducted. Two factors were considered: protein concentration and protein treatment time. Cell viability of each experimental group was normalized with the cell viability of control group (without protein treatment). Cell viabilities of all groups were close to 100%. It showed that 30Kc19α-Runx2 didn't result in cell damages or cell death of hMSCs.

There was no significant difference in cell viability among 24, 48 and 72 h protein treatment when hMSCS were exposed to same concentration of proteins. Rather, the cell viability exceeded 100% when hMSCs were treated with higher concentration of proteins (Fig. 10). We hypothesized that 30Kc19α may have effect on cell proliferation. Previous studies on 30Kc19 discovered that 30Kc19 enhances enzyme stability [8, 9]. It could be the reason for increased cell proliferation in high concentration 30Kc19α-Runx2 treated groups.

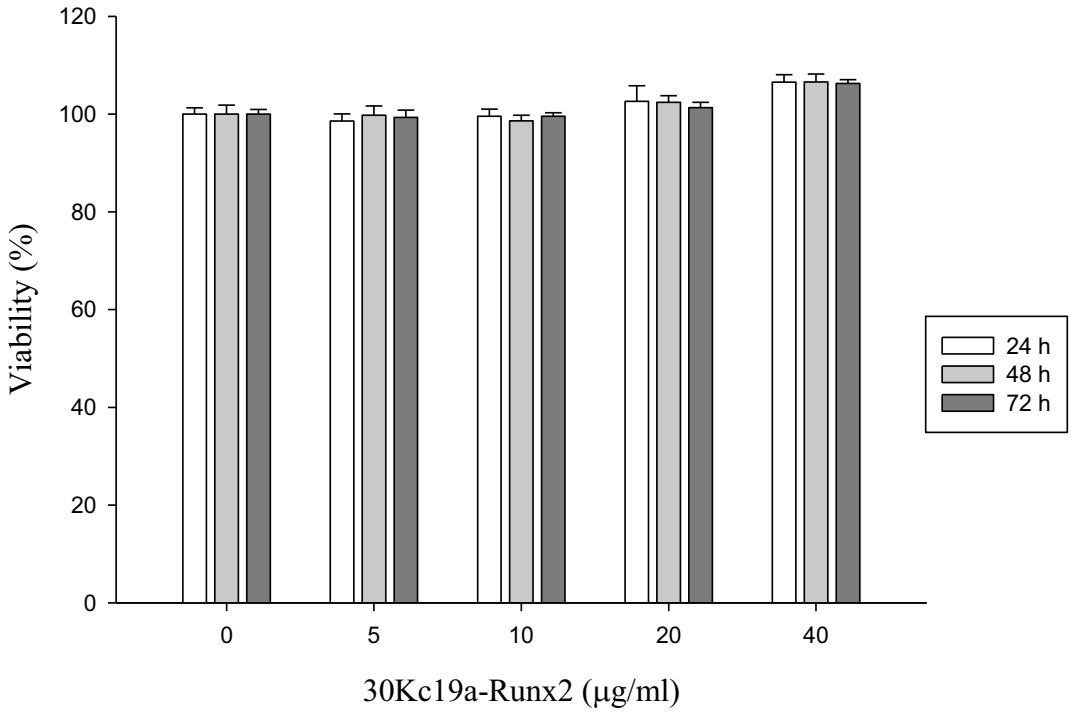


Figure 10. Effect of 30Kc19α-Runx2 on viability of hMSCs

3.4. Cell-penetrating ability of 30Kc19α-Runx2

Immunocytochemical analysis was carried out to confirm whether 30Kc19α-Runx2 could penetrate cell membrane. We planned to verify that 30Kc19α played a role in intracellular delivery of Runx2 by comparing the cell-penetrating ability of Runx2 and 30Kc19α-Runx2. However, Runx2 was not expressed in soluble form so that we couldn't use Runx2 for experiments. Therefore, we chose hMSCs which were not treated with any protein as a control group instead of hMSCs treated with Runx2. Unlike conventional optical microscopy, confocal laser scanning microscopy is capable of obtaining images of cell sectioned in multiple planes, so that we could observe labeled target molecules in cells [27]. In confocal microscopy images, blue fluorescence indicates nucleus of cells and green fluorescence indicates 30Kc19α-Runx2 labeled with Alexa Fluor® 488.

We could observe green fluorescence in the sections of hMSCs treated with 30Kc19α-Runx2 (Fig. 11). It means that 30Kc19α-Runx2 could get into cells. The proteins were not localized but spread in the cytosol and nucleus of hMSCs. In the previous study which conjugated basic fibroblast growth factor (bFGF) to 30Kc19α, 30Kc19α-bFGF fusion proteins were not observed in the nucleus of cells. 30Kc19α dragged cargo proteins into cytosols, then nuclear localization sequence in Runx2 may be involved in nuclear transport of 30Kc19α-Runx2 [28].

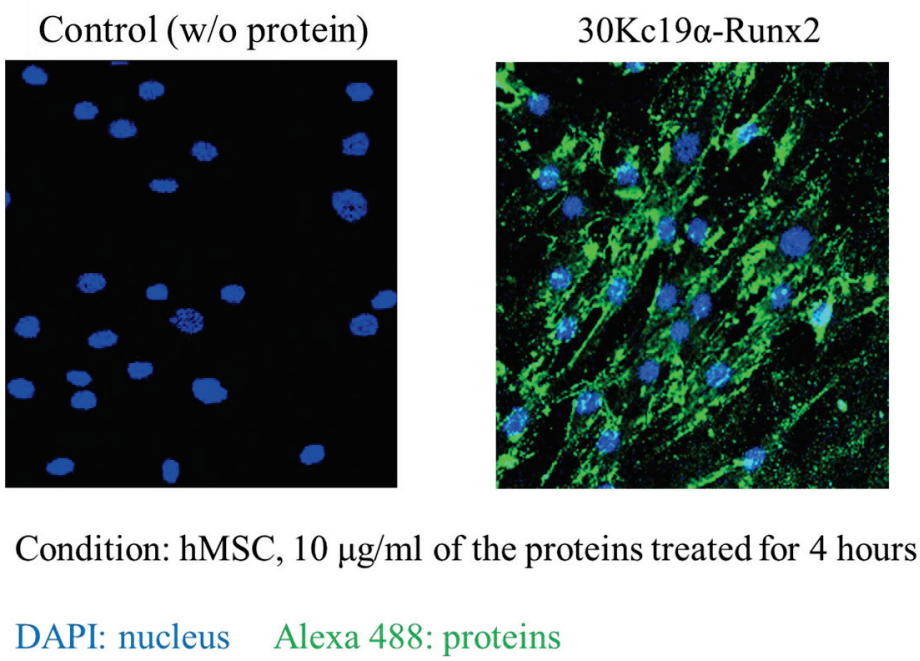


Figure 11. Confocal fluorescence images of control and 30Kc19α -Runx2 group

3.5. Effect of 30Kc19α-Runx2 on *in vitro* osteogenesis of hMSCs

Runx2-treated group had to be used as a control, but recombinant Runx2 wasn't expressed in soluble form in *E. coli* and it was already known that it couldn't penetrate hMSCs [24]. Therefore, the group treated with Runx2 was excluded from experimental groups. In order to check whether 30Kc19α has any effect on osteogenesis of hMSCs, 30Kc19α group was added as experimental group with 30Kc19α-Runx2 group.

The effect of 30Kc19α-Runx2 on osteogenic differentiation of hMSCs was verified via ALP staining and ARS staining. Both staining assays were performed because these assays detect markers observed in different stages of osteogenesis. ALP staining detects alkaline phosphatase expressed in pre-osteoblasts and osteoblasts by exhibiting a dark blue-violet hue. ARS staining stains calcium deposits produced by mature osteoblasts into red-orange (Tab. 1). Therefore, ALP staining was used to confirm early stage osteogenic differentiation, while ARS staining was used to confirm late stage osteogenic differentiation.

When differentiation of hMSCs was conducted, two kinds of OM were used: conventional OM which contains DEX and OM without DEX. DEX is a kind of glucocorticoid which regulates lineage specification of hMSCs to osteoblasts [29]. The ability of 30Kc19α-Runx2 to promote and induce osteogenesis of hMSCs was examined by treating cells with these osteogenic media.

	ALP staining	Alizarin Red S staining
Target	Alkaline phosphatase	Mineralized bone matrix
Color	Dark blue-violet	Red-orange
Stage	Pre-osteoblast & osteoblast	Mature osteoblast

Table 1. Brief description of ALP and ARS staining

3.5.1. Synergistic effect of 30Kc19α-Runx2 and DEX on osteogenesis

First, hMSCs were treated with conventional OM to induce osteogenesis in all groups. As DEX was added to OM, all groups entered osteoblastic lineage. It was verified as calcium deposits were detected not only in 30Kc19α-Runx2 group, but also in control and 30Kc19α groups by ARS staining after 3 weeks of differentiation (Fig. 13). Higher staining density was observable in 30Kc19α-Runx2 group compared to other groups when ALP and ARS staining were performed (Fig.12, 13). From the results of osteogenic differentiation assays (ALP staining and ARS staining), it was verified that 30Kc19α-Runx2 had an effect on osteogenesis of hMSCs.

In the case of 30Kc19α-Runx2 group, severe red stains were observed even when the hMSCs were differentiated in OM only for 14 days. It showed that osteogenic differentiation by 30Kc19α-Runx2 was accelerated by DEX (Fig. 13, 15). Furthermore, the significant difference in ARS staining results of the control and 30Kc19α-Runx2 group showed that 30Kc19α-Runx2 promoted osteogenesis by DEX (Fig. 13). Therefore, 30Kc19α-Runx2 and DEX had synergistic effects on osteogenesis of hMSCs.

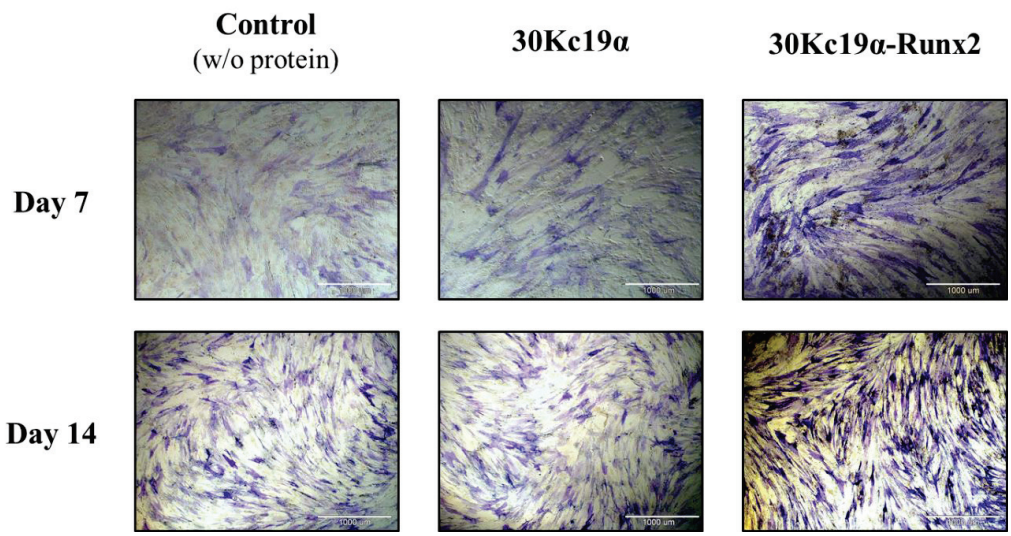


Figure 12. ALP staining results of three groups on day 7 and 14 cultured in OM

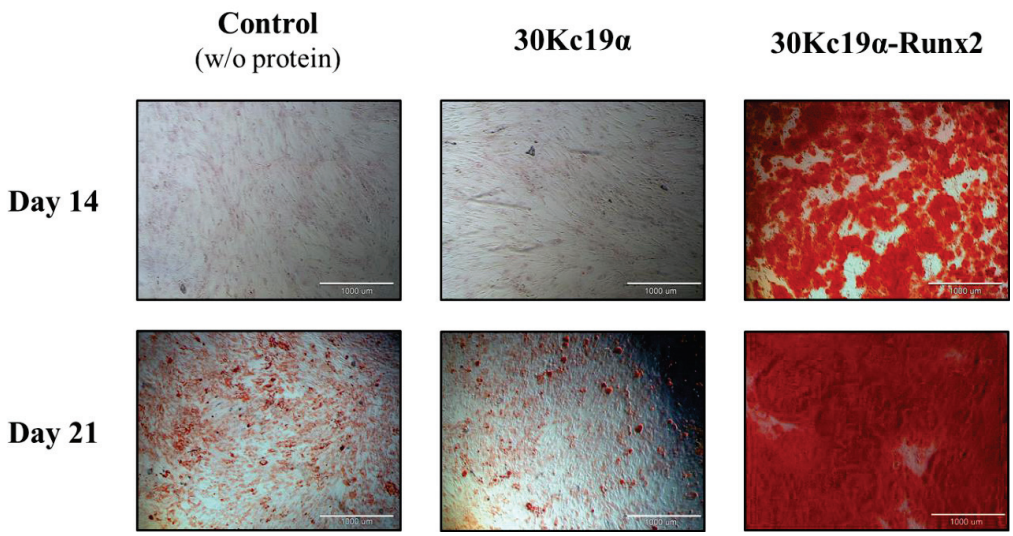


Figure 13. ARS staining results of three groups on day 14 and 21 cultured in OM

3.5.2. *In vitro* osteo-inductivity of 30Kc19α-Runx2

As DEX is an upstream component of signaling pathway, OM without DEX was used in experiments for evaluating osteo-inductivity of 30Kc19α-Runx2 [29]. ALP staining data of control, 30Kc19α and 30Kc19α-Runx2 groups cultured in OM without DEX were compared. Blue stains were observed in all groups but there was significant difference in the extent of staining among three groups. 30Kc19α-Runx2 group showed more blue stains than control and 30Kc19α groups on day 14 (Fig. 14). ARS staining data showed similar tendency to ALP staining. On day 21, only 30Kc19α-Runx2 group was stained with ARS staining. 30Kc19α-Runx2 induced osteogenic differentiation of hMSCs. It was observed that 30Kc19α was not involved in it (Fig. 15).

The reason why other two groups were also stained with ALP staining, when hMSCs were cultured in OM without DEX was that T flask culture could affect osteogenesis. The role of matrix stiffness in MSC differentiation has been demonstrated by many researches [30, 31]. MSCs cultured on stiff matrix had tendency to differentiate into osteoblasts [31]. Substrate stiffness was involved in inducing osteogenesis of all groups. However, it was obvious that the effect of 30Kc19α-Runx2 on inducing osteogenesis was somewhat greater than that of substrate stiffness, as little or no red stain was observed in the rest of the groups except the 30Kc19α-Runx2 group after ARS staining was conducted.

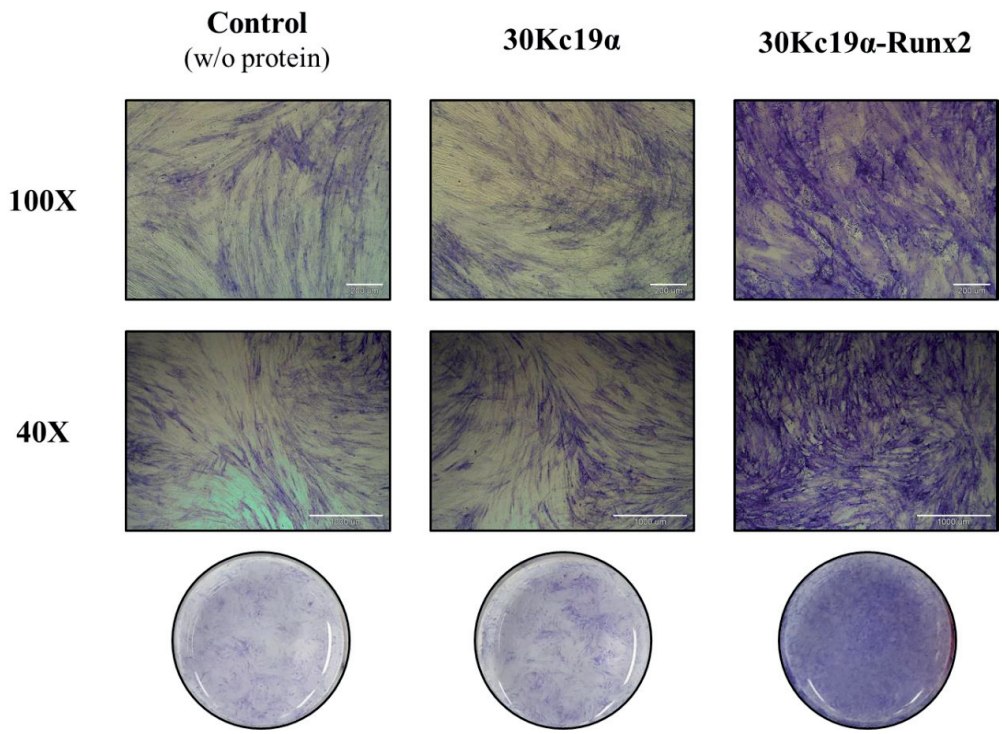


Figure 14. ALP staining results of control, 30Kc19α and 30Kc19α-Runx2 groups on day 14 cultured in OM without DEX

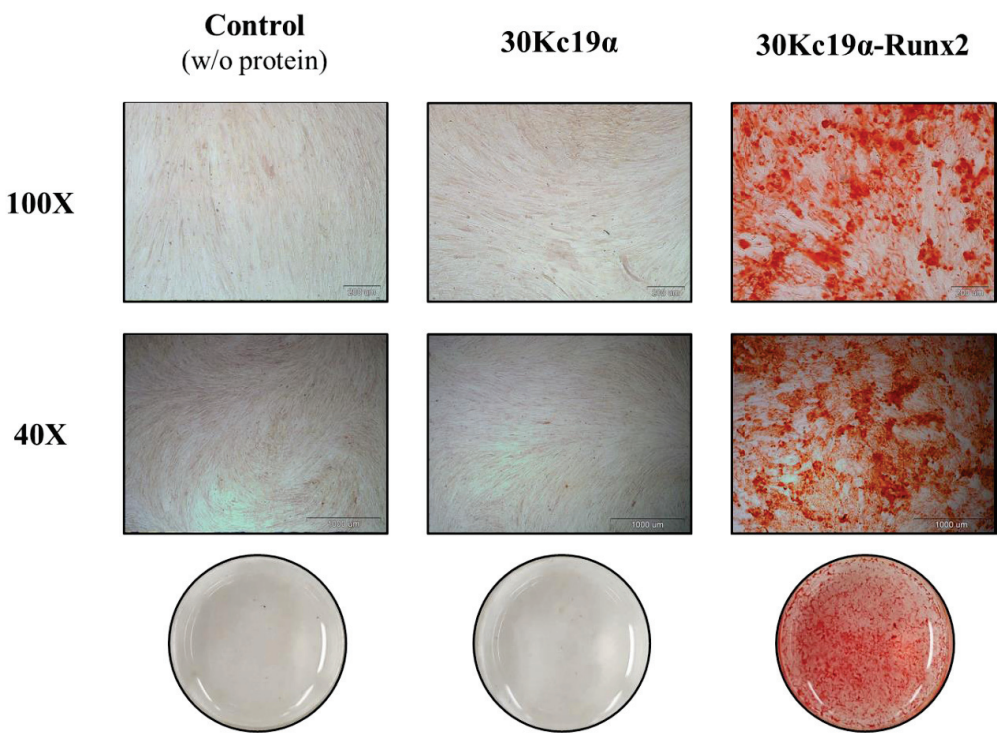


Figure 15. ARS staining results of three groups on day 21 cultured in OM without DEX

4. Conclusions

Recombinant Runx2 should be delivered into cells to role as a transcription factor. However, Runx2 itself could not penetrate cell membrane. Conjugation of Runx2 to 30Kc19α which contains CPP in it was suggested as a solution for resolving this problem. To confirm the cell penetration ability of 30Kc19α, immunocytochemistry was conducted. It was observed that 30Kc19α enabled the intracellular delivery of Runx2. Furthermore, the western blot analysis of Runx2 and 30Kc19α-Runx2 showed that Runx2 protein expressed in insoluble form was expressed in soluble form through the fusion with 30Kc19α. This result demonstrated that 30Kc19α, like 30Kc19, had the ability to enhance the solubility of expressed protein via fusion with the target protein. In order to increase portion of soluble 30Kc19α-Runx2, expression temperature was adjusted to lower temperature. The amount of 30Kc19α-Runx2 protein expressed in soluble form was greater when cultured at 25°C than at 37°C. Before preforming osteogenic differentiation of hMSCs, cells were treated with 30Kc19α-Runx2 to assess the cytotoxicity of it. Cytotoxicity of 30Kc19α-Runx2 was very low regardless of the protein concentration after protein treatment for 72 hours. Finally, the effect of fusion protein on osteogenesis of hMSCs was confirmed by ALP and ARS staining. 30Kc19α-Runx2 itself had osteo-inductivity without any help of dexamethasone and it showed synergistic effect with dexamethasone.

The cell penetrating ability of 30Kc19α enabled direct delivery of transcription factor, Runx2 into hMSCs, thereby permitting Runx2 to induce osteogenic differentiation of hMSCs. 30Kc19α delivery system is expected to induce differentiation of hMSCs into various cell types,

not limited to osteoblast. Therefore, it would be a new tool for regulating stem cell fate in tissue engineering and regenerative medicine.

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국 문 초 록

30Kc19α-Runx2 단백질을 이용한 인간 중간엽 줄기세포의 골 분화 유도

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최근 인간 중간엽 줄기세포를 골아 세포로 분화시켜 골 재생을 유도하는 연구가 많이 진행되고 있다. Runx2는 인간 중간엽 줄기세포를 골아 세포 계통으로 유도하는 주요 전사인자로 알려져 있다. 그러나 Runx2 재조합 단백질은 용해도가 낮고 세포 투과력이 낮다는 단점 때문에 골 분화에 사용하는 데 어려움이 있었다. 본 연구에서 우리는 Runx2의 수용성 발현 및 세포 내 전달을 위해 Runx2를 세포 투과 단백질인 30Kc19α와 융합시켜 인간 중간엽 줄기세포의 골 분화를 유도했다. pET-23a/*30Kc19α-Runx2* 플라스미드 제작 후, 재조합 단백질을 대장균에서 발현시키고 니켈 친화성 크로마토그래피로 정제했다. Runx2 단백질의 수용성 발현은 30Kc19α와의 융합을 통해 향상되었다. 융합 단백질은 72시간 처리 후에도 단백질 농도와 관계없이 낮은 세포 독성을 나타냈다. 또한 공초점 레이저 주사 현미경으로 30Kc19α가 Runx2를 세포 내로 끌고 들어갈 수 있음

을 관찰했다. 인간 중간엽 줄기세포의 골 분화에 대한 30Kc19α-Runx2의 효과는 ALP 염색과 ARS 염색 같은 실험들에 의해 확인되었다. 따라서 우리는 30Kc19α-Runx2 재조합 단백질이 조직 공학 및 재생 의학에서 새로운 골 분화 유도 물질로 사용될 수 있다고 기대한다.

주요단어: 줄기세포 분화, 중간엽 줄기세포, 골 분화, 전사인자, Runx2, 세포 투과 단백질, 30Kc19α

학번: 2017-25452