The role of STAT5 in osteogenesis of human bone-marrow mesenchymal stromal cells

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The role of STAT5 in osteogenesis of human bone-marrow mesenchymal stromal cells

Directed by Professor Jin Woo Lee

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The role of STAT5 in osteogenesis of human bone-marrow mesenchymal stromal cells

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Human bone-marrow mesenchymal stromal cells (MSCs) can differentiate into a variety of cell types such as adipocyte, chondrocyte, myoblast, and osteoblast, depending on induction conditions. The each process of differentiation is carried out by interaction of various proteins and hormones. The feasibility of therapeutic applications of MSCs has been raised based on these potential differentiation characteristics, however, the precise mechanism of differentiation has not yet been determined. As a possible regulator of MSCs differentiation, STAT proteins have been reported. STAT is a family of proteins consisted of 7 different types. They form either a homodimer or a heterodimer, and are phosphorylated by receptors for various growth factors and cytokines. Phosphorylation of STAT induces translocation of STAT to nuclei, then, STAT regulates the expression of target genes. In particular, STAT5 has been reported to accelerate the differentiation of MSCs to adipocytes. However, there

relationship between STAT and differentiation of MSCs into osteoblast has hardly been studied. In this study, a series of studies have been carried out to determine the mechanism how STAT5A affects the human bone-marrow MSCs differentiation into osteoblast. When MSCs were induced to differentiate into osteoblast, the expression of STAT5A and STAT5B proteins were increased compared to the control cells. Knocking-down the expression of STAT5A with specific siRNAs promoted the differentiating of MSCs into osteoblast. Expressions of Runx2 and DLX5 proteins and mRNAs, marker genes for osteoblast, were confirmed to be increased in MSCs transfected with STAT5A siRNAs. In contrast, over-expression of STAT5A during the differentiating process of MSCs into osteoblast, decreased Runx2 and DLX5 at protein and mRNA levels. The transcriptional activity of the DLX5 promoter was decreased by over-expression of STAT5A and increased by knocking-down of STAT5A with siRNAs. In summary, these data suggest that the induction of MSC differentiation into osteoblast is mediated by DLX5 which is regulated by STAT5A as a pivotal transcription factor in osteogenesis.

Key words: MSC, osteogenesis, STAT5A, DLX5

The role of STAT5 in osteogenesis of human bone-marrow mesenchymal stromal cells

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(Directed by Professor **Jin Woo Lee**)

I. INTRODUCTION

Human bone marrow mesenchymal stromal cells are capable of becoming one of a number of phenotypes, including bone and cartilage, tendon, muscle and fat[1-6]. Although they represent powerful tools for several therapeutic settings, mechanisms regulating their migration to peripheral tissues are still unknown[7].

Osteoporosis and obesity, two disorders of body composition, are growing in prevalence[8]. Interestingly, these diseases share several features including a genetic predisposition and a common progenitor cell. With aging, the composition of bone marrow shifts to favor the presence of adipocytes, osteoclast activity increases, and osteoblast function declines, resulting in osteoporosis[9]. Secondary causes of osteoporosis, including diabetes mellitus, glucocorticoids and immobility, are associated with bone-marrow adiposity[10]. An important advance in the understanding of age-related bone loss is the finding of a fat and bone

connection[8-10].

Osteoblasts are bone forming cells that are responsible for bone growth and remodeling[11]. They are derived from bone marrow mesenchymal stromal cells through a series of process including commitment, osteoprogenitor expansion, terminal differentiation and cell death[11]. Osteoblast differentiation is controlled by transcription factors Runx2 and Osterix. Runx2 and Osterix are both necessary and sufficient for osteoblast differentiation and for *in vivo* bone calcification. Mice deficient of either Runx2 or Osterix show no mature osteoblasts or calcified bones[12, 13]. Two homeotic genes, DLX and MSX, appear to regulate development of mineralized tissues, including bone, cartilage, and tooth. DLX5 represses osteocalcin gene transcription[13]. However, the coupling of increased DLX5 expression with progression of osteoblast differentiation suggests an important role in promoting expression of the mature bone cell phenotype[16]. The maximal expression of DLX5 occurs in the final stages of osteoblast differentiation in vitro when the extracellular matrix mineralizes. This pattern of DLX5 expression reflects a general role of DLX5 in lineage commitment and progression of osteoblast differentiation[16]. The function of MSX2 in osteoblasts is still controversial. One study suggested that MSX2 inhibits differentiation of osteoblast precursors and immature osteoblasts, resulting in an increase in the source of osteoblastic cells. However, other studies demonstrated that MSX2 promotes osteoblast differentiation and proliferation[13, 15].

Signal transducers and activators of transcription (STATs) are a family of latent

transcription factors of cytokine-mediated signaling events in cell growth and differentiation[16]. In mammals, there are seven STAT genes, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6[16]. There is sufficient diversity in the STAT amino acid sequences and their tissue-specific distributions to account for their diverse roles in responses to extracellular signaling proteins[16-18]. STAT proteins are inactive as transcription factors in the absence of specific receptor stimulation and are localized in the cytoplasm of unstimulated target cells[20]. Once the activated STAT dimer recognizes a target promoter, the transcription fate from this promoter is dramatically increased[22, 23]. There are no osteogenesis related experiments for STAT5A as of yet.

This study shares its purpose in finding out the relationship between STAT5A and osteogenesis. The expression of STAT5 throughout osteogenesis will be looked at, as well as its relationship with osteogenesis.

II. MATERIALS AND METHODS

1. Primary mesenchymalstromal cell (MSC) culture and cell differentiation

Bone marrow aspirates were obtained from posterior iliac crest of 10 healthy adult donors ranging from 20~69 years of age under the approval of Institutional Review Board (IRB). Mesenchymal stromal cells were specifically selected using their natural tendency to adhere to the plastic culture plate surface. After 7 days of culture in Dulbecco's Modified Eagle's medium-low glucose (DMEM-LG, Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS)(Welgene, Daegu, South Korea) and 1% antibiotic-antimycotic solution (Invitrogen, Grand Island, NY, USA), the non-adherent hematopoietic cells were removed. The cells were grown to 70% confluence through the average culture period of 10 days, then were promptly harvested by incubation with 0.25% trypsin/1mM EDTA (Invitrogen, Carlsbad, CA, USA) followed by 3 minute centrifugation at 1,300 RPM. This initial batch of cultured cells were designated as Passage 1, and the cells were subcultured in new $10cm^2$-dish up to passage 6-7. Then, cell were cultured in osteogenic medium, DMEM-low glucose supplemented with 1% antibiotic-antimycitic solution, 10% FBS, dexamethasone 10^{-7}M, β-glycerophosphate 10mM and ascorbic acid 50uM (Sigma, St.Louis, MO, USA) for 14 days.

2. **Plasmid constructs**

Expression plasmid of pCDNA-STAT5A and pCDNA-DLX5 were gifts (Dr. Yamashita H, Nagoya City University Graduate School of Medical Science, Nagoya, Japan). Human STAT5A promoter region spanning -2131/+57 bp was cloned into pGL3 basic vector and named pGL-2131. Human DLX5 promoter region spanning -1500/+141 bp was cloned into pGL3 basic vector and named pGL-1500.

3. Transient transfection and luciferase reporter assays

Hela cells in six-well plates were cultured in Dulbecco's Modified Eagle's medium-high glucose (DMEM-HG, Gibco) supplemented with 10% FBS, 1% antibiotic-antimycitic solution. Transient transfections were performed using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, 700ng luciferase reporter construct, 300ng human STAT5A and human DLX5 expression vectors, and 1ng SV-40 expression vectors and 3ul of lipofectamine 2000 were independently mixed with 250ul of opti-MEM and incubated for 5 minutes. To transfect constant amounts of DNA, sample DNAs were supplemented with control vector pCDNA3. After 5 minutes of incubation, diluted DNA were mixed with lipofectamine 2000 and were incubated for 20 minutes at room temperature. During this period, cells were washed with phosphate-buffered saline twice and 2ml of opti-MEM was added. After 20 minutes of incubation, transfection mixtures were added to the cells and incubated at 37℃ incubator in 5℃ CO2 and 95% air for 12 hours. Afterwards,

the medium was replaced with DMEM containing 10% FBS. After 24 hours, cells were harvested and lysed by 200ul reporter lysis buffer (Promega, Madison, WI, USA), and cell debris was removed by centrifugation. Luciferase activities were measured using 50ul of cell extract50ul luciferase assay reagent (Promega). Luciferase activities were normalized by renilla activities to adjust transfection efficiency. Luciferase activities exerted by the wild-type promoter constructs without expressing any receptors were taken as a control. The assays were performed in at least three experiments.

4. Inhibition of STAT5A and STAT5B

Synthetic siRNA oligonucleotides specific for regions in the STAT5A and STAT5B mRNA was designed and synthesized by Bioneer (Bioneer, Daejeon, South Korea). The silencing effects of several siRNA oligonucleotides were screened and tested initially for their ability to silence STAT5A and STAT5B expression. MSC in six-well plate at 80% confluency were transfected with siRNA oligonucleotides by lipofectamine 2000. Twenty-four hours later, the cells were subjected to the standard differentiation protocol, and various times thereafter, cell extracts were prepared for analysis. Control transfections were performed with Negative Control Duplexes (Bioneer).

STAT5 inhibitor was purchased from Merck KGaA (Merk KGaA, Darmstadt, Germany). MSCs were treated by STAT5 inhibitor.

5. Validation of gene expression in mRNA level

Total RNA from MSCs were isolated using RNAiso Plus (Takara, Shiga, Japan) reagent following the manufacturer's instruction. Briefly, 1mL of RNAiso Plus solution was added to the collected cells and was repeatedly pipette to completely dissolve cells. The cells were left on room-temperature for 10 minutes, and 200μL of chloroform was added and were vortexed until the solution became milky. The solution was then kept at room-temperature for 5 minutes, and was centrifuged at 13,000 RPM for 15 minutes at 4℃. Top liquid layer was then transferred to new tube and 500μL of 100% isopropanol was added. After brief vortex, the solution was kept at room-temperature for 10 minutes and was promptly centrifuged at 13,000 RPM for 10 minutes at 4℃. The supernatant was removed without disturbing the RNA pellet, which was then washed with cold 70% ethanol via centrifugation at 10,000 RPM for 5 minutes at 4℃. Finally, the RNA pellet was re-suspended on 30μL of diethylpyrocarbonated-water (DPEC-water). The overall quality and concentration of each RNA sample was confirmed using spectrophotometry. For cDNA reverse transcription, Omniscript Reverse-Transcription Kit (Qiagen, Venlo, Netherlands) was used. The exact PCR conditions used and the list of primer sets for specific gene amplification obtained from Gene Bank and/or the manufacturer is as listed on Table 1 and Table 2, respectively. GAPDH was used to normalize for relative expression intensity of all genes.

Table 1. PCR condition

Genes	Condition		Cycle
STAT5A	94 ℃	30sec - 60℃ 30sec - 72 ℃ - 30sec	33
STAT5B	94 ℃	30sec - 57℃ 30sec - 72 ℃ - 30sec	33
DLX5	94 ℃	30sec - 58℃ 30sec - 72 ℃ - 30sec	30
MSX2	94 ℃	30sec - 62℃ 30sec - 72 ℃ - 30sec	30
ALP	94 ℃	30sec - 52℃ 30sec - 72 ℃ - 30sec	25
GAPDH	94 ℃	30sec - 57℃ 30sec - 72 ℃ - 30sec	27

Table 2. Primer sequences for RT-PCR

Genes		Primer Sequence	Size (bp)
STAT5A	S	5‘- CGAGTGCAGTGGTGAGATCC-3'	200
	AS	5' -AACACAAGCTCATTGCTGCC-3'	
STAT5B	S	5' –CAGCCTGGACGTGCTACAGT-3'	178
	AS	5‘- TGGTCACCAGGGCTGAGATA-3'	
DLX5	S	5‘-GACAGGATCCCTATGACAGGAGTGTTTGA-3'	472
	AS	5‘-TGGACTCGAGATCTAATAAAGCGTCCCGGA-3'	
MSX2	S	5' –GCCAAGACATATGAGCCCTACCACCTG-3'	570
	AS	5' –GGACAGGTGGTACATGCCATATCCCAC-3'	
ALP	S	5' –CTACCAGCTCATGCATAACA-3'	220
	AS	5' –GACCCAATAGGTAGTCCACA-3'	
GAPDH	S	5'-GAAGGTGAAGGTCGGAGTC-3'	220
	AS	5'-GAAGATGGTGATGGGATTTC-3'	

S: Sense Strand AS: Antisense Strand

In addition to RT-PCR, quantitative real time PCR have been carried out using Takara Ex Taq^{TM} (Takara, Shiga, Japan), following the instruction as given. ABI7500 real time machine by AppliedBiosystems (ABI, Carlsbad, CA, USA) was used for all quantitative real time PCR procedures. The primers used for quantitative real time PCR as well as universal quantitative real time PCR conditions are as shown on Table 3 and Table 4, respectively. All primers were purchased from Bioneer (Bioneer, Daejeon, South Korea).

Table 3. Primer sequences for quantitative real time PCR

Gene	Strand	Primer Sequence
GAPDH	S	5' CTGCTGATGCCCCCATGTTC3'
	AS	5' ACCTTGGCCAGGGGTGCTAA3'
STAT5A	S	5' ATCTCATCTATGTGTTTCCTGACC3'
	AS	5' CACTTGCTTGATCTGTGGTTTC3'
STAT5B	S	P175658-F
	AS	P175658-R
ALP	S	P324388-F
	AS	P324388-R
Cbfa1/runx2	S	5'TGTGGCTGTTGTGATGCGTATTC3'
	AS	5'TGTTGCTGCTGCTGCTGTTG3'

S: Sense Strand AS: Antisense Strand

Table 4. Condition for quantitative real time PCR

Process	Temperature	Time
Initial Denaturation	95℃	30 sec
Denaturation	95℃	5 sec
Annealing/Extension	60℃	34 sec
Dissociation Stage		

Total of 40 cycles (Denatruation– Annealing/Extension) has been carried out

6. Western blot analysis

Lysis buffer (Promega, Madison, WI, USA) with 10μg/ml of protease and phophatase inhibitor was used for preparation of the whole cell lysates. Clarified lysates from centrifugation of samples at 13,000 rpm at 4℃ for 15 minutes were quantified using modified Bradford assay. Total 30μg aliquots of the cell lysates were separated in 10% SDS-PAGE in reducing condition. The samples were promptly transferred onto PVDF membrane (Amersham, Pharmacia, Piscataway, NJ, USA) for 120 minutes at 50V in transfer buffer containing 1.4% glycine, 20% methanol and 25mM Tris-HCL (pH 8.3). 1% skimmed milk in 1X TBST (50mMTris-HCl, 150mM NaCl, 0.1% Tween-20) was used for blocking for 1 hour at room temperature. Primary antibody was used at a concentration of 1:10,000 in 0.25% skimmed milk overnight at 4℃. Following repeated washing of the membrane with 1X TBST, the membranes were incubated with 1:5000 concentration of secondary antibody. The membranes were probed with following primary antibodies; STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, p-STAT (BD, Franklin Lakes, NJ, USA), DLX5, Runx2 and GAPDH (Santa Cruz, Santa Cruz, CA, USA). The immunoblots were visualized using ECL Plus detection kit (Amersham pharmacia, Piscataway, NJ, USA).

7. Immunocytochemistry

Serum was fixed on cover slips with 4% paraformaldehyde and 0.15% glutaraldehyde in 0.1M sodium phosphate buffer for 30 min. They were then

treated for 30 min with 50 glycine in PBS and permeabilized with 0.1% saponin. The cells were blocked with normal goat serum and incubated for 3-4 h with anti-STAT5A antibodies at 1:100 final dilutions. The cells were then incubated for 1-2 h with a goat anti-rabbit ultra-gold antibody at a 1:100 dilution. After washing, the samples were postfixes in 2% glutaraldehyde in PBS.

8. Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation (ChIP) analysis was performed, as described in the protocol included in the ChIP assay kit (Upstate Biotechnology, Charlotteswille, VA). Briefly, MSCs were maintained and induced to differentiate as described earlier. At various time points, the cells were cross-liked with 1% formaldehyde in PBS buffer. The cross-linked cells were harvested, lysed in sodium dodecyl sulfate lysis buffer, and sonicated. After incubation with salmon sperm DNA/protein A at 4℃ overnight, the DNA-protein complexes in the supernatant were immunoprecipitated with antibodies against DLX5 and STAT5A (Santa Cruz Biotechnology, Santa Cruz, CA), and the immune complexes were recovered by adding protein A-agarose. After washing the DNA-protein A complex with saline, DNA was extracted with phenol/chloroform, precipitatied, redissolved, and used as a template for polymerase chain reaction (PCR). We used the human STAT5A promoter primers (sense, ggagagaaacccagacagaatcag; and antisense, tag atctgaggtgccagagtacag), and the human DLX5 promoter primers (sense,tccctgcaccttgactccctattt; and antisense,cagtaacaccctaactcgtccaac).

9. Von Kossa and Alizarin red S staining

Monolayer cultured cells were rinsed with PBS, fixed in a solution of acetone:methanol=1:1 for 2 min, washed with distilled water and stained with 3% silver nitrate solution for 30 min.

III. RESULTS

1. The expression of STAT5 and phosphorylated STAT5 induced during human MSC osteogenesis.

During the osteogenesis of human bone marrow stromal cells (MSC), the expression of STATs and changes in phosphorylation of STAT5 were elucidated. The expression of STAT1, STAT2, and STAT3 remained unchanged both in control and osteogenic condition, while STAT4 was not detected at all. In the case of STAT5A and STAT5B, osteogenesis showed an increase in the expression compared to control (Figure 1A). We confirmed that differentiation cells were stained with Von kossa on 14 days after induction of differentiation (Figure 1B). The amount of phosphorylated from STAT5A, STAT5B and p-STAT5 was activated time-dependently (Figure 2).

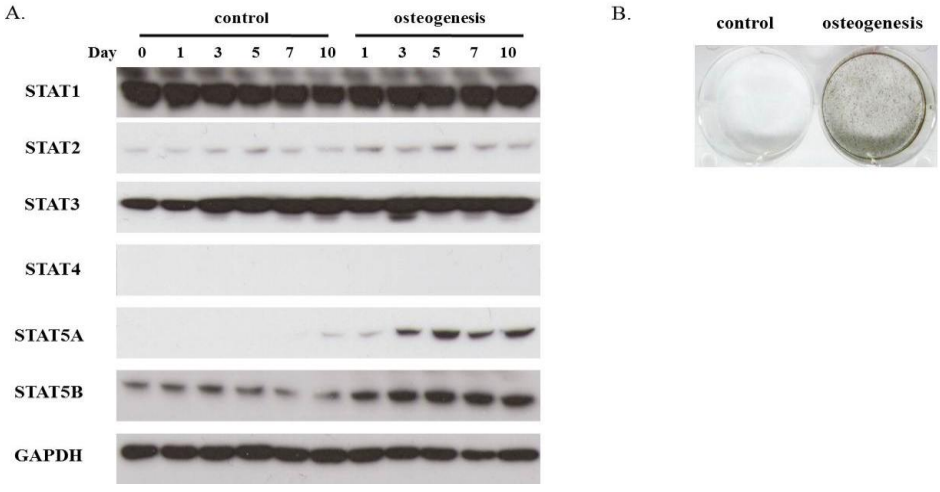

Figure 1. Expression of STAT5A and STAT5B in osteogenesis. MSC were differentiated to osteoblasts using osteogenic medium. The expression of STATs was confirmed by western blot analysis. (A) The expression of STATs family was analyzed at different time points after the induction of differentiation. (B) The cells were stained with Von Kossa on day 14 after induction of differentiation

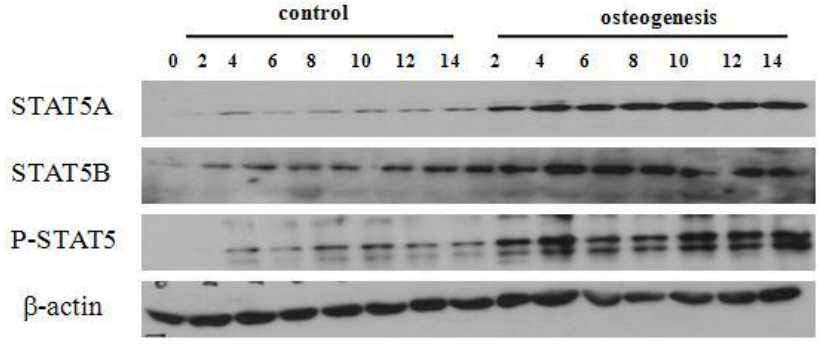

Figure 2. The expression pattern of STAT5A, STAT5B and phosphorylated STAT5 during the progression of osteognesis.

2. The expression of STAT5A, STAT5B, and key of osteogenic marker genes

From the previous studies, the expression pattern of STAT5A, STAT5B and certain key marker genes have been elucidated during MSCs osteogenesis. By RT-PCR, we have observed that the STAT5A and STAT5B's manifest was maintained time-dependently. Also, ALP, which is the key of osteogenic marker, was activated during osteogenesis. Although the expression of DLX5 was detected at late stage of osteogenesis, MSX2 showed detection in the early stages of osteogenesis (Figure 3A). The expression of STAT5A was doubled on the second day. The capacity increased regards to each day's tested period, and it maintained its capacity up to the fourteenth day. The results for STAT5B were similar in ways that it also doubled on the second day. Furthermore, STATB steadily increased until the eighth day but rapidly increased on the tenth day and on. ALP showed steady increase up to the fourteenth day during the osteogenesis (Figure 3B).

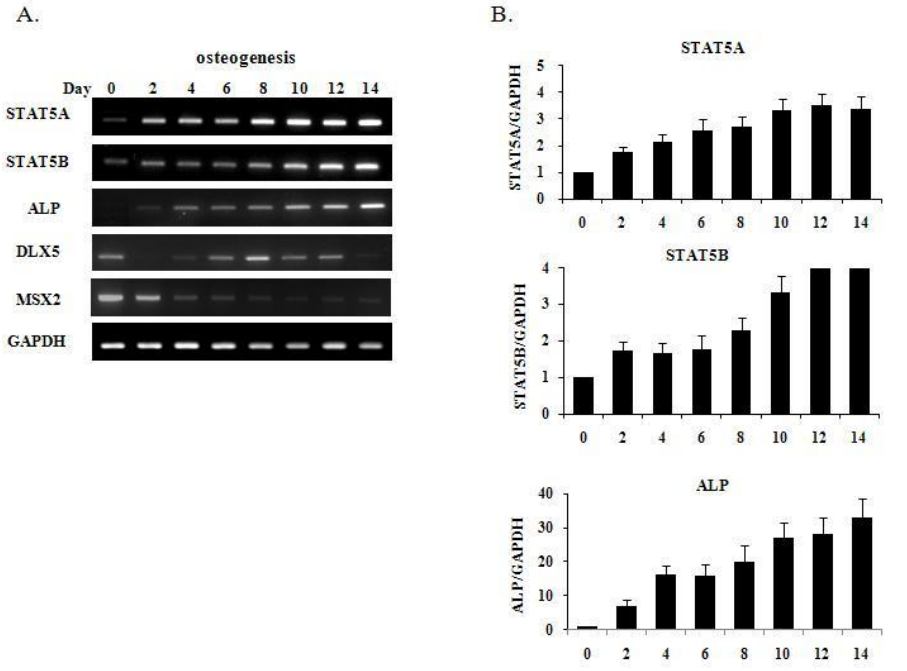

Figure 3. The expression of STAT5A, STAT5B and osteogenic transcription factors during osteogenesis by RT-PCR. MSC were incubated in DMEM-LG supplemented with 10% FBS, 10mM β-glycerophosphate and 50ug/ml ascorbic acid for 14 days in the presence of the indicated reagents. Total RNA was isolated every 2 days, and mRNA expressions of STAT5A, STAT5B, ALP, DLX5 and MSX2 was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). GAPDH expression was used as a positive control. Data shown are representative of three independent experiments.

The real time PCR was carried out to confirm the previous data. Similarly, STAT5A and STAT5B showed their expression increase in the early stages of the differentiation, and this was maintained until the late stage. Runx2, one of the key factors to controlling the osteogenesis, increased its expression by three times more after it has been induced for seven days. ALP also showed an increase, 50 times more from day 0 (Figure 4).

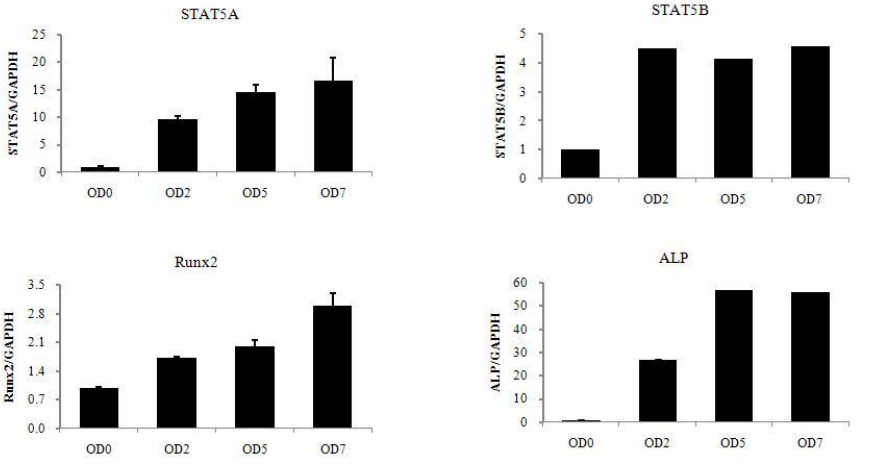

Figure 4. Expression profile of key osteogenic marker genes, STAT5A and STAT5B that have shown distinctive expression pattern in differentiated human MSCs have been quantified via real time PCR. GAPDH expression was used as a positive control. Data shown are representative of three independent experiments.

3. The existence of STAT5A during the osteogenesis within the nucleus.

To find the locations of STAT5A within the osteogenesis process, immunocytochemistry was performed. STAT5A, on day 0, was seldompresent in various locations. On day 5, its appearance increased in other locations, and on day 7 its appearance increased in previously non-existing locations. Nucleus was stained in DAPI and it was merged with STAT5A. In result, STAT5A was existent in the nucleus before the osteogenesis and it was also existent in the nucleus during the osteogenesis (Figure 5).

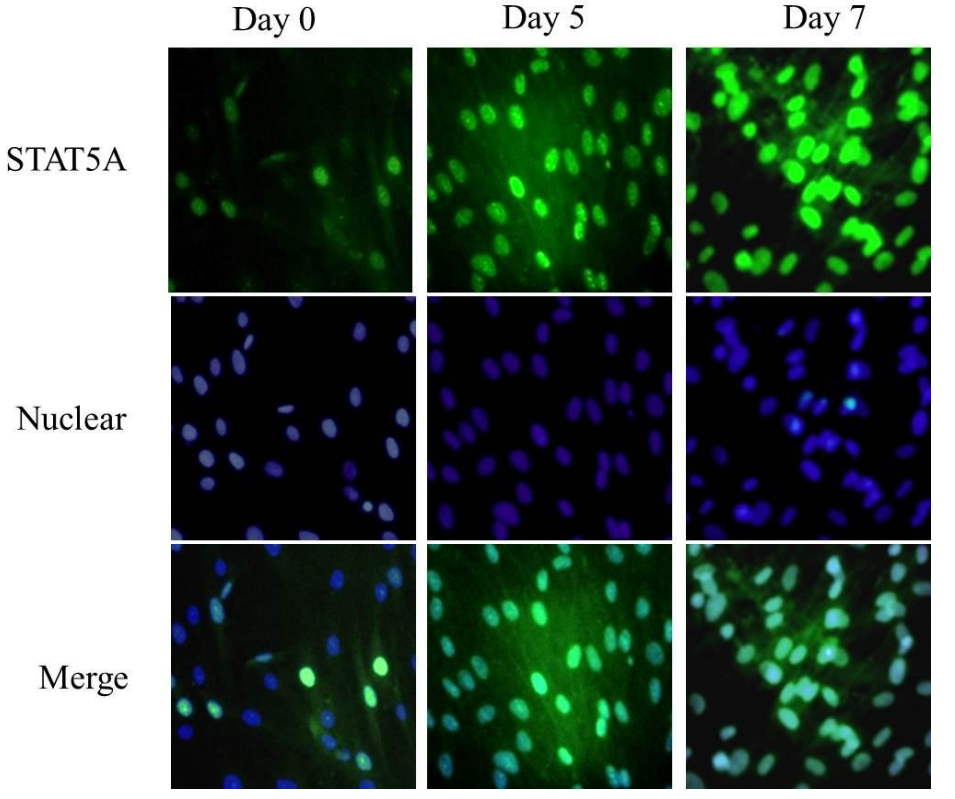

Figure 5. The localization of STAT5A during osteogenesis of human MSCs.

4. The inhibition of STAT5A induced the increased activity during MSC of osteogenesis

In order to assess the effect of STAT5A and STAT5B on osteogenesis, cells were transfected with STAT5A siRNA to block STAT5A and STAT5B siRNA to block STAT5B. To find out the progress of osteogenesis, Alizarin red S and Von kossa staining were performed at 14 day after the transfection of each siRNA. Negative siRNA were transfected as control, we were able to detect mineralization, whereas in STAT5A siRNA treated group showed complete increase in mineralization and STAT5B siRNA treated group displayed mineralization similar to control (Figure 6).

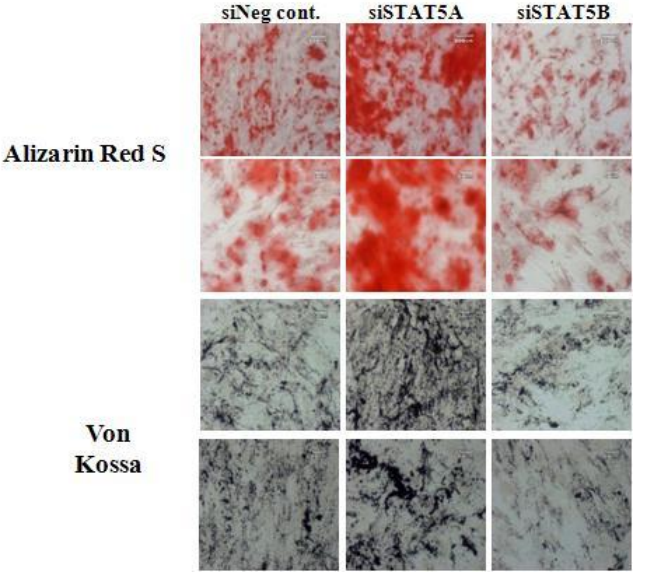

Figure 6. Effect of STAT5A and STAT5B on osteogenesis of human MSCs. siSTAT5A had effectively increased osteoblast differentiation. The cells were stained with Von kossa on day 14 after induction of differentiation.

To determine the function of STAT5 more specifically, MSC were treated by STAT5 inhibitor (N'-((4Oxo-4H-Chromen-3-yl)methylene) nicotinohydrazide), followed by osteogenesis induction. The mineralization became much clearer as the concentration of STAT5 inhibitor increased (Figure 7). Collectively, these data indicate that the presence of STAT5A is crucial in differentiation.

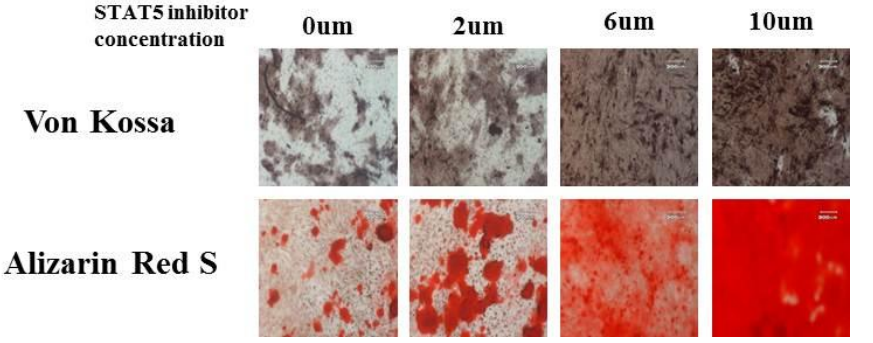

Figure 7. Effect of STAT5 on osteogenesis of human MSCs.STAT5 inhibitor (N'-((4Oxo-4H-Chromen-3-yl) methylene) nicotinohydrazide) had effectively increased osteoblast differentiation. The cells were stained with Von kossa on day 10 after induction of differentiation.

5. STAT5A affects DLX5, the key factor for osteogenesis

Previous data confirms that the importance of STAT5A during osteogenesis was MSC. Following the inhibition of STAT5A and STAT5B, we have also confirmed the expression of marker genes as DLX5 and Runx2. In control group, Negative siRNA, all of the genes such as STAT5A, STAT5B, DLX5 and Runx2 were detected whereas in STAT5A siRNA transfected group showed reduction of DLX5 and Runx2 expression. In STAT5B siRNA transfected group, STAT5B expression inhibited and DNX5 and Runx2 expression was no change compared to the control group. STAT5A expression was inhibition of 50% whereas DLX5 and Runx2 expression was increase of approximately 50% (Figure 8).

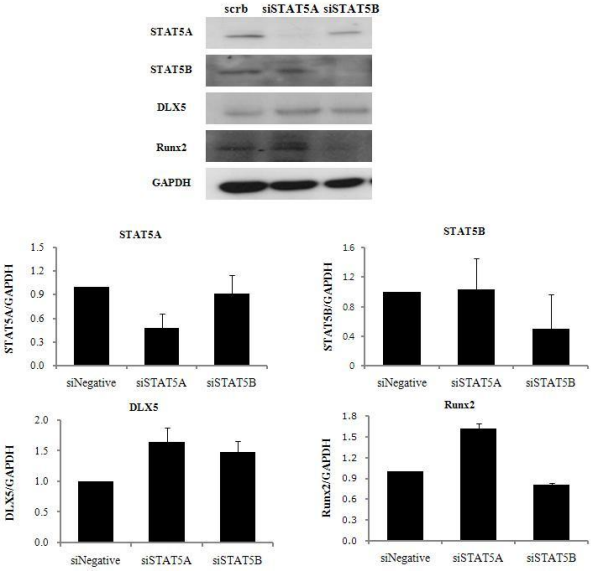

Figure 8. Expression profile of key osteogenic marker genes via siSTAT5A and siSTAT5B from human MSCs.

In order to confirm the exact regulation genes with STAT5A on osteogenesis, we performed that MSC were transfected with STAT5A and STAT5B over-expression vector. In control group where pEGFP-C1 was transfected, all of the genes such as STAT5A, STAT5B, DLX5 and Runx2 were detected. When STAT5A expression was double, DLX5 expression shown decrease of 70% and Runx2 expression was decrease of 20% (Figure 9).

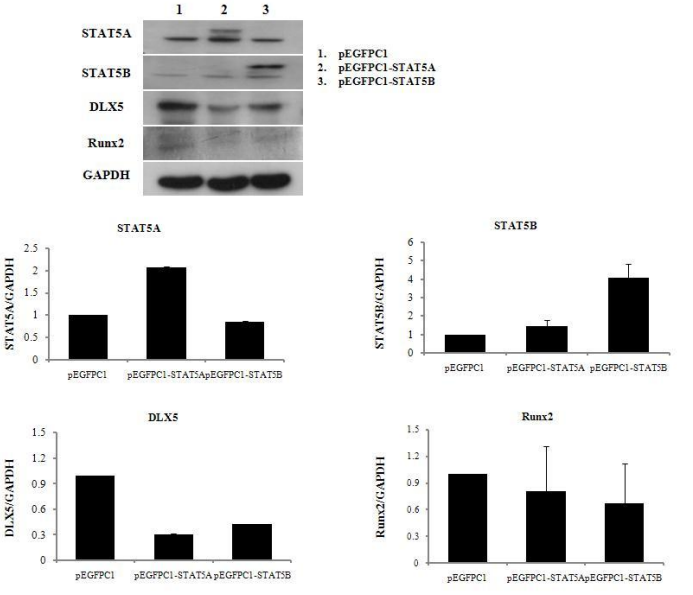

Figure 9. Expression profile of key osteogenic marker genes via over-expression of STAT5A and STAT5B from human MSCs.

6. DLX5 directly control the transcriptional activity of STAT5A

Following the observation, we then aimed to identify the exact regulation between STAT5A and DLX5. We examined luciferase assay to DLX5 affected transcriptional activity of STAT5A through DNA binding. As shown in Figure 10, we observed that DLX5 induced approximately 20 folds increase of STAT5A transcriptional activity. These results indicate that DLX5 directly interact with STAT5A promoter and increase transcriptional activity of STAT5A.

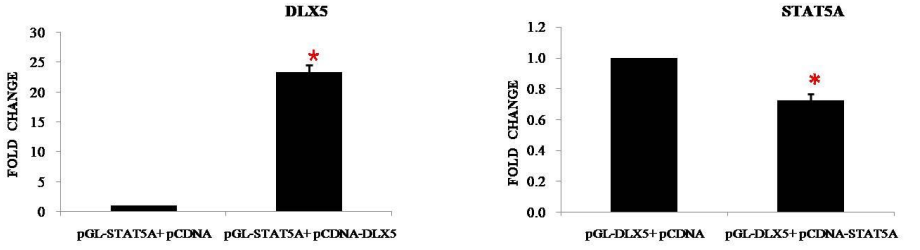

Figure 10. Transcriptional activity of STAT5A by DLX5 over-expression vector. Transcriptional activity was measured in STAT5A promoter (-2121/+88) by DLX5.

IV. DISCUSSION

Osteoporosis, decreased bone density was observed with reduced osteoblast numbers, but increased adipocyte numbers, suggesting that it is important to balance adipogenesis and osteognesis in order to maintain bone structure and volume[8-10]. Previous studies have shown that increasing expression of STAT5A during adipogenesis along with the fact that the over-expression of STAT5 accelerated the formation of adipocytes[21]. C/EBPα and C/EBPβ directly regulated the transcriptional activity of STAT5B, and PPARɣ regulated the transcriptional activity of STAT5A[21]. The inhibition of STAT5A completely blocked the adipogenesis while inhibition of STAT5B exhibited partial inhibition of adipogenesis. Previous studies established STAT5A plays a major role on adipogenesis while STAT5B has a supportive function[21]. In osteogenesis, STAT1 and STAT3 were reported to its deletion was increased mineralization and bone density[19, 22]. Disruption of the STAT1 gene in mice results in increased osteoblast replication increased mineralized nodule formation in bone marrow cultures and increased new bone formation *in vivo*[19]. And STAT1 functions as a cytoplasmic attenuator of Runx2 in osteoblast differentiation[22]. However, there unknown osteogenesis related study for STAT5A as of yet. Skeletal component cells including osteoblast, chondrocytes, adipocytes, myoblasts, tendon cells, and fibroblasts, are derived from

mesenchymal stromal cells[1-7]. The lineages are determined by different transcription factors. The transcription factors, Runx2, osterix, and β-catenin, regulate osteoblast differentiation[11]. Runx2$^{-/-}$ and Osterix$^{-/-}$ mice show complete lack of both intramembranous and endochondral ossification due to the absence of osteoblast differentiation[11-13]. The maximal expression of DLX5 occurs in the final stages of osteoblast differentiation in vitro when the extracellular matrix mineralizes. This pattern of DLX5 expression reflects a general role of DLX5 in lineage commitment and progression of osteoblast differentiation[14, 15].

So we investigated transcriptional regulation of STAT5A by DLX5 roles in osteogenesis and inhibition of STAT5A was completely increase osteoblast differentiation. Our analysis of STAT5A, STAT5B and phosphorylated STAT5 manifest was maintained during induction of osteogenesis. Using luciferase assay, we found that DLX5 bind to STAT5A promoter and presented that DLX5 regulated the transcriptional activity of STAT5A. Inhibition of STAT5A expression completely increased the osteoblast differentiation, while inhibition of STAT5B only partially increased the osteoblast differentiation. Next, we were shown that osteogenic genes expression such as Runx2 and DLX5 when STAT5A was inhibited its expression. Runx2 is a well-known master gene of osteogenesis. STAT5A expression was inhibition of 50% whereas DLX5 and Runx2 expression was increase of approximately 50%. When STAT5A expression was double, DLX5 expression shown decrease of 70% and Runx2 expression was decrease of 20%. Collectively, these data indicate that STAT5A

acts as a pivotal transcription factor in osteogenesis.

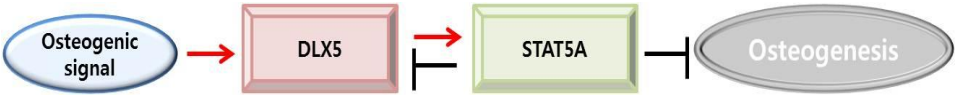

Figure 11. Effect of SAT5A on osteogenesis interacts with targeting of DLX5.

V. CONCLUSION

In this study, our purpose was to discover STAT5A's role during the osteogenesis. Furthermore, during the osteogenesis, we discovered that STAT5A and STAT5B increased and maintained its volume through protein and mRNA. The existence of STAT5A inside the nucleus during osteogenesis was confirmed through immunocytochemistry. In addition, we found that when STAT5A was inhibited osteoblast differentiation was increased. STAT5A's inhibition increased DLX5's expression, which is an osteogenic marker gene, and when STAT5A was over-expressed, the expression of DLX5 reduced. Through luciferase assay, binding of DLX5 promoter and STAT5A was observed. Overall, STAT5A and DLX5 directly bind to each other to inhibit osteogenesis inside the nucleus. For these reason, adipocyte and osteoblast control the balance of its differentiations, and will play an import role as a cell therapy for osteoporosis.

ABSTRACT (IN KOREAN)

골수유래 중간엽 줄기세포의 골분화기간 동안에 STAT5A와 STAT5B의 역할 기능 분석

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황지숙

사람의 골수유래 중간엽 줄기세포 (mesenchymal stromal cell, MSC)는 배양 조건에 따라 지방, 골, 연골로 분화되며 각각의 분화과정은 다수의 단백질과 호르몬 등의 상호작용에 의하여 이루어진다. 따라서 MSC 의 이러한 분화 특성으로 인하여 세포치료제로서의 기능성이 있으나 정확한 기전은 아직까지 규명되지 않았다. 분화과정에 작용하는 것으로 보고된 STAT 은 7 가지 종류로 구성된 단백질 군으로서, 동형접합체나 이형접합체를 이루며, 다양한 사이토카인 수용체에 의하여 인산화되어 핵으로 이동한 후 특정유전자의 promoter 부위에 결합하여 유전자 발현을 조절한다. 특히 STAT5 는 중간엽 줄기세포가 지방세포로의 분화를 촉진시키는 것으로 보고되었다. 그러나 STAT 과 MSC 의 골아세포로의 분화 관계에 대한 연구는 거의 이루어지지 않았다. 이에 본 연구에서는

STAT5A 가 사람 MSC 의 골아세포로의 분화과정 에서 어떠한 작용을 하는지 밝히고자 하였다. 골아세포로 분화가 유도된 MSC 세포군과 대조군에서 STAT 의 발현변화를 분석한 결과 STAT5A 와 STAT5B 의 발현이 골아세포 분화가 유도된 경우 대조군에 비하여 증가되었다. 골아세포 분화기간 동안 STAT5A 의 발현을 RNAi 를 통해 억제한 결과, 비교군과 대비하여 골아세포 분화가 증가되는 것을 확인하였다. 골아세포 표지유전자로 잘 알려진 Runx2 와 DLX5 의 발현이 단백질과 mRNA 수준에서 증가되는 것을 확인하였다. 반대로 골아세포 분화기간 동안 STAT5A 를 과발현 시켰을 경우, Runx2 와 DLX5 의 단백질 및 mRNA 의 발현이 감소되는 것을 확인하였다. DLX5 유전자의 promoter 부위를 함유한 luciferase reporter 실험을 통하여 STAT5A 에 의하여 DLX5 의 전사 활성이 감소되는 것을 확인하였다. 이러한 결과를 토대로 MSC 의 골아세포로의 분화 과정에서 STAT5A 는 DLX5 의 발현을 전사 수준에서의 조절함으로써 골아세포 분화 유도를 감소시킬 수 있음을 확인하였다.

핵심 되는 말: 중간엽 줄기세포, 골분화, STAT5A, DLX5

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