Exploring upregulated genes during osteogenic differentiation of hMSCs

Sekyung Ahn, Jaesuk Rim, Jongjin Kwon, Euiseok Lee, Hyonseok Jang

Department of Oral & Maxillofacial Surgery, College of Medicine, Korea University, Korea

Abstract

Human bone marrow mesenchymal stem cells are thought to be multipotent cells, which are present in adult marrow, that can replicate as undifferentiated cells and that have the potential to differentiate to lineages of mesenchymal tissues, including bone, cartilage, fat, tenden, muscle, and marrow stroma.

Cells that have the characteristics of human mesenchymal stem cells could be isolated from marrow aspirates of human and animals. This study was designed to identify and characterize genes specifically expressed by osteogenic supplements -treated cells by suppression subtractive hybridization(SSH) method.

The results were as follows:

1. 2 genes were upregulated genes in osteogenic diffeentiation of hMSCs, which is further proved by Northern blot analysis.
2. IGFBP-2 has been identified playing an important role in bone formation.
3. HF1 was also upregulated during osteogenic differentiation, but its role in bone formation is not clear yet

Key words

Bone marrow mesenchymal stem cells, Osteogenic supplements, Suppression subtractive hybridization(SSH), IGFBP-2 , HF1

INTRODUCTION

Human mesenchymal stem cells (hMSCs) are multipotent, capable of differentiating into at least osteogenic, chondrogenic and adipogenic cells when cultured in proper conditions[1-3]. When cultured in the presence of dexamethosone, ascorbic acid, and β-glycerophosphate (osteogenic supplements, OS), hMSCs differentiate into bone-forming osteoblasts, expressing osteoblastic markers (Alkaline phosphatase(ALP), Bone sialoprotein(BSP), Osteocalcin(OC), type I collagen), and producing bone-like nodules with a mineralized extracellular matrix containing hydroxyapatite[4]. It was presumed that osteoinductive effects were due to physiological concentration of dexamethosone (10^{-7} or 10^{-8} M), because dexamethosone is potent regulator of cellular growth and differentiation[5,6]. However, the underlying molecular mechanisms of OS-induced osteogenic differentiation are unclear yet.

The hypothesis of this study is that OS may induce hMSCs to express multiple genes that are not expressed or at low levels without OS. These differentially expressed genes and corresponding encoded proteins may play an important role in osteogenesis. Exploring these genes will help to uncover the whole secrets of osteoblastic differentiation of hMSCs. The objective of

* Corresponding author

Hyonseok Jang
Department of OMFS, Korea University Ansan Hospital
516, Gozan-dong, Danwon-gu, Ansan, Gyeonggi, 425-707, Korea
Tel: 82-31-412-5370, 5956 Fax: 82-31-401-7125
E-mail: omfs1109@korea.ac.kr

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this study is to make the subtracted cDNA library using suppression subtractive hybridization method to identify and characterize genes specifically expressed by OS treated cells.

MATERIALS AND METHODS

1. Cell culture

Human stem cells (P3) were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 units / ml streptomycin / penicillin, and plated on 150mm dishes. The cells were cultured in 10% CO_2 at 37℃ with 95% humidity. At confluence period, Cells were digested with tripsin and then plated proportionally into 150mm tissue-culture dishes with the density of 5.0×10^4 cells/ml. At control group, cells were continually cultured in DMEM at the same conditions. As for test groups, 10^{-7}M dexamethosone, 0.2mM ascorbic acid and 10mM β-glycerophosphate were added into DMEM. Medium was changed every day. At day 14, cells were digested by tripsin and collected for RNA extraction.

2. RNA extraction

At day 14, culture medium was removed. Cells were washed with PBS twice and digested by tripsin. Total RNA was extracted with Tri-Reagent/chloroform solution, precipitated with isopropanol and washed with ethanol according to the manufacturer's instructions (SIGMA). RNA was then dissolved in RNase-free sterile water. After DNase treatment, total RNA was dissolved again and quantitated by measuring absorbance at 260nm. Quality of total RNA was evaluated by running a formaldehyde-containing 1% agarose gel.

3. polyA+ RNA purification

PolyA$^+$ RNA was purified using Oligotex mRNA purification kit (QIAGEN) following the manufacturer's instruction, precipitated with glycogen/NH_4OAc and dissolved in RNase-free sterile water. After quantification by measuring absorbance at 260nm, aliquots (0.2μg) were used for formaldyhyde-containing 1% agarose gel electrophoresis.

4. Suppression substraction hybridization (SSH)

Subtractive hybridization is a method for construction of cDNA libraries enriched for differentially expressed genes. Usually, tester is a cDNA population where the genes of interest are expressed, and driver is a cDNA population where these genes are not expressed or expressed at the lower level.

In this study, cDNA subtraction was made using Clontech PCR select cDNA subtraction kit according to the manufacturer's protocol. First- and second-strand cDNAs were synthesized using cDNA synthesis primer (appendix 1). cDNA ends were blunt-ended by T4 DNA polymerase. The resulting cDNA pallet was dissolved in 50μl sterile water for each group and digested by Rsa I overnight. The cDNA was then phenol/chloroform extracted, ethanol precipitated and resuspended in 3.5μl sterile water.

0.5μl of digested cDNA was diluted in 2.5μl sterile water to prepare tester cDNA. 1μl of diluted cDNA was ligated to 1μl of 10μM adaptor1 and adaptor 2R (appendix 1) in separate ligation reaction in a 5μl reaction volume at 16℃ overnight, using 200units of T4 DNA ligase. 0.5μl of EDTA/Glycogen mix was added and heat at 72℃ for 5 minutes to inactivate the ligase. Ligation efficiency was analysized by PCR using G3PDH 3' primer with either G3PDH 5' primer or PCR primer 1 (appendix 1).

Bi-directional subtractive hybridization was conducted. Forward subtracted library was enriched for the cDNAs up-regulated during osteogenesis, whereas reverse subtracted library enriched for down-regulated cDNAs. For the forward subtraction, control (Ctl) group served as driver cDNA, and OS-treated (Dex) group served as tester. For the reverse subtraction, Ctl group acted as tester and Dex group as driver. 1μl of driver cDNA was mixed with either adaptor1-ligated tester or adaptor 2R-ligated tester and 0.75μl of hybridization buffer. Solutions were overlaid with one drop of mineral oil. DNAs were denatured at 98℃ for 1.5 minutes and then allowed to anneal overnight at 68℃. After the first hybridization, two solutions were mixed in the presence of heat-denatured driver cDNA and then hybridized for additional 20 hours at 68℃. The final hybridization was then diluted in 100μl of dilution buffer and heated at 68℃ for 7 minutes and stored at -20℃.

After hybridization, two PCR amplifications were conducted for each subtraction. The primary PCR contained

1μl diluted subtracted cDNA, 1μl of PCR primer 1 and 0.5μl of 50X advantage cDNA polymerase mix (CLONTECH), 0.5μl 10mM dNTP mix, 2.5μl of 10X PCR buffer and 19.5μl of sterile water. The program was 72℃ for 2 minutes, and 27 cycles at (94℃ 10 seconds, 66℃ 20 seconds, and 72℃ 1.5 minutes). The amplified PCR products were diluted for 10-fold in sterile water. Aliquots (1 μl) of products were then used as the template in the second PCR amplifications. Master mix (containing 18.5μl μl sterile water, 2.5μl of 2.5μl of 10X PCR buffer, 0.5μl 10mM dNTP mix, 1.0μl of nested PCR primer 1, 1.0μl of nested PCR primer 2R, and 0.5μl of 50X advantage cDNA polymerase mix) was added. The PCR parameter was 94℃ 10 seconds, 68℃ 20 seconds, and 72℃ 1.5 minutes for 19 cycles for the forward subtraction group and 11 cycles for reverse subtraction group. 2.0% agarose gel electrophoresis was conducted to analyze PCR products. The final PCR products were purified using Qiaquick PCR purification microcentrifuge kit according to the manufacturer's protocol.

5. Cloning and subtracted cDNA sequence analysis

Purified secondary PCR products were ligated with pETBlue-1 AccepTor Vector (NOVAGEN) at 16℃ for 2 hours following manufacturer's instruction. Ligated vectors were then transformed into DH5α™ Competent Cells by heat shock following the manufacturer's protocol (GIBCOBRL). Cells were then shaking at 37℃ for 1 hour for expression. After expression, the reaction was diluted with 10 fold LB and 100μl of diluted reaction was spread on 100mm agar dish and incubated at 37℃ overnight for cloning. Individual clones were picked up and cultured in LB with ampicillin (50μg /ml) with constant agitation at 37℃ for 5 hours. 1μl of cultures were used for PCR amplification of cDNA inserts. The remaining cultures were added 50% glycerol to the final concentration of 7% glycerol and stored at -70℃. For PCR, 1μl of cultures were mixed with master mix containing 5μl 10X PCR buffer, 2μl of 10μM nested PCR primer 1 and 2R or vector specific primers U25 and R26 (Apendix), 4μl 2.5mM dNTP, 0.75μl 1.5mM $MgCl_2$, 0.4μl Taq cDNA polymerase (Invitrogen) and 34.85μl sterile water. The PCR program was 95℃ 1 minute and then 25 cycles at 94℃ 10 seconds, 68℃ 20 seconds and 72℃ 1.5 minutes. 2% agarose gel electrophoresis was conducted to analyze the PCR products.

PCR products were purified using Qiaquick PCR purification microcentrifuge kit and then quantitated by measuring absorbance at 260nm. cDNA sequencing was performed by automated cDNA sequencing .

6. Northern blot analysis

Total RNA was separated by formaldehyde-containing 1.2% agarose gel electrophesis for both Ctl and Dex group (2μg/well). Gel was then soaked in ddH_2O for 30minutes to diffuse formaldehyde. Total RNA bands were transferred to nylon membrane in 20X SSC buffer and then fixed by heating the membrane at 37℃ for 3 hours.

10ng (2μl) of purified colony PCR products were labeled with 32P using Rediprime™ II Random Prime Labelling System (Amersham Pharmacia Biotech) and then purified by Quick Spin Column for Radiolabeled RNA Purification according to manufacturer's instructions (Roche Diagnostics Corporation). After quantification, the probes were denatured at 98℃ for 3 minutes and then chilled in ice for at least 5 minutes before hybridization to avoid reannealing. Denatured probes were then hybridized with membrane in Expresshyb hybridization buffer (Clontech) in the presence of 0.1mg/ml sperm DNA at 68℃ for 16 hours. After hybridization, membranes were washed four times in low stringency buffer (2X SSC and 0.5% SDS) and twice in high stringency buffer (0.2X SSC and 0.5% SDS). Hybridized membranes were then exposed overnight to PhosphoImagen Screen.

RESULTS

From the electrophoresis results of total RNA for both Ctl and Dex groups, the intensity of 28S to 18S RNA bands was 2:1 (Fig. 1-A). $polyA^+$ RNAs for both Ctl and Dex groups were purified. Formaldehyde/agarose gel electrophoresis of $polyA^+$ RNA from the Dex group was conducted (Fig. 1-B). Electrophoresis of poly A^+ RNA from Ctl group was not conducted because of low quantity.

$polyA^+$ RNA were reverse transcribed, Rsa I digested and ligated to adaptors. Fig. 2 shown was the ligation efficiency analysis. After 22 PCR cycles, Ctl group showed bands (lane 1-4), whereas Dex group did not (lane 5-8). When adding 5 more cycles, bands were also

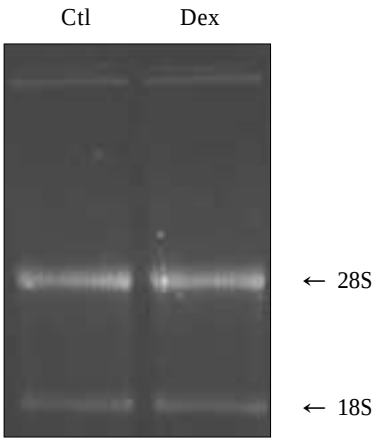

Fig. 1-A. Formaldehyde/agarose gel analysis of total RNA from control (Ctl) and OS-treated (Dex) groups.

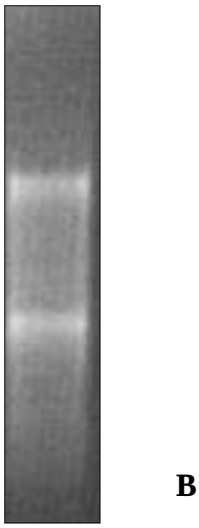

Fig. 1-B. mRNA gel of Dex group.

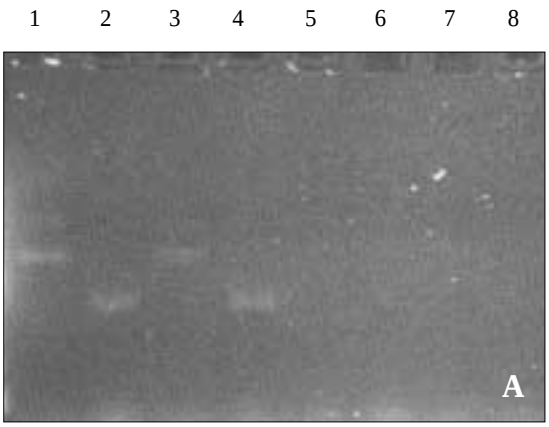

Fig. 2-A. Ligation efficiency after 22 PCR cycles.

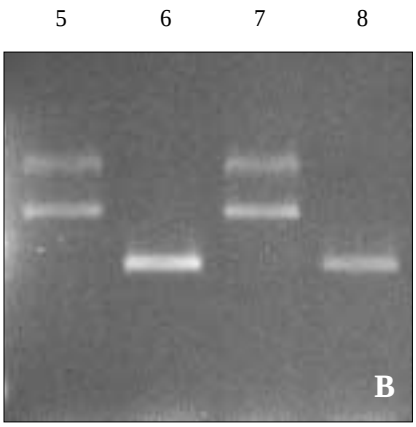

Fig. 2-B. Ligation efficiency after 27 PCR cycles.

Lanes 1-4 were control group and lanes 5-8 were Dex group. Lane 1,5: PCR products using Adapter 1-ligated cDNA as template and G3PDH 3' Primer and PCR Primer 1. Lane 2,6: PCR products using Adapter 1-ligated cDNA as template and G3PDH 3' and 5' Primer. Lane 3,7: PCR products using Adapter 2R-ligated cDNA as template and G3PDH 3' Primer and PCR Primer 1. Lane 4,8: PCR products using Adapter 2R-ligated cDNA as template and G3PDH 3' and 5' Primer.

shown up (lane 5'-8') in Dex group. This indicated that adaptors were ligated to both groups, but the quantity was different between two groups. Two bands in lane 1, 3 and 5,7 may be caused by partially digested cDNA.

Subtractive hybridization was performed and subtracted cDNA were amplified (Fig. 3). Lane 1 showed the amplified subtracted cDNA, enriched for up-regulated genes (forward subtraction). Lane 3 showed the amplified subtracted cDNA, enriched for down-regulated genes (reverse subtraction). Lane 2 and lane 4 showed the corresponding PCR products for unsubtracted cDNAs, and lane 5 was the positive control of subtracted samples from the kit.

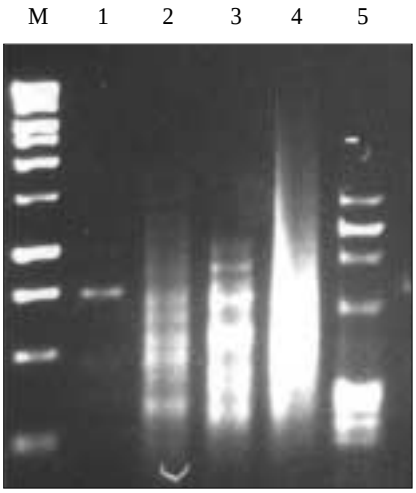

Fig. 3. Secondary PCR products. Lane M: size marker. Lane 1: subtracted Dex tester cDNA with 19 secondary PCR cycles. Lane2: unsubtracted Dex tester cDNA. Lane 3: subtracted Ctl tester cDNA. Lane 4: unsubtracted Ctl tester cDNA. Lane 5: subtracted skeletal muscle tester cDNA containing 0.2% of X 174/Has III digested cDNA. Primary PCR was 27 cycles for all. Secondary PCR cycles from lane 2-5 were 11.

The subtracted cDNAs were cloned. The inserts from both forward and reverse subtracted libraries were amplified, using adaptor primers (Fig. 4). For the clone #6, vector specific primers were also used for PCR amplification (Fig. 4-C). A larger cDNA fragment was produced in the latter case compared with the PCR products using nested primers, since some vector sequence was included.

Four amplified inserts from forward subtracted library were radioactively labeled and hybridized to the Northern blots containing total RNAs from both Ctl and Dex groups. Northern blot analysis revealed up-regulation of clone 1, 4 and 6 (Fig. 5). Corresponding gene sequences are shown in appendix 2. However, four randomly chosen clones from the reverse subtracted library did not show differential expression.

Comparison of the query sequences to the working draft sequences of the human genome showed that clone #1 sequence was overlapped with human factor (HF-1), which encodes a regulatory protein of the complement

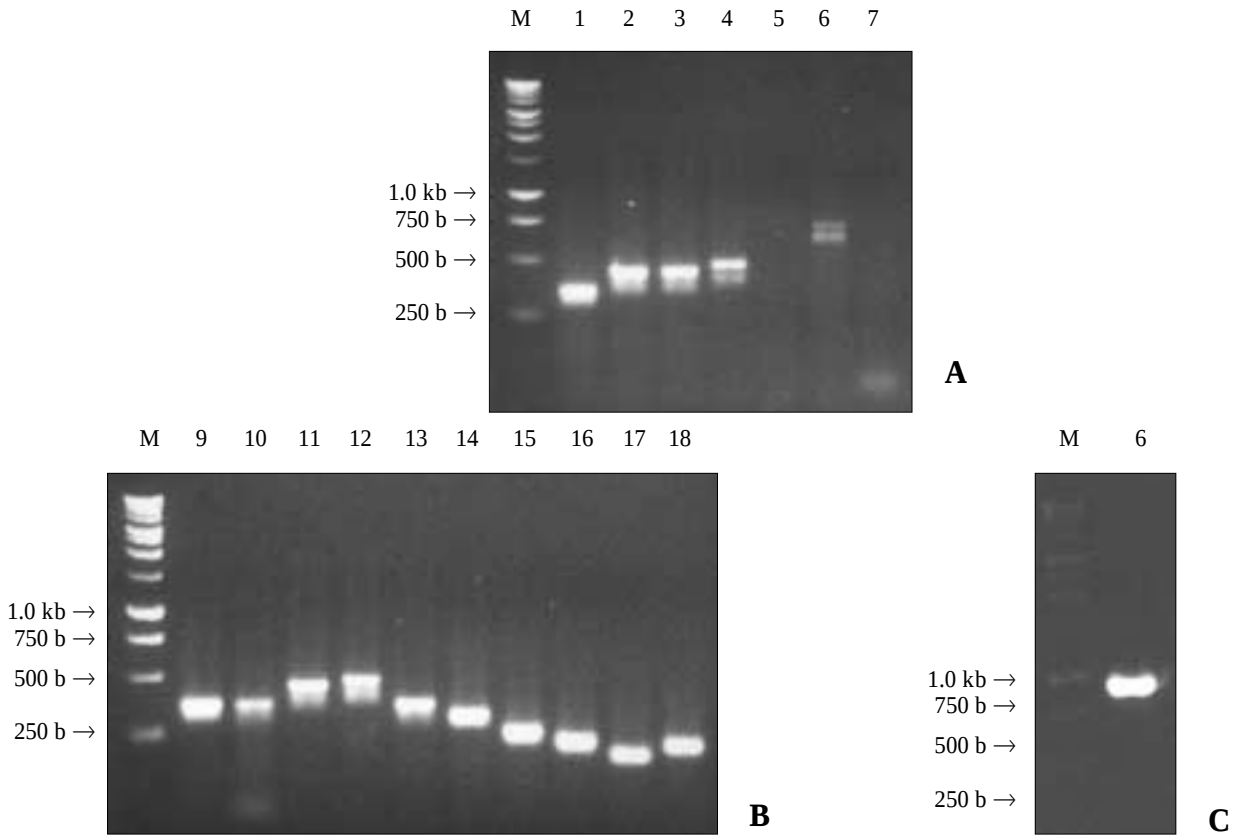

Fig. 4-A. Colony PCR products of forward subtraction (Dex group as the tester). Lane M: size marker. Lanes 1-7: different clones.

Fig. 4-B. Clony PCR products of reverse subtraction (Ctl group as the tester). Lane M: size marker. Lanes 9-18: different clones.

Fig. 4-C. Clone #6 PCR products using vector specific primers U25 and R26. Lane M: size marker. Lanes 6": colony PCR product of clone.

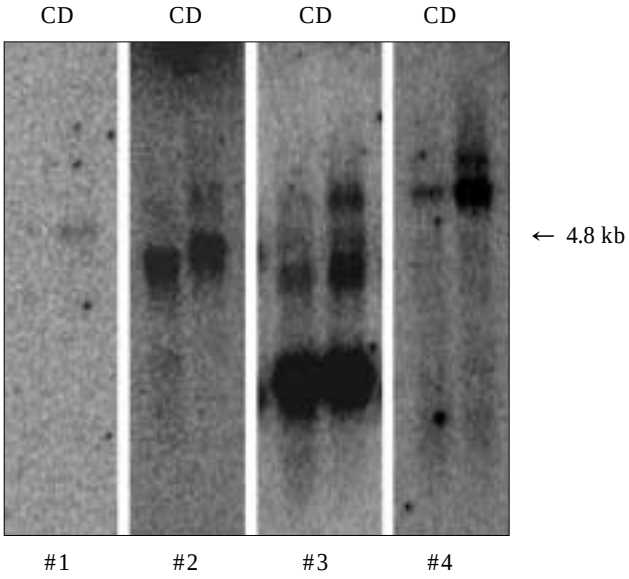

Fig. 5. Northern blot analysis showed upregulated cDNAs (clone #1, #4, #6) and non-differentially expressed cDNA (clone #2), in OS-treated (D) group compared with control (C) group. (3 days exposure)

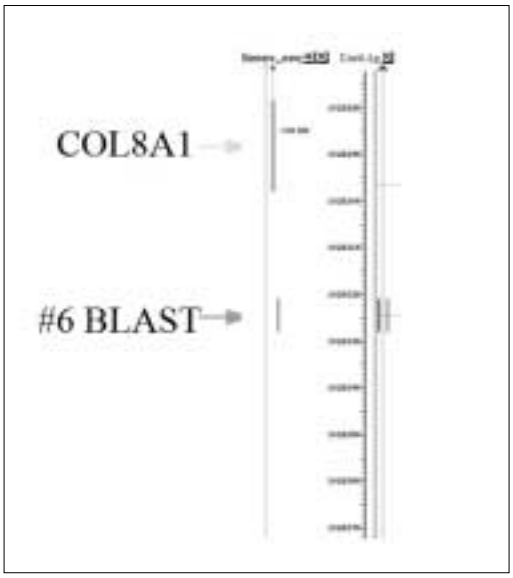

Fig. 6. sequence query of clone #6 with NCBI gene bank, showed that #6 blast hit gene was located 2.5kb to 3' of COL3A1 gene.

system. However, the relationship between HF-1 and bone formation was not clear yet. Clone #4 sequence was identical with the gene encoding insulin-like growth factor binding protein 2 (IGFBP-2), which binds to and modulates bioactivity of insulin-like growth factor (IGFs)[7]. It has been demonstrated that IGFs are among the most abundant growth factors synthesized by osteoblasts, and stimulate osteoblast proliferation and matrix protein synthesis[8,9]. Dexamethasone has also been shown to regulate the expression of IGF-I, IGF-II and IGFBPs before[10,11], which seems consistent with results in this study. There was no matched gene sequence in the NCBI gene bank for clone #6. However, genomic map overview showed that it is located 2.5kb to the 3' end of COL8A1 (Fig. 6), gene encoding the collagen VIII μ1 subunit. Northern blot analysis showed that RNA corresponding to the clone #6 was larger than 5kb. It indicates that gene corresponding to clone #6 may be either novel gene or 3' fragment of COL8A1 gene. However, further studies need to be conducted to verify this deduction.

DISCUSSION

The aim of this study is to identify genes upregulated during the process of osteogenic differentiation of hMSCs. In vitro, hMSCs could not differentiate into osteoblasts spontaneously. However, in the presence of osteogenic supplements (OS), they could express osteoblastic markers, such as ALP, BMPs, BSP, OC et al, and form bone-like nodules[4,12]. So the cell model in this study is reliable to study osteogenic differentiation and could provide somewhat molecular evidence for bone engineering.

Surpresssion subtractive hybridization (SSH) has been proved to be an efficient method for the generation of cDNA libraries[13]. It is used to selectively amplify target (differentially expressed) cDNA fragments and simultaneously suppress non-target DNA amplification. 1-2μg of poly A^+ RNAs from the two cell populations are required under the standard procedure. In this study, about 1μg of poly A^+ RNAs from both Dex and Ctl groups were used, however based on the ligation efficiency results, we concluded that some cDNAs from Dex group were probably

lost during cDNA extraction / precipitation. It resulted in low complexity of forward subtracted library yielded only several cDNAs (see Fig. 3, lane 1) and low product of differentially expressed cDNAs in reverse subtracted library.

During the process of osteogenic differentiation of hMSCs, there should be hundreds of genes up-regulated and also some genes down-regulated, which may form a spatial and temporal modulation to bone formation[14]. This study only provided tiny contributes to the whole story of osteogenic differentiation of hMSCs and bone formation. The further testing of random clones from both forward and reverse subtracted library is required. Alternately, the new subtraction can be done using optical cDNA concentration. When a number of differentially expressed genes are identified, the future analysis should be conducted to reveal the function of these genes in bone formation and bone remodeling.

CONCLUSIONS

Three genes were upregulated in osteogenic diffeentiation of hMSCs, which is further proved by Northern blot analysis. In these three genes, IGFBP-2 has been identified playing an important role in bone formation. It binds to and modulates bioactivity of insulin growth factors, which the most abundant growth factors synthesized by osteoblasts and stimulate osteoblast proliferation and matrix protein synthesis. HF1 was also upregulated during osteogenic differentiation, but its role in bone formation is not clear yet. Sequence from clone 6 is interested, because it is strongly upregulated in the process of osteogenic differention. But we don't know yet which gene it is. Maybe it is a novel gene.

Shortcoming in this project is that only several cDNAs was yielded in forward subtraction, because some cDNAs from dex group were probably lost during cDNA extraction / precipitation. So further analysis of differentially expressed random clones is required, or new subtraction should be conducted using optimal cDNA concentration

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Appendix:

1. Primers used in this study:

cDNA synthesis primer: 5' -TTTTGTACAAGCTT30N1N-3'
Adaptor 1: 5' - CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT - 3'
3' - GGCCCGTCCA - 5
PCR primer 1: 5' - CTAATACGACTCACTATAGGGC - 3'
Nested PCR primer 1: 5' - TCGAGCGGCCGCCCGGGCAGGT - 3'
Adaptor 2R: 5' - CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT - 3'
3' - GCCGGCTCCA - 5'
Nested PCR primer 2R: 5' - AGCGTGGTCGCGGCCGAGGT - 3'
G3PDH 5' primer: 5' - ACCACAGTCCATGCCATCAC - 3'
G3PDH 3' primer: 5' -TCCACCACCCTGTTGCTGTA - 3'
U25: 5' - CCAgggTTTTCCCAgTCACgACgTT - 3'
R26: 5' - ggAAACAgCTATgACCATgATTACgC - 3'

2. cDNA sequences for clone #1, #4 and #6:

Clone#1:ACAGTATTCTTGATTCTATATATCGCTATTTTAGAATCCATTACATGTATTGT
ATGTAACCTATTTTTAAAGATTTGCGGAACAAATACATATTTTTCCTATTTCAGAAACAG
ATTGTCTCAGTTTACCTAGCTTTGAAAATGCCATACCCATGGGAGAGAAGAAGGATGTG
TATAAGGCGGGTGAGCAAGTGACTTACACTTGTGCAACATATTACAAAATGGATGGAG
CCAGTAATGTAACATGCATTAATAGCAGATGGACAGGAAGGCCAACATGCAGAGACAC
C T C C T G T G T G A A T C C G C C C A C A G T A C C T C G G C C G C G C C C A C G C T A
Clone#4:ACAACCTCAAACAGTGCAAGNATGTCTCTGAACGGGCAGCGTGGGGAGT
GCTGGTGTGTGAACCCCAACACCGGGAAGCTGATCCAGGGGAGCCCCCACCATCCGG
GGGGACCCCGAGTGTCATCTCTTCTACAATGAGCAGCAGGAGGCTCGCGGGGTGCAC
ACCCAGCGGATGCAGTAGACCGCAGCCAGCCGGTGCCTGGCGCCCCTGCCCCCCGCC
CCTCTCCAAACACCGGCAGAAAACGGAGAGTGCTTGGGTGGTGGGTGCTGGAGGATT
TTCCAGTTCTGACACACGTATTTATATTTGGAAAGAGACCAGCACCGAGCTCGGCACCT
CCCCGGCCTCTCTCTTCCCAGCTGCAGATGCCACACCTGCTCCTTCTTGCTTTCCCCGG
GGGAGGAAGGGGGTTGTGGTCGGGGAGCTGGGGTACCTCGGCCGCACCCACGCTA
Clone#6:GTAAAACGACGGCCAGTGAATTGTGCGGCCATTTAGGTGACACTATAGAA
TACAGCGGCCGCGAGCTCGGGCCCCCACACGTGTGGTCTAGAGCTAGCCTAGGCTCGA
GAAGCTTGTCGACGAATTCAGATTCGAGCGGCCGCCCGGGCAGGTACATGTGTGGGCT
TTTAATTCCCACCAAGAAAGAGAGAAATTATCTTTTTAGTTAAAACCAAATTTCACTTT
TCAAAATATCTTCCAACTTATTTATTGGTTGTCACTCAATTGCCTATATATATATATGTG
TGTGTGTGTGCGCGTGAGCGCACGTGTGTGTATGCGTGCGCATGTGTGTGTATGTGTATTA
TCAGACATAGGTTTCTAACTTTTAGATAGAAGAGGAGCAACATCTATGCCAAATACTGT
GCATTCTACANTGGTGCTAATCTCANACCTAAATGATACTCCATTTAATTTAAAAAAGA
GTTTTAAATAATTATCTATGTGCCTGTATTTCCCTTTTGAGTGCTGCACAACATGTTAACA
TATTAGTGTAAAAGCAGATGAAACAACCACGTGTTCTAAAGTCTAGGGATTGNGCTATA
ATCCCTATTTAGTTCAAAATTAACCAGAATTCTTNCATGTGAAATGGACCACACTCATAT
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TAACGCGTCTGCAGCATGCGTGGTACCGAAGCTTTCCCTATAGTGAGTCGTATTAGAGNC
TTG