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NR2F1 mediated down-regulation of osteoblast differentiation was rescued by Bone morphogenetic protein-2 (BMP-2) in human MSC

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Abstract

Endochondral ossification is the process by which long bones are formed; the process of long bone formation is regulated by numerous factors such as transcription factors, cytokines, and extracellular matrix molecules. Human hormone nuclear receptors (hHNR) are a family of ligand-regulated transcription factors that are activated by steroid hormones, such as estrogen and progesterone, and various lipid-soluble signals, including retinoic acid, oxysterols, and thyroid hormone. Whole genome microarray data from our previous study revealed that most hHNR’s are up-regulated during osteoblast differentiation in hMSCS. NR2F1 was among the highest expressed hHNR during osteogenesis, NR2F1 belongs to the steroid/thyroid hormone nuclear receptor superfamily. NR2F1 is designated as an orphan nuclear receptor because its ligands are unknown. NR2F1 plays a wide range of roles, including cell differentiation, cancer progression, and central and peripheral neurogenesis. Identifying signaling networks involved in osteoblast differentiation is important in orchestrating new therapeutic and clinical applications in bone biology. This study aimed to identify alterations in signaling networks mediated by NR2F1 in osteoblast differentiation. siRNA-mediated down-regulation of NR2F1 leads to impairment in the differentiation of hBMSC-TERT to osteoblast; gene-expression results confirmed the down-regulation of osteoblast markers such as RUNX2, ALPL, OSC, and BSP. Global whole gene expression analysis revealed that most down-regulated genes were associated with osteoblast differentiation (DDIT3, BMP2). Pathway analysis revealed prominent signaling pathways that were down-regulated, including the TGFβ pathway and MAPK pathway. Functional studies on NR2F1 transfected cells, during osteoblast differentiation in combination with TGFβ1 and BMP-2, showed that TGFβ1 does not recover osteoblast differentiation, whereas BMP-2 rescues osteoblast differentiation in NR2F1 siRNA transfected cells. Thus, our results showed that BMP-2 could intervene in NR2F1 down-regulated signaling pathways to recover osteoblast differentiation.
Keywords: Human bone marrow MSCs, osteogenesis, NR2F1, TGFb1 signaling, DDIT3, BMP2 signaling

Introduction

Bones are hard and flexible tissues, capable of minor shape changes without fracture; these elastic properties marks the salient features of bones (Seeman, 2008). Even though bones are hard and flexible tissues, they keep on remodeling throughout the lifespan of animals during growth and fracture (Seeman, 2002). Bones are made up of two cell types namely osteoblasts and osteoclasts; coordination between these two cells is mandatory for bone development and bone turnover. Bone development is achieved by two different ways; endochondral ossification and intramembranous ossification. Osteoblasts are involved in both types of ossification process. Therefore, studying the factors that influence osteoblast differentiation has become imperative in recent decades. One of the predominant precursor cells of osteoblasts are the mesenchymal stem cells in the bone marrow (Karsenty et al., 1999). The cascade of differentiation of osteoblasts from mesenchymal stem cells is crucial and is influenced by numerous factors, ranging from paracrine to endocrine secretions to transcription factors. Bone homeostasis is well and rigorously maintained, and any disturbance in bone homeostasis will lead to disease conditions. One of the primary bone disease conditions is osteoporosis, which leads to a decrease in bone mass and fracture, mainly in elderly people and post-menopausal woman (Weitzmann and Pacifici, 2006). Nuclear receptors are a family of ligand-regulated transcription factors that are activated by steroid hormones, such as estrogen and progesterone, and various lipid-soluble signals, including retinoic acid, oxysterols, and thyroid hormone (Mangelsdorf et al., 1995). The chicken ovalbumin upstream promoter-transcriptional factors (COUP-TFs) belong to the steroid/thyroid hormone receptor superfamily (Sagami et al., 1986). The COUP-TFs are denoted as orphan nuclear receptors since their ligands are unknown. Currently, only two COUP-TF homologs have been identified—COUP-TFI (EAR3) and COUP-TFII (ARP-1), also known as nuclear receptor 2 families 1 and 2 (NR2F1 and 2) (Ladias and Karathanasis, 1991; Miyajima et al., 1988; Wang et al.). COUP-TFs play important roles in many
developmental processes. Here, our focus is COUP-TFI or NR2F1. From this point onward, we will only mention NR2F1 instead of COUP-TFI. NR2F1 has a wide range of functions, ranging from disease progression to cell differentiation. Maria Soledad et al. reported that NR2F1 directs tumor cells to dormancy and defers their progression (Sosa et al., 2015). NR2F1 is also known to play vital roles in neurogenesis, especially differentiation of neurons and oligodendrocytes (Neuman et al., 1995; Yamaguchi et al., 2004).

Our previous study on human MSCs have shown that hHNR’s play a role during osteoblast differentiation, where NR2F1 was one of the most up-regulated and its role in osteoblast differentiation is not completely elucidated. Hence, we selected to study the role of NR2F1 during osteoblast differentiation in hMSC.

Materials and methods

Cell Culture
We employed hMSC-TERT cell lines which were formed from primary normal human mesenchymal stem cells (MSCs) by overexpressing the human telomerase reverse transcriptase gene (hTERT). For the current experiments, we employed a sub-clone derived from hMSC-TERT, called CL1, which exhibits enhanced differentiation potential (they form mineralized nodules at day 7 of differentiation) these cells have been extensively characterized and they exhibit cellular and molecular phenotypes similar to the primary hMSC (Elsafadi, 2016). For ease, we will refer to this cell line as hMSC for the remaining part of the manuscript. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 0.25 mol/l D-Glucose, 0.004 mol/l L-Glutamine, 0.006 mol/l Sodium Pyruvate, 10% Fetal Bovine Serum (FBS), 1x penicillin-streptomycin (Pen-strep), and non-essential amino acids (all purchased from Gibco-Invitrogen, USA).

siRNA-mediated transfection of hMSC
For siRNA transfection, cells in logarithmic growth phase were transfected by forward transfection with Ambion in vivo pre-designed NR2F1-siRNA (2.5e-7 mol/l)
(siRNA ID: s224723, Part Number. 4457308, Ambion, The RNA Company, USA) and negative control siRNA # 2 siRNA (Cat # 4390846) using a Lipofectamine RNAiMAX Reagent (Invitrogen, CA, USA) plus serum-free OptiMEM®I medium (Invitrogen, CA, USA) under the conditions described by the manufacturer. After 24 h of transfection, the cells were induced to osteogenic differentiation for additional one week. siRNA transfection efficiency was validated after 3 days of transfection using qRT-PCR with a corresponding primer.

**AlamarBlue Cell Viability/Proliferation Assay**

After siRNA transfection, cell viability was measured on day 3, 5, and 7 with the Alamar Blue assay according to the manufacturer's recommendations (AbD Serotec, Raleigh, NC, USA). In brief, to culture the cells in a 96-well plate, 10 μl of Alamar Blue substrate was added and the plates were incubated in the dark at 37°C for 1 h. Reading was subsequently taken by measuring the fluorescence of the cells (Ex 530 nm/Em 590 nm) with the BioTek Synergy II microplate reader (BioTek Inc., Winooski, VT, USA).

**In vitro osteoblast differentiation**

Cells were grown in standard DMEM growth medium in 24-well plates at 0.05 × 10^6 cells/ml. At 70-80% confluence, the cells were cultured in DMEM supplemented with osteoblast induction mixture containing 10% FBS, 1% Pen-strep, 0.003 mol/l L-ascorbic acid (Wako Chemicals, Neuss, Germany), 0.01 mol/l β-glycerophosphate (Sigma), 1e-8 mol/l calcitriol (1α,25-dihydroxy vitamin D3; Sigma), and 1e-8 mol/l dexamethasone (Sigma). The media were replaced three times per week. For the in vitro functional studies, TGFβ1 (10 ng/ml, Peprotec 100-21C) or BMP-2 (1000 ng/ml, Peprotec Catalogue Number: 120-02C) was used with the NR2F1 transfected cells in osteoblast differentiation medium, and NR2F1 transfected cells, without TGFβ1 or BMP-2, in osteoblast differentiation medium were used as controls.
Cytochemical staining

Alizarin Red S staining for mineralized matrix

Cells cultured in osteoblast differentiation media were washed with PBS and then fixed with 70% ice-cold ethanol at -20 °C for 30 min. After removing the fixative, the cell layer was rinsed in distilled water and stained with 2% Alizarin Red S Staining Kit (ScienCell, Research Laboratories, Cat. No. 0223) for 20 min at room temperature. The excess dye was washed off with water at room temperature.

OsteolImage Mineralization Assay

The mineralized matrix was quantified with an OsteolImage Mineralization Assay Kit (Lonza, USA, Cat. No. PA-1503). The culture media was removed and the cells were washed once with PBS and fixed with 70% cold ethanol for 20 min. An appropriate amount, as recommended by the manufacturer, of the diluted staining reagent was added and the plates were incubated in the dark for 30 min at room temperature. The cells were washed and stained. Quantitation was performed using a fluorescence plate reader at 492/520 excitation/emission wavelengths.

ALP Quantification assay

For the quantification of ALP expression, we used the Abcam ALP quantification kit and followed the manufacturer's instructions.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted using innuPREP RNA Mini Kit (Analytik Jena, Germany, REF No: 845-KS-2040250) as recommended by the manufacturer. Total RNA was quantified by Nanodrop spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific, USA). Complementary DNA (cDNA) was synthesized from 1 μg of the RNA with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) using Labnet, Multigene thermal cycler according to the manufacturer's instructions. Relative levels of mRNA were determined from the cDNA with RT-PCR (Applied Biosystems Real-Time PCR Detection System) using a Fast SYBR Green PCR kit
(Applied Biosystems, UK) according to the manufacturer's instructions. Following normalization to the reference gene, GAPDH, quantification of gene expression was carried out using a comparative CT method, where ΔCT is the difference between the CT values of the target and reference genes (for primer sequence see Elsafadi, et al., 2016).

**DNA microarray global gene expression profiling**

Total RNA was extracted using innuPREP RNA Mini Kit (Analytik Jena, Germany, REF No: 845-KS-2040250) as recommended by the manufacturer. 50 ng of total RNA was labeled and hybridized with the Agilent Human SurePrint G3 Human GE 8 x 60 k v16 microarray chip (Agilent Technologies). All microarray experiments were conducted at the Microarray Core Facility (Stem Cell Unit, Anatomy Department, College of Medicine, King Saud University). Normalization and data analyses were conducted using the GeneSpring GX software (Agilent Technologies). Pathway analysis was conducted using the Single Experiment Pathway analysis feature in GeneSpring 12.0 (Agilent Technologies). A two-fold cutoff with P < 0.05 was used.

**Statistical Analysis**

All of the results were presented as the mean and standard deviation (SD) of at least three independent experiments. Student's t-test was used to test the differences between groups. P < 0.05 was considered statistically significant.

**RESULTS**

**Expression of human hormone nuclear receptors and temporal expression of NR2F1 during osteoblast differentiation**

We have in our previous work described expression of multiple novel genes found during osteoblast differentiation of human MSC (hMSCs). In addition to this we discovered a wide variety of hormone nuclear receptors expression during
osteoblast differentiation. We found ten hHNR’s were highly up-regulated and five hHNR’s were down-regulated during osteoblast differentiation (Fig. 1A). NR2F1 was about 8 fold up-regulated and among the highest ones, NR2F1 is an orphan receptor and its role during osteogenesis is not known (Fig. 1A). To understand the temporal expression of NR2F1 during osteogenesis we performed real-time PCR at different times points, we discovered that NR2F1 gene expression gradually increase and have a maximum expression at day 5 and then the expression decreases at day 7 (Fig. 1B).

**Characterization of NR2F1-deficient cells**

hMSC-TERT cells were knocked down with NR2F1-siRNA. After 3 days of transfection, we validated the NR2F1-siRNA transfection efficiency with qRT-PCR and it exhibited more than 70% down-regulation of the NR2F1 gene (Fig. 2A). Next, we examined the proliferation efficiency of NR2F1-siRNA transfected cells by performing Alamar Blue proliferation assay on days 3, 5, and 7. NR2F1-siRNA transfected cells showed a slight reduction in proliferation compared with control scrambled siRNA-transfected cells (Fig. 2B).
NR2F1-deficient cells showed reduced osteoblast differentiation

To elucidate the osteoblast differentiation efficiency of NR2F1-siRNA transfection, the transfected cells were cultured in osteoblast differentiation media for 7 days. After 7 days of culturing in the osteoblast differentiation media, we analyzed the ALP expression for NR2F1-siRNA transfected cells, which showed a significant down-regulation, both quantitatively (Fig 2C) and qualitatively (Fig 2D). When we looked for mineralized matrix formation by visualizing with Alizarin red staining and Osteoimage staining, we discovered that there was almost no mineralization (Fig. 2G).
E-F). In addition, known osteoblast gene expression markers including RUNX2, ALPL, OSC, and BSP were all down-regulated.

**NR2F1-deficient cells’ control over osteoblast favors signaling pathways**

To determine the role of NR2F1 in osteoblast differentiation, we performed a global gene expression profile and pathway analysis using the Genespring software. Hierarchical clustering revealed that control cells and NR2F1-deficient cells separately clustered completely. Many genes were affected by NR2F1 knockdown (Fig. 3A), in Table 1 we have listed 30 top most down-regulated genes. Bioinformatic analysis showed that 4 out of 10 pathways are associated with osteoblast differentiation; they are TGFβ signaling pathway, MAPK signaling pathway, endochondral ossification pathway, and IL-6 pathway (Fig. 3B). To confirm the microarray pathway analysis data, we performed qRT-PCR and the results showed good concordance between microarrays for selected genes from each pathway (Fig. 3C-F, Supplementary Table 1).

<table>
<thead>
<tr>
<th>Table -1 Global gene expression of the first thirty down-regulated genes NR2F1 vs. scramble (SCR) control.</th>
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<tbody>
<tr>
<td><strong>Gene Symbol</strong></td>
</tr>
<tr>
<td>DDIT3</td>
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<tr>
<td>YARS</td>
</tr>
<tr>
<td>FAM134C</td>
</tr>
<tr>
<td>SDF2</td>
</tr>
<tr>
<td>CXCL3</td>
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<tr>
<td>EEF1A1</td>
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<tr>
<td>FDPSP2</td>
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<td>CA12</td>
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<td>C3</td>
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<td>POLR1C</td>
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<td>IFIT3</td>
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<td>CXCL2</td>
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<td>HADHA</td>
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<tr>
<td>DPCD</td>
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<tr>
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<tr>
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<td>SSR2</td>
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<td>CXCL2</td>
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<td>LDHA</td>
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**FIG. 3: Gene expression profiling of NR2F1 depleted cells**

(A) Hierarchical clustering of parental scrambled cells and NR2F1 transfected cells based on differentially expressed mRNA levels. Each row represents one replica and each column represents a transcript. Expression level of each gene in a single sample is depicted according to the color scale. (B) Pie chart illustrating the distribution of the top pathways designations for the deregulated genes in NR2F1 siRNA transfected cells. (c) qRT-PCR
validation TGFβ pathway genes from Genespring microarray pathway analysis software. (D) qRT-PCR validation MAPK pathway genes from Genespring microarray pathway analysis software. (E) qRT-PCR validation endochondral ossification genes from Genespring microarray pathway analysis software. (F) qRT-PCR validation IL-6 pathway genes from Genespring microarray pathway analysis software. All data are presented as mean ± SD, n = 3. Each experiment was performed at least two times; *P < 0.05; **P < 0.01.

**Functional studies on NR2F1 depleted cells**

Bioinformatics pathway analysis showed that 4 out of 10 pathways are associated with osteoblast differentiation. For the functional studies, we analyzed these four pathways since we suspected that NR2F1 down-regulated osteoblast differentiation through any of the four pathways, namely TGFβ signaling pathway, MAPK signaling pathway, endochondral ossification pathway, and IL-6 pathway (Supplementary Table 2). Initially, we studied the TGFβ1 pathway since it is the most prevalent signaling pathway. NR2F1 transfected cells, after seven days of differentiation in osteoblast differentiation medium, were evaluated with ALPL staining for differentiation. The results of the ALPL staining showed that TGFβ1 (10 ng/ml) did not recover osteoblast differentiation (Fig. 4A). Cell viability study with Alamar Blue staining indicated that TGFβ1 recovered proliferation of NR2F1 transfected cells (Fig. 4B). In the second step of the functional study, we used BMP-2 to induce both the BMP and MAPK signaling pathways since our microarray data showed that the top-most down-regulated gene, DDIT3, is under the control of the MAPK signaling pathway. In addition, some studies have also reiterated the connection between BMP-2 and DDIT3. Taking all these into consideration, we used BMP-2 to recover NR2F1-induced down-regulation of osteoblast differentiation. NR2F1 transfected cells, after seven days of differentiation in osteoblast differentiation medium containing rhBMP-2 and after being stained with ALPL staining, were used to evaluate the differentiation. BMP-2 recovered osteoblast differentiation as shown by the ALPL staining (Fig. 4C) and the qRT-PCR for RUNX2 (Fig 4D). We evaluated the MAPK signaling pathway genes, DDIT3 and JUN, both of which are up-regulated in NR2F1 transfected cells cultured with BMP-2 in osteoblast differentiation medium.
Here, NR2F1 transfected cells, cultured in osteoblast differentiation medium without BMP-2, were used as controls (Fig. 4D).

**FIG. 4: Effect of TGFβ1 and BMP2 on NR2F1 depleted cells**

(A) Quantification of ALPL activity in NR2F1 transfected cells cultured in OS differentiation media with TGFβ1 (10ng/μl), NR2F1 transfected cells cultured in OS medium used as a control. (B) Alamar blue cell viability quantification of NR2F1 transfected cells cultured with TGFβ1 (10ng/μl) at day 5 and day 7 of OS differentiation. (C) Quantification of ALPL activity in NR2F1 transfected cells cultured in OS differentiation media with BMP2 (1000ng/μl), NR2F1 transfected cells cultured in OS medium used as a control (D) qRT-PCR of NR2F1 transfected cells cultured in OS differentiation media with BMP2 (1000ng/μl), NR2F1 transfected cells cultured in OS medium used as a control. (E) MAPK signaling pathway including highlighted (yellow) genes from gene expression microarray data down-regulated genes NR2F1 vs SCR control siRNA. All data are presented as mean ± SD, n = 3. Each experiment was performed at least two times; *P < 0.05; **P < 0.01.

**Discussion**

Identifying novel mechanisms and regulatory signaling networks in osteoblast differentiation are prime requests in the clinical use of mesenchymal stem cells in
regenerative medicine. NR2F1 is an orphan nuclear receptor, yet its ligands are yet to be identified.

In this study, we elucidated the role of NR2F1 in osteoblast differentiation signaling networks. Knockdown of NR2F1 showed a slight reduction in the proliferation of NR2F1 transfected cells; this is because NR2F1 is known to control cell dormancy. Interestingly, TGFβ1 recovered the proliferation mediated by NR2F1 down-regulation (Neuman et al., 1995; Kim et al., 2012).

Our results with NR2F1 transfected cells during osteoblast differentiation revealed impairment in osteoblast differentiation, which was confirmed by ALPL expression, reduction in matrix mineralization, and reduction in osteoblast key markers genes expressions. In addition, global gene expression analysis showed that down-regulated signaling pathways were associated with osteoblast differentiation. TGFβ pathway is one of the prominent signaling pathways known to play a significant role in osteoblast differentiation (Zhou et al., 2011; Lee et al., 2000). Functional studies with TGFβ1 to recover NR2F1-induced down-regulation of osteoblast differentiation was unsuccessful. In addition, we induced BMP signaling, as another agent, to recover osteoblast differentiation. We discovered that induction of BMP signaling recovered osteoblast differentiation induced by NR2F1. As it is known, BMP2 induces MAPK pathway. Therefore, the MAPK pathway was one of the most down-regulated pathways, and one of the top-most down-regulated genes was DDIT3 (Huang et al., 2014). DDIT3 is one of the genes in the MAPK pathway and is known to play a key role in osteoblast differentiation (Huang et al., 2014). The BMP-2 gene was among the down-regulated genes in the microarray data (Supplementary Table 2). Our results of BMP-2-induced osteoblast differentiation of NR2F1 cells exhibited increased ALPL expression and increased RUNX-2, DDIT3, and JUN genes expressions. Hence, BMP-2 affects DDIT3 which in turn modulates osteoblast differentiation (Pereira et al., 2004; Shirakawa et al., 2006).

**Conclusion**
In conclusion, our study reported that NR2F1 mediated the down-regulation of osteoblast differentiation in hBMSCs, which was rescued by BMP-2 induction. In addition, BMP-2 recovered NR2F1-mediated down-regulation of osteoblast differentiation through the MAPK pathway by inducing DDIT3 and JUN.

Conflict of Interest

The authors declare no conflict of interest

Acknowledgments

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References


Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. Mol Cell Biol. 20(23), 8783-8792.


**Supplementary Figure 1.** Graph shows post confluent cultures of osteoblast differentiated NR2F1 transfected cells showed impaired cell growth, cells were counted at different time points after osteoblast differentiation.