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Transcriptional profiling of human femoral mesenchymal stem cells in osteoporosis and its association with adipogenesis

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ABSTRACT

Genetic alterations are major contributing factors in the development of osteoporosis. Osteoblasts and adipocytes share a common origin, mesenchymal stem cells (MSCs), and their genetic determinants might be important in the relationship between osteoporosis and obesity.

In the present study, we aimed to isolate differentially expressed genes (DEGs) in osteoporosis and normal controls using human MSCs, and elucidate the common pathways and genes related to osteoporosis and adipogenesis. Human MSCs were obtained from the bone marrow of femurs from postmenopausal women during orthopedic surgeries. RNA sequencing (RNA-seq) was carried out using next-generation sequencing (NGS) technology. DEGs were identified using RNA-seq data. Ingenuity pathway analysis (IPA) was used to elucidate the common pathway related to osteoporosis and adipogenesis. Candidate genes for the common pathway were validated with other independent osteoporosis and obese subjects using RT-PCR (reverse transcription-polymerase chain reaction) analysis.

Fifty-three DEGs were identified between postmenopausal osteoporosis patients and normal bone mineral density (BMD) controls. Most of the genetic changes were related to the differentiation of cells. The nuclear receptor subfamily 4 group A (NR4A) family was identified as possible common genes related to osteogenesis and adipogenesis. The expression level of the mRNA of NR4A1 was significantly higher in osteoporosis patients than in controls ($p = 0.018$). The expression level of the mRNA of NR4A2 was significantly higher in obese patients than in controls ($p = 0.041$). Some genetic changes in MSCs are involved in the pathophysiology of osteoporosis. The NR4A family might comprise common genes related to osteoporosis and obesity.

1. Introduction

Osteoporosis is a common disease characterized by low bone mass and deteriorated microarchitecture, resulting in fragility fractures (Rachner et al., 2011; Langdahl, 2015). Although multiple risk factors such as advanced age, long-term glucocorticoid use, low body weight, tobacco use, or excess alcohol use contribute to the development of osteoporosis (O'Connor, 2016), approximately 60% of peak bone mass is genetically determined and is a major contributing factor for the development of osteoporosis (Bonjour and Chevalley, 2007). However, the osteoporosis-related genes that have been identified to date using current advanced technologies explain no more than 10% of the

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; BMD, bone mineral density; BMI, body mass index; CLEC2B, C-type lectin domain family 2 member B; CRH, corticotropin-releasing hormone; CVs, coefficients of variation; DEGs, differentially expressed genes; FBS, fetal bovine serum; FGFR2, fibroblast growth factor receptor 2; HBEGF, heparin-binding EGF-like growth factor; IPA, ingenuity pathway analysis; ISCD, the international society for clinical densitometry; LOC105374013, endogenous retrovirus group K member 5 Gag polyprotein; LRP5, Low-density lipoprotein receptor-related protein 5; MMP1, matrix metallopeptidase 1; MMP13, matrix metallopeptidase 13; MSCs, mesenchymal stem cells; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NGS, next-generation sequencing; NR4A1, nuclear receptor subfamily 4 group A member 1; NR4A2, nuclear receptor subfamily 4 group A member 2; NR4A3, nuclear receptor subfamily 4 group A member 3; POMC, proopiomelanocortin; RNA-seq, RNA sequencing; RT-PCR, Semi-quantitative reverse transcription-polymerase chain reaction; Runx2, Runt-related transcription factor 2; SEMs, standard error of means; SEPT, septin 7; SLC26A7, solute carrier family 26 member 7; SPOCK3, sparc/osteonectin, cwcv, and kazal-like domains proteoglycan 3; STAT3, Signal transducer and activator of transcription 3; TMN, the trimmed mean of M-values normalization method; TZDs, thiazolidinediones

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variation in bone mineral density (BMD) in any individual human population (Deng et al., 2011). Causal genes and molecular mechanisms leading to osteoporosis still remain to be elucidated.

Bone-forming osteoblasts are derived from mesenchymal stem cells (MSCs). The allocation of MSCs into osteoblasts is accelerated after skeletal injury and during the rapid growth phase of puberty (Rosen and Klibanski, 2009). Although MSCs are the source of osteoblast, it is not currently well known if individual intrinsic genetic differences in these cells contribute to osteoporotic bone loss (Benisch et al., 2012).

MSCs also differentiate into adipocytes (Valtieri and Sorrentino, 2008). Two disorders of body composition, obesity and osteoporosis, have increased in prevalence during the last three decades (Rosen and Klibanski, 2009). There has been a great increase in the understanding of interactions between bone and fat (Rosen, 2008). Bone and fat cells share a common origin, and their fates are interrelated in a specific manner (Rosen and Klibanski, 2009). There are many clinical experiences related to these phenomena. For example, glucocorticoids markedly enhance marrow adipogenesis at the expense of osteoblast differentiation (Mazziotti et al., 2007). Thiazolidinediones (TZDs) reduce bone mass and increase the risk of osteoporotic fractures because MSCs switch into the adipocyte lineage (Lecka-Czernik et al., 1999). Genetic determinants might be important in the switch of stem cells for both physiologic and pathologic states (Rosen and Klibanski, 2009).

Recently, whole-transcriptome sequencing using next-generation sequencing (NGS) technologies or RNA sequencing (RNA-seq) has started to reveal the complex landscape and dynamics of the transcriptome at a remarkable sensitivity and accuracy (Ozsolak and Milos, 2011; Wang et al., 2009). RNA-seq enables not only the identification of differentially expressed genes (DEGs) but also the precise quantitative determination of exon and isoform (alternative splicing) expression, along with the unbiased characterization of novel exons and novel transcript clusters (Marioni et al., 2008).

In the present study, we isolated human MSCs from the bone marrow of typical severe osteoporosis patients and controls with normal BMD and identified DEGs using NGS technology. Subsequently, we elucidated the common pathway and genes related to osteoporosis and adipogenesis using ingenuity pathway analysis (IPA) of the identified DEGs.

2. Materials and methods

2.1. Study subjects

This study was approved by the Ajou University Hospital Institutional Review Board (AJIRB-GEN-GEN-11-332), and full informed written consent was obtained from all of the subjects. Human MSCs were obtained from the bone marrow of femurs during total hip or knee arthroplasty due to osteoarthritis or hip fracture. All of the subjects were postmenopausal women aged ≥ 60 years. Subjects with other metabolic disorders and/or secondary causes of osteoporosis were not included. Height and body weight were measured using standard methods, with the subjects wearing light clothing. The body mass index (BMI) was calculated as the weight divided by the height squared (kg/m^2). Densitometric examinations were performed using a Lunar iDXA apparatus (GE Lunar, Madison, WI, USA). For lumbar spine BMD, when specific vertebrae were not suitable for analysis due to compression fracture or degenerative changes or any other reasons, it was calculated excluding the affected vertebrae. The coefficients of variation (CVs) for BMD were 0.975% (L1-L4), 0.888% (femur neck), and 0.738% (total hip). 30 subjects had been previously scanned twice for precision testing according to the International Society for Clinical Densitometry (ISCD) recommendations (Schousboe et al., 2013). Patients were diagnosed with a vertebral fracture when at least one of the three height measurements decreased by > 25% compared to the nearest uncompressed vertebral body. All of the subjects in the severe osteoporosis group had vertebral fractures or hip fractures and low BMD (the T-score of the lumbar spine, total hip, or femoral neck was − 2.5 or less). All of the subjects in the control group had no fracture and normal BMD (the T-scores of the lumbar spine, total hip, and femoral neck were − 1.0 or above). For the validation of candidate common genes related to osteoporosis and adipogenesis with other independent subjects, two osteoporosis patients with hip fractures low BMD (T-score ≤ − 2.5) and controls with normal BMD, two obese patients and non-obese controls with similar BMDs (T-score < − 1 and < − 2.5) were recruited. Obesity was defined as BMI ≥ 25 kg/m^2, according to the Korean Society for the Study of Obesity (Oh et al., 2004). Fasting blood samples were drawn from the antecubital area between 08:00 and 11:00 h. The concentrations of calcium and phosphorus were measured using automated techniques. The concentration of 25-hydroxyvitamin D (25(OH)D) was assayed using a radioimmunoassay kit (DiaSorin, Stillwater, MN, USA).

2.2. Human MSC culture

MSCs were isolated by negative immunoselection (RosetteSep Isolation Kit, STEMCELL Technologies Inc., Vancouver, Canada) according to the manufacturer's instructions. Briefly, the cells were incubated for 25 min with the depletion cocktail of tetrameric antibodies at room temperature. Next, the cells were diluted with phosphate-buffered saline and isolated by density-gradient centrifugation. The isolated MSCs were plated into vented 25 cm^2 tissue culture flasks (Thermo Scientific, Waltham, MA USA) with Complete MesenCult® Medium (Human) and MesenCult® MSC Basal Medium (Human) mixed with Mesenchymal Stem Cell Stimulatory Supplements (Human) (STEMCELL Technologies Inc., Vancouver, Canada) and antibiotics (Welgene, Gyeongsan, South Korea). The flasks were incubated at 37 °C in a humidified atmosphere containing 5% CO_2; after 7 days, half of the medium was replaced with fresh medium. Then the cells were cultured with a half-medium change every week until the fibroblast-like cells at the base of the flask reached confluence. On reaching confluence, the adherent cells were detached using 0.25% trypsin and were re-seeded at 1×10^5 cells per 25 cm^2 flask (4000 per 1 cm^2; first passage). These cells were incubated again until confluence and were once again trypsinized and re-seeded at 1×10^5 cells per 25 cm^2 flask (4000 per 1 cm^2; second passage). At the end of the second passage, once the cells reached confluence, they were trypsinized and either cryo-preserved or used immediately (Angelopoulou et al., 2003).

2.3. Mouse MSC preparation

Femurs were isolated from 22-week-old ovariectomized and sham-operated DDY female mice. Bone marrow was flushed with α-MEM (Gibco, Franklin Lakes, NJ, USA) containing 10% fetal bovine serum (FBS) (Gibco) and was maintained in α-MEM containing 10% FBS media. The animal research procedures were approved by the Animal Care and Use Committee of the Ajou University School of Medicine (IACUC No. 2014-0066), and all experiments were conducted in accordance with the institutional guidelines established by the committee. All efforts were made to minimize animal suffering and to reduce the number of mice used.

2.4. RNA isolation

Total RNA was isolated using the RNeasy Micro Kit (Qiagen, Valencia, CA) and was treated with the Turbo DNA-freeTM Kit (Life Technologies, Grand Island, NY, USA) to remove potential contaminating DNA that may lead to false-positive amplification. The quality and purity of RNA were confirmed using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). The resulting intact RNA was used in RNA-seq and QPCR analysis.