

Investigation of transcriptome mechanism associated with osteoporosis explored by microarray analysis

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Abstract. Microarray data of osteoporosis (OP) were analyzed based on prediction of transcription factors (TFs) or their targets as well as influences of TFs or TF network to uncover key TFs in OP. The microarray data E-GEOD-35956 was downloaded from the GPL570 platform. Differentially expressed genes (DEGs) with logarithm of fold change (llogFCI) >2 and P-value <0.05 were identified between OP samples and normal controls. TF genes were screened from the DEGs based on ITPP, Marbach 2016, TRRUST databases. TF targets were enriched from DEGs using Fisher's exact test. TF targets were selected based on their impact factors. TF targets were chosen from TF network analysis. Finally, key TFs were identified by based on TFs coverage. A total of 300 DEGs were obtained. There were no TF genes screened from the DEGs. In total 165, 87 and 178 TF targets were screened from DEGs respectively based on Fisher's exact test, influence of TFs or TF network analysis. According to the optimal TF set with TFs having maximum coverage of DEGs, 178 TF targets was the most. Thus, the optimal sets of TFs were *FOXO1*, *KLF16*, *RXRA*, *RARA*, *HNF4A*, *CEBPB*, *ESR1*, *SOX8*, *ZNF219*, and *SPI1*. Altogether, these results suggested identified crucial TFs in OP might play a significant role in OP development, showing these key TFs probably would aid in unveiling the underlying molecular mechanisms and may be therapeutic targets, diagnostic or prognostic biomarkers for OP.

Introduction

Osteoporosis (OP) is a common metabolic skeletal disease in the elderly population featured by osteopenia and microstructure, mainly due to the imbalance between bone resorption and

bone formation, resulting in increased morbidity and high cost of health care. Current existing therapeutic agents specific to OP are mainly antiresorptive drugs that repress bone resorptive action of osteoclasts. Largely owing to that the mechanisms of excessive bone resorption have been thoroughly explored (1). However, the medicinal efficacy on bone mass and strength of patients remains limited. Thus, it is imperative to address this public health concern, and identify its underlying molecular mechanisms.

The cellular and molecular processes involved in pathophysiology of OP are relatively complicated regulatory networks, involving numerous genes, factors and several pathways. In molecular regulatory networks, it is considered that alterations of gene expression elicit abnormal protein function and pathways turbulence, thus occurrence and development of disease. It is well-known that substantial genes were regulated by transcription factors (TFs), recognized as the master regulators binding to DNA sequences specifically and thereby modulating gene transcription. The transcriptional regulation elicited by TFs is of importance to normal function of the organism, with transcriptional regulation central to cell cycle growth and survive (2), cell differentiation (3), cell adhesion (4) and cell homeostasis (5). However, TF failure leads to nearly 1/3 of human developmental diseases ascribed to TF misregulations (6,7). Thus, TF prediction is a pivotal step to comprehending sophisticated regulatory molecular networks. To date, effective screening methods of TFs related to OP remained largely lacking. OP mediated by osteoblasts is not able to balance bone absorption mediated by osteoclasts, leading to decrease in bone mass and low bone mineral density. Osteoblasts, as bone-forming cells, derive from mesenchymal stem cells (MSCs) which is the source for bone remodeling, besides, the differentiated osteoblasts are decreased from MSCs with aging concerning series of molecular mechanisms (8). However, research on osteoblast action involving underlying mechanisms are relatively ignored.

Therefore, the present study was designed to investigate the mechanistic TFs for OP progression using bioinformatics methods based on gene expression data from the MSC of OP patients. TF targets enrichment analysis was performed for the chosen differentially expressed genes (DEGs). Then, analysis of TF impact factors (IFs) was conducted for DEGs. Moreover, influence of TF network analysis was executed for DEGs.

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Finally, the obtained TFs based on TF targets prediction and influence of TFs targets or TF network were analyzed comprehensively to achieve the key TFs, which might contribute to future therapy of OP.

Materials and methods

Microarray data collection and preprocessing. The gene expression profile data GSE35956 were extracted from the study of Benisch *et al.* (9), which was based on Gene Expression Omnibus database. Human mesenchymal stem cells (hMSC) of elderly patients (79-94 years) suffering from OP were isolated from femoral heads after low-energy fracture of the femoral neck. A total of 5 OP patients (elderly) and 5 control group (middle-aged) were included. Then, the raw data were normalized and converted into expression values by the robust multi-array average (RMA) algorithm (www.bioconductor.org) and R statistical software (version 3.0.0; R Project for Statistical Computing, <https://www.r-project.org/>).

DEG analysis. Multiple linear regression package, limma package (10,11) was used to determine the DEGs between the OP patients and normal controls. Multiple testing corrections were performed using the Bayesian method. Only genes with logarithm of fold change ($\log_{2}FC$) ≥ 2 and P-value < 0.05 were chosen as the DEGs. To ensure the stability of the object in the screening process, all the DEGs were chosen if their number was over 300 with their $\log_{2}FC$ beyond 2 while the top 300 DEGs were chosen if their the number was below 300 with their $\log_{2}FC$ beyond 2.

TF genes or TF target prediction. It is known that each TF, as a protein, has a corresponding regulatory gene, which mainly binds to specific DNA sequences and thus exerts its regulatory role in incomputable biological process. Hence, TF gene was identified first based on mapping DEGs to TF databases including ITPP (12), Tissue-specific regulatory circuits (13), TRRUST (14) databases involved in TFs genes, their downstream regulated genes (target genes) and binding sites. Then, once no TF genes existed, we speculated potential effect of TF by analyzing whether TF targets contained DEGs using Fisher's exact test, which could reflect that the more TF targets were enriched, the more crucial the TFs were. Noticeably, the abundant TF targets appeared due to likelihood that TF targets were widely regulated by TFs such as TFs involved in regulating cell cycle. Thus, such TFs were needed for the following study combined with disease state.

The influence of TF analysis. IFs of TFs were assessed by calculating G-score according to its metric. By comprehensively considering the G-score (the forum was shown as follow), average G-score and numbers of regulated genes, the IF (expressed in P-value) of TF was gained. The lower of this value is, the bigger of TF influence is. According to the P-value, the crucial TFs targets were predicted.

$$G_s^x = |L_s^x|(-\log_{10} P_s^x)$$

The influence of TF network analysis. Considering the possibility that TFs could form a co-expression relationship with its target genes, to assess the importance of TFs, Search Tool

for the Retrieval of Interacting Genes/Proteins (STRING) and the afore-mentioned 3 databases were used to calculate the influence of TFs on local network neighborhood using the weighted sum method (15). Subsequently, G-score of network was calculated and the crucial TF targets were determined by its ranking.

Identification of optimal set of TFs. To select the optimal set of TFs with the greatest combined influence, TFs with maximum coverage of DEGs were determined. Top TFs obtained based on above third methods (TF targets enrichment analysis, TF targets influences analysis, TF network influences analysis) were integrated to obtain the optimal crucial TFs.

Results

Data preprocessing and DEG identification. After data preprocessing, a total of 20,514 genes were obtained from 5 OP patients (elderly) and 5 control groups (middle-aged). A total of 300 DEGs were obtained, namely, *LYG2*, *PPEF1*, *TTC16*, *LOC100134368*, *LOC100505716*, *CKM*, *LOC100506272*, *ADAMTS7*, *MAB21L2*, *NR0B1*, which were all upregulated in OP group (Table I).

TF genes or TF targets prediction. The result of TF genes enrichment analysis suggested that no DEGs were identified as TF genes. In the following, we analyzed whether TF targets were contained in DEGs using Fisher's exact test. As shown in Table II, 165 TFs targets including *CKM*, *CD74*, *ADAMTS*, *ADAMTS7*, *NPAS1* were enriched, correspondingly, the top 10 TFs were *WT1*, *ZBTB7A*, *CACBP*, *ZNF281*, *ZBTB47*, *ZNF219*, *PATZ1*, *TFAP2C*, *HGS*, and *KLF16*.

The influence of TF analysis. According to the G-score, average G-score, numbers of regulated genes and the P-value of TF comprehensively, 87 TF targets from DEGs were obtained, correspondingly, the top 10 crucial TFs were *FKBP8*, *SPI*, *LMO4*, *KLF3*, *KHDRBS1*, *ZFPM1*, *EML3*, *ZNF580*, *ZBTB45*, and *WDTX1* (Table III).

The influence of TF network analysis. Based on the G-score of network ranking, 178 TF targets were identified from DEGs, correspondingly, the top 10 influential TFs included *FOXO1*, *KLF16*, *RXRA*, *RARA*, *HNF4A*, *CEBPB*, *ESR1*, *SOX8*, *ZNF219*, and *SPI* (Table IV).

Optimal set of TF identification. Ultimately, the above third methods were integrated with the greatest combined influence to attain the optimal set of TFs with maximum coverage of DEGs. The PPI network analysis based on the G-score of network efficacy is better than others methods due to the number of the 178 TF targets was more than the number of 165 or 87 TF targets, as shown in Fig. 1. Thus, the optimal set of TFs were *FOXO1*, *KLF16*, *RXRA*, *RARA*, *HNF4A*, *CEBPB*, *ESR1*, *SOX8*, *ZNF219*, and *SPI*.

Discussion

Bioinformatics methods were utilized to identify crucial TFs for clinical OP treatment. The results suggested that 300 DEGs

Table I. The top 10 DEGs.

Gene name	logFC	AveExpr	t	P-value	adj.P.Val
<i>LYG2</i>	3.822863546	4.898607116	11.79654581	3.09E-07	0.006343212
<i>PPEF1</i>	2.967917113	5.306881431	8.769700405	4.84E-06	0.049630867
<i>TTC16</i>	3.371397328	5.527493053	8.198929385	8.85E-06	0.054473657
<i>LOC100134368</i>	2.877190274	5.538742477	7.729046328	1.49E-05	0.054473657
<i>LOC100505716</i>	3.098691591	5.203848263	7.677271035	1.58E-05	0.054473657
<i>CKM</i>	3.47319398	6.247940415	7.670112434	1.59E-05	0.054473657
<i>LOC100506272</i>	2.368835964	4.8244862	7.19255559	2.78E-05	0.081533396
<i>ADAMTS7</i>	1.372895995	7.074901655	6.9525671	3.72E-05	0.092051022
<i>MAB21L2</i>	3.654174352	9.046481165	6.727015578	4.91E-05	0.092051022
<i>NR0B1</i>	1.911083095	5.997674202	6.593036972	5.82E-05	0.092051022

DEGs, differentially expressed genes; logFC, logarithm of fold change.

Table II. Top 10 TF enrichment analysis of DEGs.

TF_name	TF_P-value	FDR_p	num_genes
WT1	6.52E-08	7.48E-05	97
ZBTB7A	1.83E-05	0.007169601	48
CACBP	2.16E-05	0.007169601	118
ZNF281	2.50E-05	0.007169601	111
ZBTB47	3.30E-05	0.00756938	6
ZNF219	3.96E-05	0.00756938	100
PATZ1	0.000106106	0.015445541	79
TFAP2C	0.000128968	0.015445541	112
HGS	0.000132363	0.015445541	5
KLF16	0.000153328	0.015445541	107
EGR	0.000157328	0.015445541	127

TFs, transcription factors; DEGs, differentially expressed genes.

Table III. Top 10 TFs identified from DEGs based on their influence.

TF_gene	G-score	ave_score	num_genes	rank_p
<i>FKBP8</i>	1.629660153	1.629660153	1	0
<i>SPI</i>	2063.253105	0.206263432	10003	0
<i>LMO4</i>	5.595214111	1.398803528	4	0.02001
<i>KLF3</i>	5.506357538	1.376589384	4	0.02405
<i>KHDRBS1</i>	2.696028355	1.348014178	2	0.02983
<i>ZFPM1</i>	1.310340162	1.310340162	1	0.03837
<i>EML3</i>	1.28108726	1.28108726	1	0.04572
<i>ZNF580</i>	2.548392738	1.274196369	2	0.04752
<i>ZBTB45</i>	2.477553068	1.238776534	2	0.05746
<i>WDTC1</i>	1.178355662	1.178355662	1	0.07665

TFs, transcription factors; DEGs, differentially expressed genes.

Table IV. Top 10 TFs identified from DEGs based on their G-scores of TF network.

TF_gene	G_net_score
<i>FOXO1</i>	328.9369128
<i>KLF16</i>	323.1693838
<i>RXRA</i>	312.8356811
<i>RARA</i>	310.4836752
<i>HNF4A</i>	300.1282162
<i>CEBPB</i>	297.0888622
<i>ESR1</i>	291.421492
<i>SOX8</i>	291.0030097
<i>ZNF219</i>	290.6270745
<i>SPI</i>	285.5734732
<i>NFIC</i>	283.8264335

TFs, transcription factors; DEGs, differentially expressed genes.

from DEGs were enriched based on TF genes enrichment analysis, 87 TF targets from DEGs were attained by their IF analysis and 178 TF targets from DEGs were achieved by TF network influence analysis. According to the optimal TF set with TFs having maximum coverage of DEGs, 178 TF targets were the most. Thus, the optimal sets of TFs were *FOXO1*, *KLF16*, *RXRA*, *RARA*, *HNF4A*, *CEBPB*, *ESR1*, *SOX8*, *ZNF219*, and *SPI*. These TFs identified from third bioinformatics methods are possibly capable of aiding in a better understanding of the underlying molecular mechanisms, implying these crucial TFs are likely to be the potential therapy target for OP.

It is well-established that TF, specific to binding to its target gene, thus exerts inhibitory or facilitative role in gene expression, showing a significant part in the multitude of biological processes involved in diseases (15-17). Particularly, it is well known that runt-related TF-2 (Runx-2), served as a master member of osteogenic differentiation specific TFs during the early stage of osteogenesis, upregulates the transcription of various mineralized related protein genes in osteoblasts and chondrocytes, and thus promotes these cells to differentiate into osteoblasts (18,19). Hence, Runx-2 is also proved to play a

such as upregulated *LYG2*, *PPEF1*, *TTC16* were gained in OP group compared with control group. A total of 165 TF targets

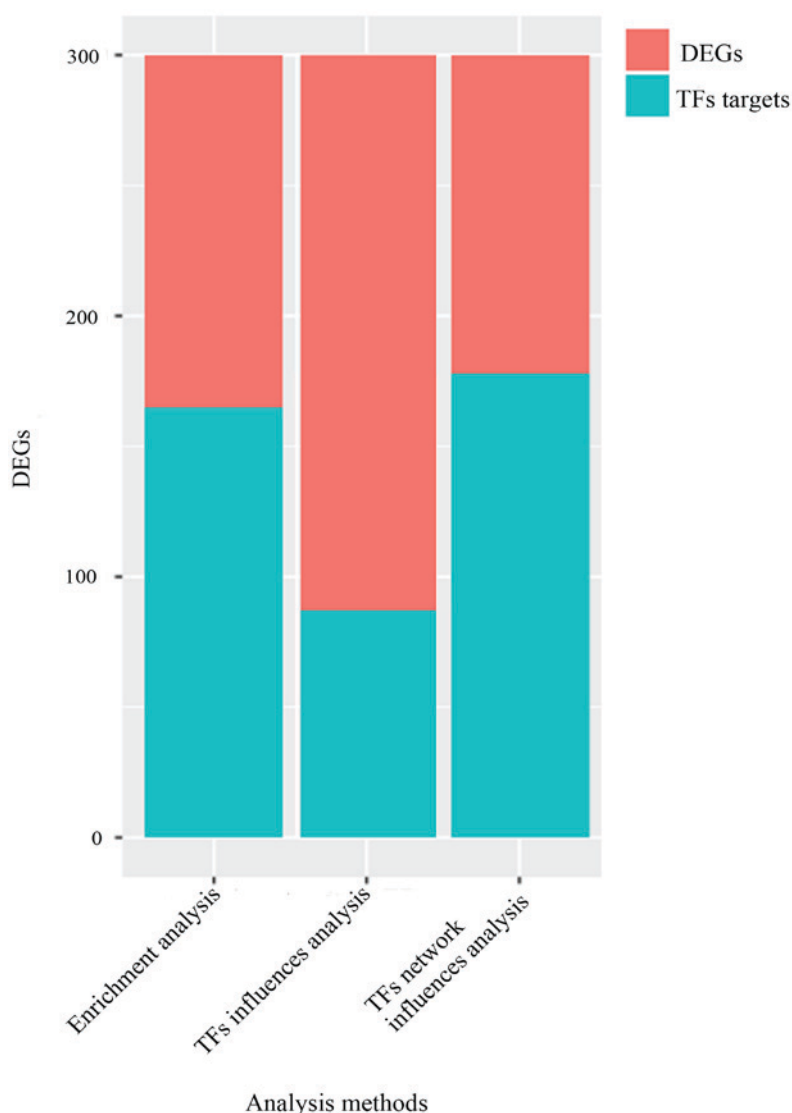


Figure 1. Distribution scale schematic illustrates the overlap of TF target DEGs from third methods: enrichment analysis, IF analysis, TF network analysis. TF, transcription factor; DEGs, differentially expressed genes; IF, impact factor.

key role in bone metabolism regulation and bone development, indicating it is of great significance to prevent and treat OP. Osterix, a zinc-finger-containing TF, is an essential osteogenic marker for the differentiation of preosteoblasts into mature osteoblasts during the late stage of osteoblast differentiation, which is required for bone formation (20,21). It is reported that nuclear receptors, emerged as a family of TFs, are fundamental regulators of maintaining bone development and remodeling, besides, several drugs targeting it are widely applied in treating bone diseases such as OP via regulating rates of bone formation and resorption (22). Although some TFs are proved to play an essential role in bone metabolism, global prediction of TFs in OP are still relatively scarce.

Thus, in the present study, comprehensive bioinformatics methods were introduced to obtain key TFs from a global point of view. First, 300 DEGs were obtained including top 10 DEGs such as *LYG2*, *PPEF1*, *TTC16* all upregulated in OP according to 5 OP samples compared with 5 normal samples. Following, based on third bioinformatics methods, 165 TF targets, 87 TF targets and 178 TF targets were identified, respectively. The 178 TF targets had the most coverage of TFs, thus, the optimal

TF set of these TF targets were *FOXO1*, *KLF16*, *RXRA*, *RARA*, *HNF4A*, *CEBPB*, *ESR1*, *SOX8*, *ZNF219*, and *SPI1*. It is suggested that deletion of forkhead box O1 (FoxO1), one of members of the Forkhead box O (FOXO) family of TFs, could lead to a bone formation increase, which was maintained up to 24 months in mice while a lower number of adipocytes in the bone marrow of Foxo-deleted mice at this late age was presented (23), indicating FoxO1 possibly plays a crucial regulatory role in bone metabolism. It has also been demonstrated that Retinoid-X receptor- α (RXRA), one of nuclear hormone superfamily, is an essential cofactor in the action of 1,25-dihydroxyvitamin D (1,25[OH]₂-vitamin D) and epigenetically regulates activation of vitamin D, indicating it may influence bone mineral accrual (24-26). It is shown that CCAAT/enhancer-binding protein β (CEBPB), a TF, reduced bone mass in knockout mice (27). It has been found that estrogen receptor α (ESR1) is associated with low mineral osseous densitometry in women after menopause (28). Schmidt *et al* have shown that Sox8-deficient mouse exhibit a severely impaired bone formation, which is modulated by a strongly reduced expression of runt-related TF 2 (29). Taken together, the above evidence indicates

that the comprehensive bioinformatics analysis of TFs from a point of view provided by our study may aid in a better understanding of the molecular mechanism of OP. Albeit a novel insight of comprehending OP molecular pathogenesis emerged, many further studies are required due to the presence of some limitations in this study. Relatively small sample number and datasets, verification of *in vitro* and *in vivo* experiments are needed in further investigations.

In conclusion, our study provided novel insight into the mechanism by which crucial TFs were identified by comprehensive bioinformatics analysis of OP microarray data. These selected TFs are possible potential targets in the management of OP.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

CSW conceived the study and drafted the manuscript. YLiu, YLi and XL performed the experiments, analyzed the data and revised the manuscript. All authors read and approved the final study.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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