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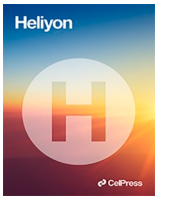

Transcriptome analysis of osteogenic differentiation of human maxillary sinus mesenchymal stem cells using RNA-Seq

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ABSTRACT

Recent studies have demonstrated that human maxillary sinus mesenchymal stem cells (hMSMSCs) have osteogenic potential and can be osteogenically induced. Here, we investigated pivotal molecular functions and candidates that contribute to the osteogenic differentiation of hMSMSCs. Human maxillary sinus membranes were harvested from 3 patients with jaw deformities. hMSMSCs from human maxillary sinus membranes were osteogenically induced for 0 or 21 days. Subsequently, their functional profiles were analysed by RNA sequencing and validated by quantitative PCR. Compared with control hMSMSCs, osteogenically induced hMSMSCs showed (1) osteogenic differentiation phenotype, as evidenced by the cell nodes, alizarin red staining, osteogenesis-related protein, and RNA expression; (2) accelerated osteogenic process of ossification and calcium signalling, as demonstrated by Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway; (3) enriched osteogenesis gene expression of SMOC2, OMD, IGF1, JUNB, BMP5, ADRA1A, and IGF2, which was validated by quantitative PCR. Based on by these results, we demonstrated that accelerated ossification process, calcium signalling, and upregulation of SMOC2, OMD, IGF1, JUNB, BMP5, ADRA1A and IGF2, may contribute to the osteogenic differentiation of hMSMSCs.

1. Introduction

Grafting the floor of the maxillary sinus has proven to be a valuable technique for the reconstruction of vertical bone height for posterior maxillary implant placement [1,2]. The maxillary sinus membrane, also known as the Schneiderian membrane, is essential for spatial osteogenesis after the maxillary sinus floor lift. During the grafting process, the maxillary sinus membrane is compromised to establish a new space, that can provide a barrier and osteogenic potential functions [1]. Human maxillary sinus mesenchymal stem cells (hMSMSCs) derived from the maxillary sinus membrane have inherent properties that endow them with osteogenic differentiation capabilities [3]. Our previous studies demonstrated the genuine differentiation phenotype of hMSMSCs *in vitro* [4] and intuitive bone augmentation *in vivo* [5]. Recently, Samer et al. (2013) used hMSMSCs as a model to reveal their osteogenic potency, revealing

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that hMSMSCs can result in more bone formation in OraGraft and ProOsteon transplants when compared to maxillary tuberosity bone marrow osteoprogenitor cells [6]. Another interesting study reported that hMSMSCs are potential contributors to the osteogenic process, accompanied by an inflammatory response upon stimulation with recombinant human bone morphogenetic protein-2 [7].

The aforementioned studies revealed that human maxillary sinus or Schneiderian membrane mesenchymal stem cells, may exhibit intrinstic osteogenic differentiation which contributes to new bone formation after maxillary sinus floor lift [8]. In this study, we used osteogenic differentiation of hMSMSCs as a model for maxillary sinus floor mucosal osteogenesis. RNA sequencing (RNA-seq) was used to analyse gene expression profiles of hMSMSCs induced for osteogenic differentiation. Candidate genes were validated using descriptive assays.

The aim of this study was to identify the essential molecular functions and candidate genes that contribute to the osteogenic capacity of hMSMSCs. We used a combination of molecular and genetic techniques to investigate the underlying mechanisms of hMSMSC osteogenic differentiation. Our results reveal several key molecular functions and candidate genes that may play a crucial role in the osteogenic capacity of hMSMSCs. These findings provide important insights into the potential therapeutic applications of hMSMSCs in bone tissue engineering and regeneration.

2. Materials and methods

2.1. Human maxillary sinus membrane samples

Maxillary sinus membrane samples were obtained from patients with jaw deformities (n = 3) who underwent orthognathic surgery at the Department of Stomatology, First Affiliated Hospital, Sun Yat-sen University (Guangzhou, China). Subjects with allergic symptoms and chronic inflammation in the sinus or nasal cavities and smokers were excluded. Membranes on the floor of the maxillary sinus were separated and collected during surgery. All samples were used to the establish an *in vitro* culture. This study was approved by the Institutional Review Board of First Affiliated Hospital, Sun Yat-sen University. Written informed consent was obtained from all the participants.

2.2. hMSMSCs isolation, cultures and osteogenesis induction

To isolate hMSMSCs, maxillary sinus membrane tissues were extensively rinsed in PBS and digested. Similar to previous methods [6], the maxillary sinus membrane was first treated with dispase-II (Roche, USA) for 1 h to eliminate epithelial structures. The digested tissue was then minced into small pieces and treated with 0.06% collagenase-I (Sigma Aldrich, USA) for 1 h at 37 °C, under a slow rotation. The tissue fluid was then passed through a sterile screen at slow speed. Finally, the harvested cells were resuspended in α-MEM medium (Gibco, USA).

For cultures of hMSMSCs, the harvested cells were cultured in α-MEM medium supplemented with 10% FBS (Invitrogen, USA) and 100 units/ml of penicillin-streptomycin (Invitrogen, USA). After 5–7 days of culture medium screening, cell density was approximately 80%, and the collected cells were primary hMSMSCs.

For flow cytometry analysis, 1×10^5 cells (P1) were harvested, washed, and suspended in PBS with 3% FBS and then incubated with antibodies to human CD34, CD73, CD90 (Biolegend, USA) and STRO-1 (Abcam, UK) at 4 °C in the dark. Cell suspensions without antibodies were used as controls. After washing with PBS, samples were analysed using a flow cytometer (Beckman Coulter).

To induce osteogenesis in hMSMSCs, primary cells were expanded to the next passage (P1). Initially, the P1 cells were expanded to 70–80% cell density. Half of the cultured cells were used as controls (D0) and the other half were cultured for 21 days with stemPro® osteogenesis differentiation kit (Gibco, USA), which were harvested as the OD group (D21).

2.3. Evaluation of cell morphology, alizarin red and alkaline phosphatase staining

hMSMSCs were seeded and expanded on a specific coverslip (Corning, USA) in 6 well plates. For morphological analysis, the cells were washed twice with PBS and fixed with 4% paraformaldehyde for 15min. The cell nodes with the morphology of colony growth trend were record using an optical microscope (Leica, USA) 10× and 40× objective lens [9].

For alizarin red staining analysis, the cells were fixed with 4% paraformaldehyde for 15 min and rinsed with DPBS. In accordanceing to the manufacturer's instructions, the cells were stained with 1% alizarin red solution for 30 min at 37 °C (Solarbio life science, China). The amount of alizarin red stain was quantified by measuring absorbance of the solution at 562 nm using an Alizarin Red Assay Kit (Beyotime, China).

For alkaline phosphatase staining, the cells were fixed with 4% paraformaldehyde for 15 min and rinsed with DPBS. An alkaline phosphatase staining kit (Solarbio Life Science, China) was used according to the manufacturer's instructions. An alkaline phosphatase assay kit (Beyotime, China) was used to quantify ALP activity at 405 nm.

Images of the slides were obtained using an optical microscope (Leica, USA) 10× and 40× objective lens. Three pictures were randomly taken at each time point for quantification.

2.4. RNA-seq experiment

hMSMSCs from Ctr group and OD group were harvested using TRIzol reagent (Qiagen, Hilden, German). Total RNA was extracted from hMSMSCs using the PureLink™ RNA kit (Thermo Fisher Scientific, USA) with the manufacturer's instructions. Library

preparation was performed according to the manufacturer's protocol using a TruSeq Stranded mRNA HT Sample Prep Kit (Illumina, USA). cDNA libraries were then constructed with rRNA elimination and sequenced using the Illumina HiSeq 2500 platform as 150-bp pair ended reads.

2.5. RNA-seq data analysis

Using STAR version 2.5.0a, the Fastq files were aligned to the hg19 reference genome for analysis. By matching htseq-counts, read overlapping genes were annotated (Ensembl build GRCh37.87). Differentially expressed genes were selected using a cut-off at a P value less than 0.05 (FDR adjusted for multiple testing) and the absolute value of log2 (fold change) exceeding than or equal to 1.

For enrichment analysis, DE genes were imported into Metascape and used to identify seed genes by analysing GO terms and KEGG pathways. For enrichment analysis, DE genes from the intra or inter-group comparisons were imported into Metascape (metascape.org) for GO terms and KEGG pathway analysis.

2.6. Quantitative RT-PCR

According to the manufacturer's protocol, total RNA of hMSMSCs from Ctr (n = 3) and OD (n = 3) groups was extracted using mirVana™ miRNA Isolation Kit (Life Technologies, USA). Using a PrimeScript RT kit (Takara, Japan), 500 ng of total RNA was reverse-transcribed into complementary DNA. One microlitre of cDNA was quantified using with SYBR Green Master Kit (Roche, Switzerland). Relative expression of mRNAs was calculated by the 2-ΔΔCT method. All data were normalized to PGK1 expression of the house-keeping gene. The primer sequences are listed in Table 1.

2.7. Western blot

Proteins were extracted from Ctr (n = 3) and OD (n = 3) groups. Next, ALP (Thermo Fisher Scientific, USA), RUNX2 (Abcam, UK), OSX (Abcam, UK), and GAPDH (Cell Signaling Technology, USA) proteins were detected in hMSMSCs by Western blot NuPAGE system (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Briefly, 25 μg of proteins was resolved on 10% SDS-PAGE gel, and the proteins were then transferred onto a polyvinylidene difluoride membrane (Millipore, USA). Primary antibodies of ALP, RUNX2, OSX and GAPDH were incubated overnight at 4 °C. HRP-conjugated secondary antibody (Cell Signaling Technology, USA) was then added to the membrane for 1 h at 37 °C. Target proteins were detected by chemiluminescence (Millipore, USA). GAPDH was used as an internal control to normalize the target proteins. All data were obtained using ImageJ Software.

2.8. Statistical analysis

All data are expressed as mean ± standard deviation. The student's unpaired *t*-test was used for all statistical analyses. Statistical analysis was performed using SPSS software, and statistical significance was set at $P < 0.05$.

3. Results

3.1. Positive osteogenic differentiation direction of hMSMSCs under induced conditions

hMSMSCs under growth conditions (Ctr group) maintained the typical "fibroblast-like" shape, but hMSMSCs under osteogenic stimulation condition (OD group) exhibited an irregular arrangement and had a "cell node" appearance within 3.77 ± 1.09 nodes per view (Fig. 1a). Flow cytometric analysis revealed that hMSMSCs were negative for CD34 and positive for CD73, CD105, and STRO-1, representing the characteristic phenotype of MSC (Fig. s1). By evaluating the Alizarin Red staining of hMSMSCs, we found that the OD

Table 1
Primers for quantitative real-time polymerase chain reaction.

Gene	Forward (5′-3′)	Reverse (5′-3′)
ALP	GGCCTGTACCATACAAGCCC	CCACGTAGACGAGGTAGTTGTG
RUNX2	TCAACGATCTGAGATTTGTGGG	GGGGAGGATTTGTGAAGACGG
OSX	CCTCTGCGGGACTCAACAAC	AGCCCATTAGTGCTTGTAAAGG
SMOC2	ATGACGACGGCACCTACAG	TCGCGTTGGGGTAACTTTTCA
OMD	TTCGTCCCAAAGTCGTCCG	TGGATGGTGATGGTCTGGTGAT
IGF1	GCTCTTCAGTTCGTGTGTGGA	GCCTCCTTAGATCACAGCTCC
JUNB	ACAAACTCCTGAAACCGAGCC	CGAGCCCTGACCAGAAAAGTA
BMP5	CTCTACAATGCCATGACCAATGA	CCGAGATAACTGTATGCGACGA
ADRA1A	TGCCAGATCAACGAGGAGC	GGCGTTTTTCCGATGGATGC
IGF2	GTGGCATCGTTGAGGAGTG	CACGTCCCTCTCGGACTTG
ADRA1B	TCTGGCGGTCATTCTAGTCAT	GGTGTCCTCGTGAAAGTTCTTG
COL10A1	CATAAAAGGCCCACTACCCAAC	ACCTTGCTCTCCTCTTACTGC
BMP6	TGTTGGACACCCGTGTAGTAT	AACCCACAGATTGCTAGTGGC
PGK1	CTCAACAACATGGAGATTGG	CTTTGGACATTAGGTCTTTGAC

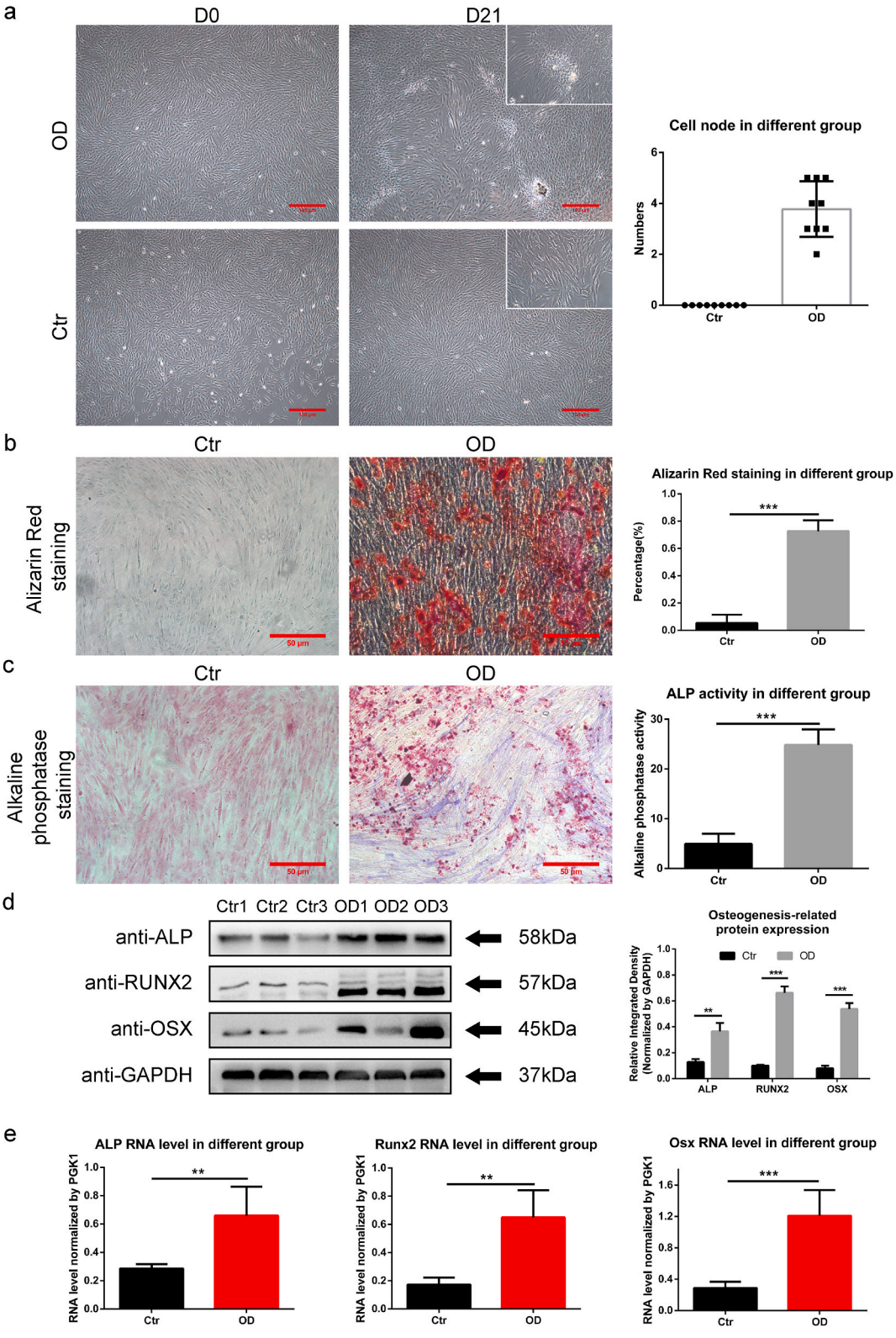

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Fig. 1. The osteogenic dynamic in hMSMSCs. **a** Difference of cell morphology and cell node numbers of hMSMSCs between OD and Ctr groups in osteogenic differentiation process (n = 3). **b-c** Osteogenic differentiation of hMSMSCs was demonstrated by Alizarin Red S staining and Alkaline phosphatase staining in OD and Ctr groups (n = 3). **d** ALP, RUNX2, and OSX protein levels were detected by Western blot analysis and they were compared in hMSMSCs between OD and Ctr groups (n = 3). **e** ALP, RUNX2, and OSX RNA expression level were determined in hMSMSCs OD and Ctr groups by quantitative RT-PCR analysis (n = 3). Data are presented as means ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

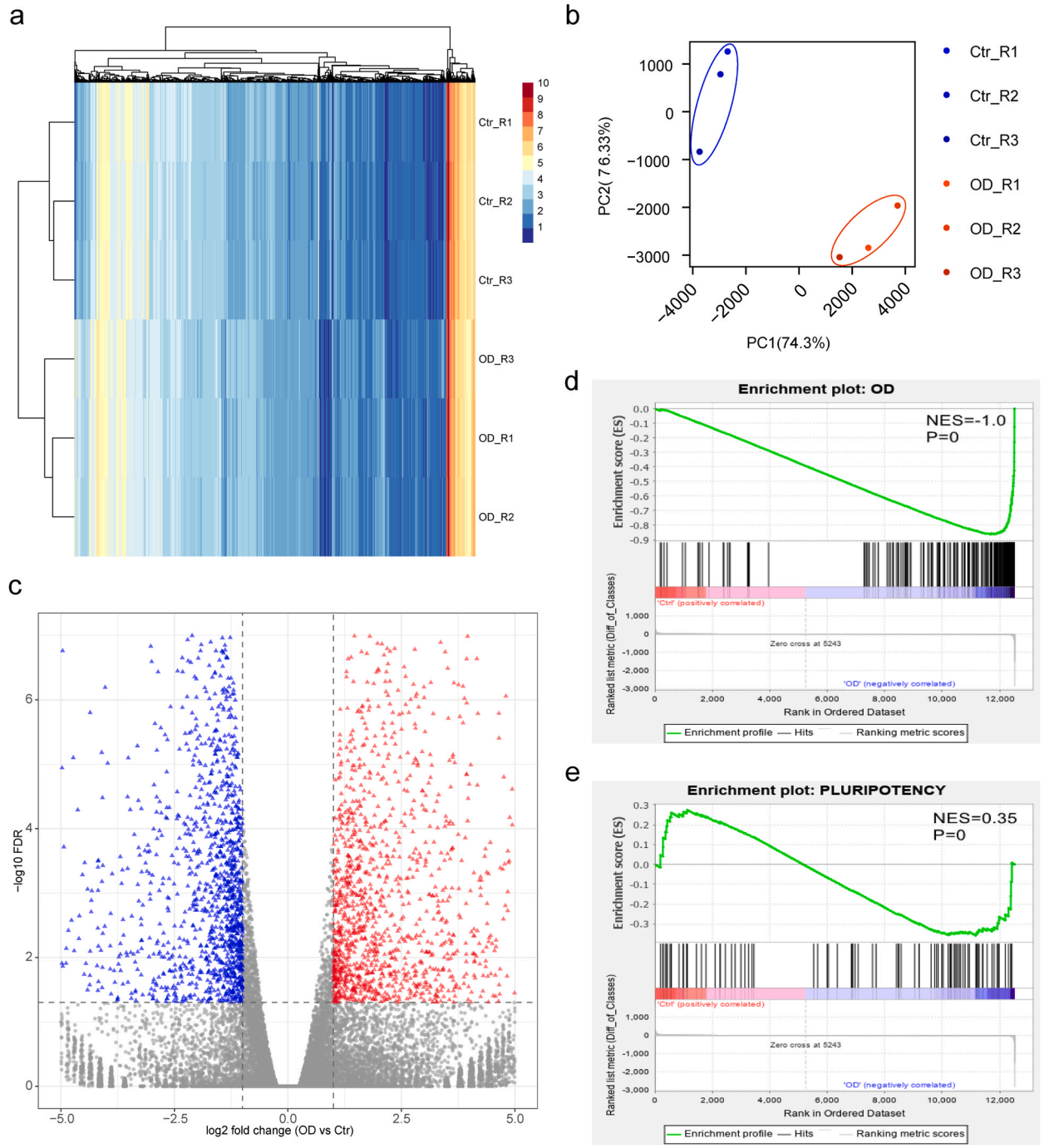
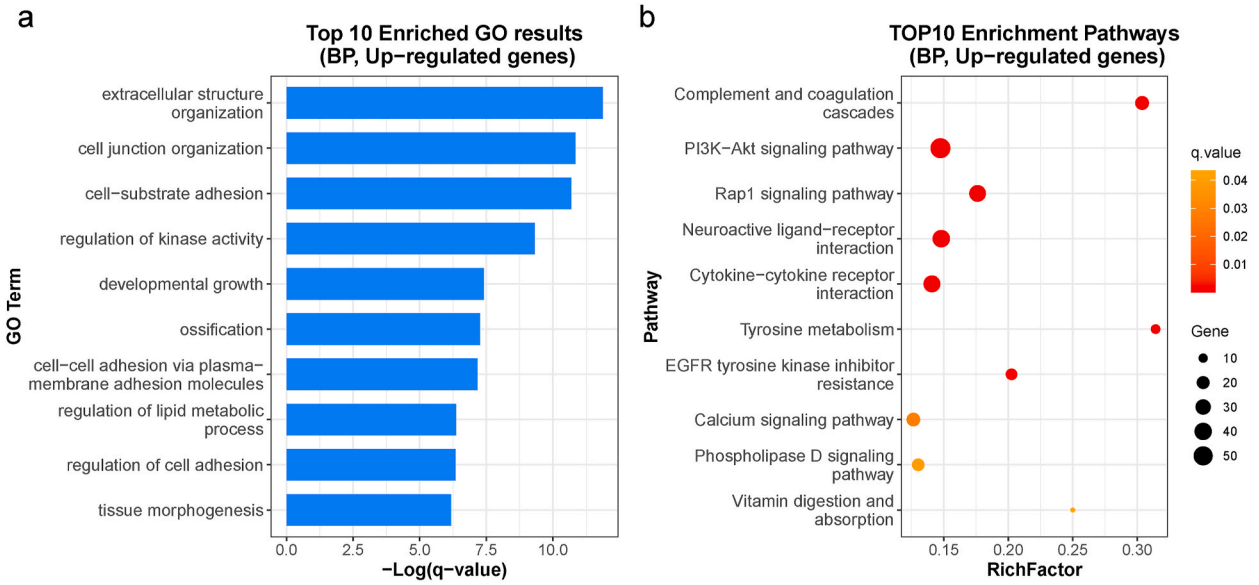


Fig. 2. Global transcriptome analysis in hMSMSCs. **a** The heatmap shows the K-means clustering of transformed expression values in OD and Ctr groups derived hMSMSCs(n = 3). **b** Principal component analysis plot denotes the classifier of OD and Ctr groups derived cells (n = 3). **c** MA plot reveals the numbers and distribution of DEGs between OD and Ctr groups derived cells (n = 3). **d** Gene set enrichment analysis of osteogenic differentiation (OD) and pluripotency of OD versus Ctr group.

group had more calcium nodules than the Ctr group undergoing osteogenic stimulation (Fig. 1b). A comparison of alkaline phosphatase staining, demonstrated that the OD group contained more calcium than the Ctr group (Fig. 1c). In addition, both the mRNA and protein levels of ALP, Runx2and Osx were significantly increased in cells from the OD group compared those from the Ctr group (Fig. 1d–e). Therefore, hMSMSCs under osteogenic stimulation exhibited a positive osteogenic differentiation direction, consistent with a previous report [4].



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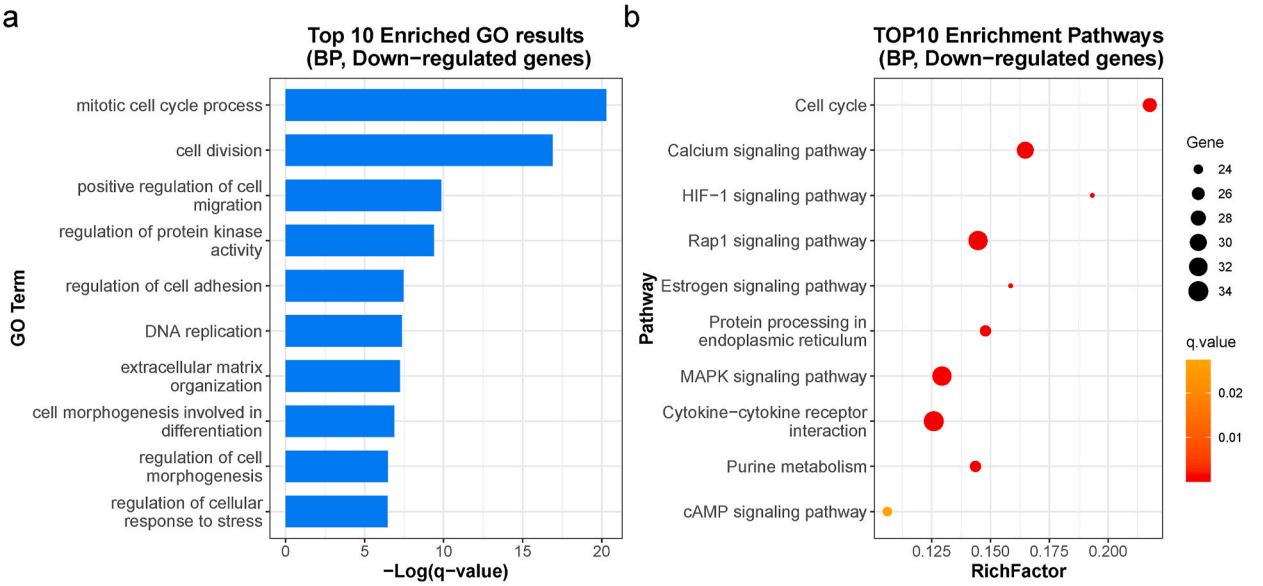
Top 20 up-regulated genes during osteogenic differentiation of MSMSCs

Gene	Annotation	Log_2FC	FDR
ZBTB16	zinc finger and BTB domain containing 16	13.78	6.77E-66
SMOC2	SPARC related modular calcium binding 2	12.9	7.85E-14
NPY2R	neuropeptide Y receptor Y2	10.77	3.06E-20
ADH1B	alcohol dehydrogenase 1B (class I)%2C beta polypeptide	10.7	1.02E-39
APOB	apolipoprotein B	10.56	1.25E-46
OMD	osteomodulin	10.15	3.50E-22
PIP	prolactin-induced protein	9.9	6.31E-07
C7	complement 7	9.81	1.94E-12
ACKR1	atypical chemokine receptor 1 (Duffy blood group)	9.7	1.35E-06
AOX3P	aldehyde oxidase 3%2C pseudogene	9.32	6.56E-08
CNTFR	ciliary neurotrophic factor receptor	9	1.95E-02
FCRL6	Fc receptor-like 6	8.8	3.38E-10
CP	ceruloplasmin (ferroxidase)	8.67	6.18E-36
STEAP4	STEAP4 metalloreductase	8.55	5.61E-33
OCA2	oculocutaneous albinism II	8.54	3.22E-12
CYP4B1	cytochrome P450 family 4 subfamily B member 1	8.54	3.29E-06
SLC14A2-AS1	SLC14A2 antisense RNA 1	8.49	9.78E-06
REM1	RAS (RAD and GEM)-like GTP-binding 1	8.42	3.01E-08
IGF1	insulin like growth factor 1	8.27	4.28E-03
ITLN1	intelectin 1	7.99	3.20E-04

Fig. 3. Biological functional enrichment analysis of Upregulated DEGs. **a** Top 10 enriched GO terms for Upregulated signature genes of hMSMSCs within OD and Ctr groups. **b** Top 10 enriched KEGG pathway for Upregulated signature genes of hMSMSCs within OD and Ctr groups. The size of dot shows the number of genes. Rich Factor is the ratio between genes enriched in this pathway and all the annotated genes in this pathway. The lower the q-value, the more significant the enrichment of genes. **c** Table listing shared the Top 20 Upregulated genes of hMSMSCs in OD versus Ctr group.

3.2. Global transcriptome analysis in hMSMSCs under osteogenic differentiation

To reveal transcriptional differences in hMSMSCs during osteogenic differentiation, RNA-seq was performed on D0 and D21 during osteogenic stimulation process. Based on the hierarchical cluster of transcripts, the total gene profiles were classified into different clusters, and all samples were arranged into two different groups (OD and Ctr groups). A subset of upregulated genes was clustered in the OD group and down-regulated genes were clustered in the Ctr group (Fig. 2a). Principal component analyses (PCA) demonstrated



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Top 20 down-regulated genes during osteogenic differentiation of MSMSCs

Gene	Annotation	Log_2FC	FDR
PKP1	plakophilin 1	-9.82	7.78E-15
PTPRN	protein tyrosine phosphatase%2C receptor type%2C N	-8.74	2.19E-17
DDN	dendrin	-7.05	2.31E-04
NPPB	natriuretic peptide B	-6.96	4.01E-02
ZP1	zona pellucida glycoprotein 1	-6.92	2.62E-04
IRF5	interferon regulatory factor 5	-6.71	4.95E-12
MUC12	mucin 12%2C cell surface associated	-6.68	7.06E-06
CGA	glycoprotein hormones%2C alpha polypeptide	-6.65	1.56E-05
CARD11	caspase recruitment domain family member 11	-6.63	1.51E-03
TIE1	tyrosine kinase with immunoglobulin-like and EGF-like domains 1	-6.55	7.50E-04
SERPINA9	serpin peptidase inhibitor%2C clade A 2C member 9	-6.41	1.48E-02
CPA5	carboxypeptidase A5	-6.4	1.56E-02
PPM1E	protein phosphatase%2C Mg2+/Mn2+ dependent 1E	-6.3	6.66E-03
TRBJ2-3	T cell receptor beta joining 2-3	-6.29	6.40E-07
KCNK5	potassium channel%2C two pore domain subfamily K%2C member	-6.26	1.96E-02
MMP1	matrix metallopeptidase 1	-6.17	9.00E-15
SLIT2-IT1	SLIT2 intronic transcript 1	-6.13	2.21E-02
LPAR3	lysophosphatidic acid receptor 3	-6.02	4.25E-03
SNRPEP4	small nuclear ribonucleoprotein polypeptide E pseudogene 4	-5.96	5.46E-03
IL17B	interleukin 17B	-5.95	5.31E-03

Fig. 4. Enrichment analysis of Downregulated DEGs. **a** Top 10 enriched GO terms for Down-regulated signature genes of hMSMSCs within OD and Ctr groups. **b** Top 10 enriched KEGG pathway for Downregulated signature genes of hMSMSCs within OD and Ctr groups. The size of dot shows the number of genes. Rich Factor is the ratio between genes enriched in this pathway and all the annotated genes in this pathway. The lower the q-value, the more significant the enrichment of genes. **C** Table listing shared the Top 20 Downregulated genes of hMSMSCs in OD versus Ctr group.

that the stimulation treatment increased the separation of osteogenic differentiation cell populations from baseline cells *in vivo* (Fig. 2b). The cell population shift also indirectly indicated that the specific direction of hMSMSCs changed after osteogenic stimulation.

3906 differentially expressed genes (DEGs) in hMSMSCs from the OD and Ctr groups were screened. To clarify differences in transcriptional profiles, the DEGs in each group were arranged in a volcanic pattern, with 2067 up-regulated and 1839 down-regulated DEGs. The DEGs were mainly distributed in regions with 1-to-2.5-fold change. Of note, the upregulated DEGs that were distributed in the region exceed 2.5-fold change were more abundant than downregulated DEGs (Fig. 2c). To further explore the role of expression profiles in hMSMSCs, we performed gene set enrichment analysis (GSEA) on the osteoblast differentiation and embryonic mesenchymal stem cell pluripotency signalling pathways. The OD differentiation signalling pathway was significantly enriched in the osteogenic group (Fig. 2d). In contrast, the degree of enrichment of the pluripotency signalling pathway in embryonic mesenchymal stem cells in the Ctr group was significantly higher than that in the OD group (Fig. 2e). The multidimensional distribution of DEGs indicated that the transcriptional profile of hMSMSCs changed during osteogenic differentiation, which affected the conversion of the expression levels of associated genes.

3.3. Biological functional enrichment analysis of up-regulated DEGs in hMSMSCs

We sought to clarify the effects of upregulated DEGs on the regulation of hMSMSCs under osteogenic conditions. The top 10 enriched Gene Ontology (GO) terms of biological processes were selected to facilitate analysis based on the upregulated DEGs. Upregulated DEGs were mainly associated with extracellular structural organization and ossification (Fig. 3a). Furthermore, KEGG enrichment analysis showed that these upregulated DEGs were involved in signaling pathways associated with osteogenic differentiation, including the PI3K-Akt, Rap1 and calcium signalling pathways, EGFR tyrosine kinase inhibitor resistance, and Vitamin Wet and absorption (Fig. 3b). Compared with the OD and Ctr groups, GO and KEGG enrichment analysis were consistent with the cell phenotypic results.

We subsequently analyzed the Top20 significant upregulated DEGs among OD- and Ctr-derived hMSMSCs based on their changes during osteogenic stimulation. The 20 most differentiated genes were annotated (Fig. 3c). These included five osteogenic differentiation-related genes: SPARC-related modular calcium binding 2(SMOC2), osteomodulin (OMD), RAS (RAD and GEM)-like GTP-binding 1 (REM1), insulin-like growth factor 1 (IGF1), and intelectin 1 (ITLN1). Of these, SMOC2 (12.90-fold change), REM1 (8.42-fold change), and ITLN1 (7.99-fold change) are involved in calcium formation. These genes ranked 2nd, 18th, and 20th, respectively. Additionally, OMD (10.15-fold change) and IGF1 (8.27-fold change) were associated with osteogenesis. These genes were ranked 6th and 19th, respectively. Therefore, both biological process and top 20 gene ranking analyses demonstrated that alterations in upregulated DEGs were distinct functional characteristics of hMSMSCs during osteogenic stimulation.

3.4. Biological functional enrichment analysis of downregulated DEGs in hMSMSCs

Subsequently, we focused on the enrichment and regulatory role of down-regulated DEGs during the osteogenic induction of MSMSCs. GO enrichment analysis revealed that many genes were enriched in the biological processes of cell cycle, division, and adhesion. Moreover, some of the genes were enriched in the biological processes related to cell morphological differentiation (Fig. 4a). These enrichment analyses also indicated that hMSMSCs maintained cell division and homeostasis process under the growth conditions. Similarly, KEGG pathway enrichment analysis (Fig. 4b) showed that the downregulated DEGs were mainly enriched in the following signalling pathways: cell cycle (−8.21-fold change), calcium signaling pathway (−6.18-fold change), and HIF-1 signalling pathway (−6.11-fold change). Among them, many cell cycle-related genes were enriched in the Ctr group, indicating that hMSMSCs have a strong potential to maintain cell division and proliferation. Notably, the calcium signalling pathway was also enriched in the downregulated DEGs. These genes may have negative regulatory role in calcium salt deposition.

We further discovered the Top20 significant downregulated DEGs between OD- and Ctr-derived hMSMSCs during osteogenic stimulation. The 20 most differentially downregulated genes were labelled (Fig. 4c). These included four cell cycle and growth-related genes: protein tyrosine phosphatase2C receptor type2C N(PTPRN), interferon regulatory factor 5(IRF5), tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (TIE1), and matrix metallopeptidase 1 (MMP1). PTPRN (−8.74-fold change), IRF5 (−6.71-fold change), TIE1 (−6.55-fold change) and MMP1(−6.17-fold change) ranked 2nd,6th, 10th, and 16th, respectively. These genes may have negative regulatory roles in calcium salt deposition. Combined with the upregulated, downregulated DEGs enrichment and ranking analysis indicated that hMSMSCs may switch from maintaining the cell cycle and division to osteogenic differentiation after osteogenic induction.

3.5. Genetic analysis of osteogenic differentiation in hMSMSCs

Finally, we selected biological processes related to osteogenic differentiation and their related genes for analysis. Two major functional categories were evaluated: ossification and the calcium signalling pathway (Fig. 5a). Genes associated with ossification were all upregulated, whereas those related to the calcium signalling pathway were partially downregulated, indicating the complexity and variety of the regulation of calcium formation. Cluster heatmap analysis also showed that osteogenesis and calcium signalling pathway-related genes (SMOC2, OMD, IGF1, JUNB, BMP5, ADRA1A, IGF2, ADRA1B, COL10A1, and BMP6) were enriched in the OD group compared to the Ctr group (Fig. 5b). qRT-PCR demonstrated that the levels of expression osteogenesis-related (SMOC2, OMD, IGF1, JUNB, BMP5, and IGF2) and calcium signalling pathway-related genes (ADRA1A) were higher in the OD group wthan the Ctr

group. In addition, levels of expression of ADRA1B, COL10A1, and BMP6 were not significantly different between the OD and Ctr groups (Fig. 5c). Taken together, these results demonstrated that osteogenesis and calcium signalling pathway-related genes (SMOC2, OMD, IGF1, JUNB, BMP5, ADRA1A, and IGF2) are required to sustain the osteogenic differentiation activity of hMSSMCs.

4. Discussion

New bone formation in the maxillary sinus floor region is key to ensuring initial stability after dental implantation, and is also the basis of the success rate of dental implantation in this operation [8]. One of the factors that determine the new bone formation in this region is the osteogenic differentiation potential of human maxillary sinus mesenchymal stem cells [10,11]. Some clinical and animal studies have found that the mucosa close to the bone surface of the maxillary sinus floor, also known as the Schneiderian sinus membrane, is a special layer with specific osteogenic potential [12,13]. However, the genetic changes underlying hMSMSCs during osteogenic differentiation remain unclear. In this study, we investigated gene expression profiles and analysed the enrichment of DEGs in hMSMSCs at the beginning and end of cell differentiation. Thus, our findings provide a novel insight into the regulatory mechanisms and differentiation potential of hMSMSCs during osteogenic differentiation. Anatomically, the maxillary sinus floor area is a complex structure, and the formation of new bone can be induced by mesenchymal stem cells derived from the maxillary sinus mucosa and osteogenic differentiation with mineralization [14]. Emerging evidence indicates that human maxillary sinus stem cells may have multipotency differentiation features, which may contribute to osteogenic differentiation [7,15]. Based on cell culture and differentiation model *in vitro*, we found that human maxillary sinus mucosal-derived cells appeared fibroblastic, and the cells showed an osteoblastic or osteoprogenitor appearance after osteogenic induction. Alizarin red and alkaline phosphatase staining showed abundant calcium salt deposition under hMSMSC-inductive conditions. Furthermore, the RNA and protein levels of ALP, Runx2, and Osx were increased in the culture system. These *in vitro* findings are almost concordant with previous study [16]. Therefore, these results revealed that hMSMSCs possess osteogenic potential and could be oriented toward osteogenic differentiation by an osteogenic stimulus

a

Functional categories	-Log(p-value)	Genes with fold changes
Ossification	9.88	OMD (10.15), IGF1 (8.27), JUNB (7.20), WNT11(4.58), IGF2(4.51), COL10A1(4.35), BMP6(4.17), ITGB6(3.05), BCL2(1.46)
Calcium signaling Pathway	6.18	SMOC2(12.90), ADRA1A (4.73), ADRA1B (4.36), CACNA1G(4.35), RYR1(3.67), PTGER3(3.60), PTGFR (2.77), GNAL (2.14), CD38(1.60), PTK2B (1.55), ITPKB (1.15), CGA(-6.65),LPAR3(-6.01), CAMK2A(-5.00), SLC8A3(-4.84), GRIN1(-4.5), PTGER1 (-3.52), GALR2(-2.68), ATGR1(-2.09), CALM3(-1.10)

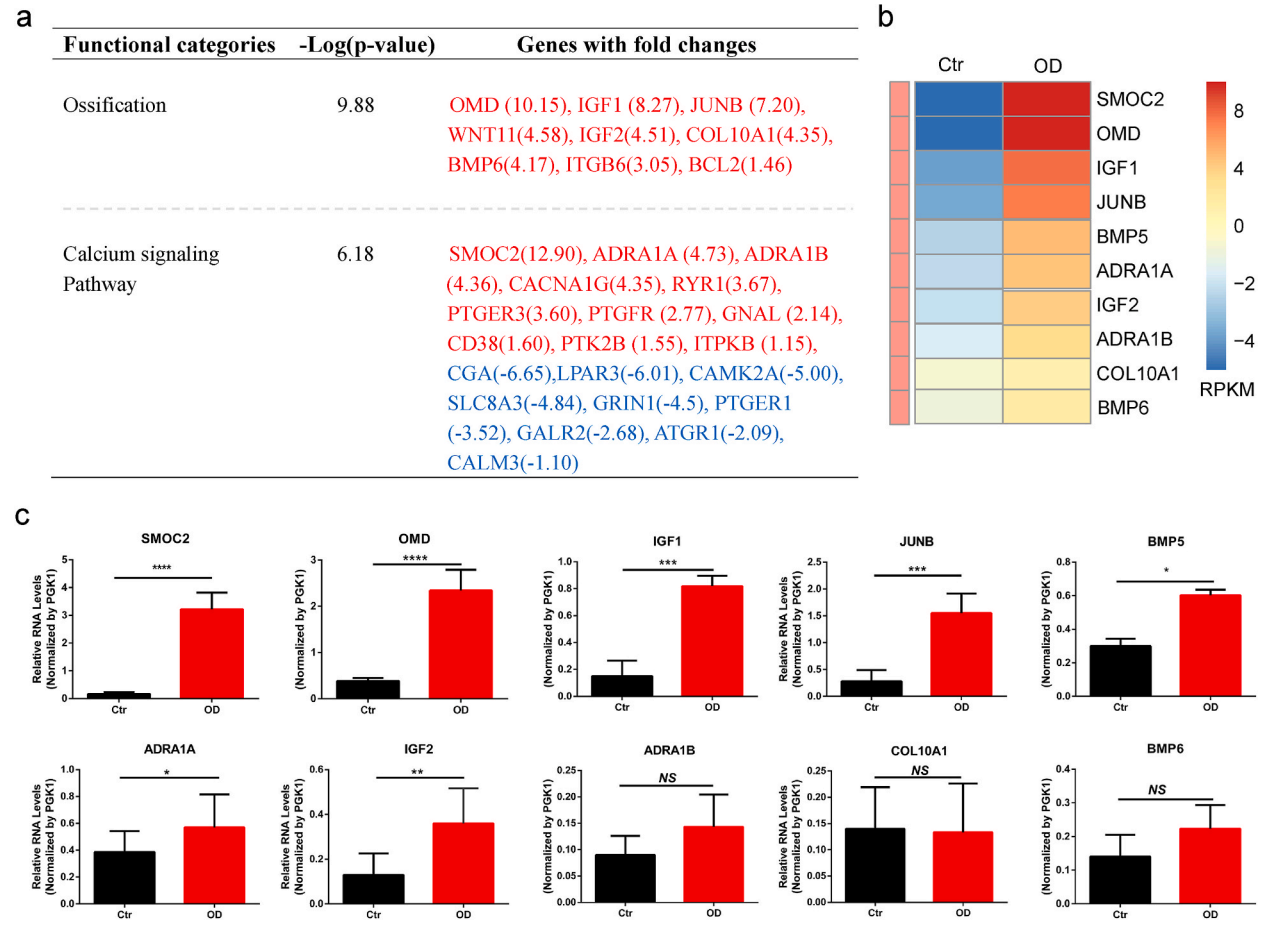

Fig. 5. Osteogenic related signature genes profile in hMSMSCs. **a** Interested GO term and pathway of osteogenic differentiation in hMSMSCs. **b** Clustered heatmap showing selected genes relevant to osteogenic differentiation function in hMSMSCs. **c** RNA expression level of selected genes determined in hMSMSCs between OD and Ctr Groups by RT-PCR analysis (n = 3). Data are presented as means ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

in vitro.

The intrinsic attributes of hMSMSCs are altered by specific inductive stimuli [17]. Hence, the study of intrinsic transcriptional profiles is the key to understanding the osteogenic differentiation process. Osteogenic differentiation is a complex process. Firstly, numerous cytokines, signalling pathways, and small molecules induce stem cells to differentiate into osteoblasts. Osteoblasts then mature and secrete the extracellular matrix, promoting mineralisation into the bone [18,19]. The gene expression profile plays an important role in the regulation of transcriptional mechanisms. Aforementioned researches demonstrated that BMP, FGF, and other genetic families could contribute to the osteogenic differentiation of hMSMSCs [20–22]. We found that the expression levels of OMD, IGF1, JUNB, BMP5, IGF2, COL10A1, an BMP6 were upregulated during ossification. This indicates that these osteogenesis-related signalling genes were activated and directed hMSMSCs differentiation toward osteogenesis. Meanwhile, the upregulated expression of SMOC2, ADRA1A, and ADRA1B, and the downregulated of CGA, LPAR3 in the calcium signalling pathway indicate that the mechanisms of calcium regulation mechanism are important components of osteogenic differentiation. In addition, we found that the PI3K-Akt signalling pathway was highly enriched in the osteogenic differentiation of hMSMSCs. The PI3K-Akt signaling pathway occurs mainly through the activation of PI3K, which then combines with Akt to form a complex, which positively activates the Wnt signalling pathway and promotes the expression of genes related to osteogenic differentiation [23,24]. This also indicates that the hMSMSC differentiation is not determined by a single signal or biological process. The internal regulatory network and mechanism of osteogenic differentiation of hMSMSCs are complex, and may depend on superior transcriptional control. Changes in the external environment can affect the diversity of cellular transcriptional profiles, and drive specific cellular differentiation processes.

Compared to other studies, we found that hMSMSCs were tissue-specific. Bayarsaihan and Mina et al. (2022) demonstrated that dental pulp-derived MSCs dominate eight distinct cell clusters with key genes involved in osteoblast differentiation (RUNX2, SP7, SATB2, HAND2, MEF2C, COLLA1 and ALPL) [25]. The striking heterogeneity among dental pulp-derived MSCs reflects various stages of osteogenic differentiation. Another comparative study [26]demonstrated that MSCs derived from tissues of the maxillofacial region, including the jaw bone and periodontal ligament, were HOX-negative and showed high expression levels of specific genes, including MSX1, NCAM1, LHX8, BARX1, FOXF1, S100A4, ZNF185, and NPTX1. The core regulatory gene differences suggest that MSCs retain their original memory.

The process of osteogenic differentiation encompasses multiple phases, such as the proliferation of the MSC population, matrix maturation, and mineralization. In our study, we observe that genes involved in mitotic cell cycle process and cell division, such as RHOB and BIRC5 are downregulated. These genes play a crucial role in driving the active proliferation of MSCs during the early stages of osteogenic differentiation. Similarly, during the matrix maturation phase, genes associated with extracellular structure organization and tissue morphogenesis, such as SMOC2 and ACTC1, undergo significant changes in expression. These genes contribute to the organization and maturation of the bone matrix. In addition, genes involved in bone mineralization, such as ACVR2B and IGF1 are upregulated. These genes may contribute to the maintenance of mineralization state by modulating the availability and distribution of essential minerals required for mineral deposition and maturation. Although the study only encompasses two time points, it enables a partial assessment of gene expression changes during the osteogenic differentiation phases. Further exploration with additional time points would have allowed for a more comprehensive understanding of the gene expression dynamics throughout the entire osteogenic differentiation process.

Of note, because of the limitations of the study design, most results are descriptive or based on bioinformatic analyses, and we did not perform a comprehensive investigation to reveal core regulatory mechanisms. Hence, more functional and sequencing experiments, for instance, single cell RNA-Seq, are required in future studies to address this issue.

5. Conclusions

Based on RNA-Seq, DEGs related to the osteogenic differentiation of hMSMSCs were identified. Biological functional analyses have revealed that extracellular structural organisation, ossification, and calcium signalling pathways plays important roles in t osteogenic differentiation. Furthermore, seven related genes (SMOC2, OMD, IGF1, JUNB, BMP5, ADRA1A, and IGF2) were found that may be involved in the regulation of osteogenic differentiation. These differentially expressed genes provide insights into the screening of osteoblast-related small molecules. This has important application prospects in bone augmentation for dental implantation. Nonetheless, our findings provide an expression profile, and enhance the current understanding of the mechanisms of osteogenic differentiation, and identify a potential target for modified osteogenesis in the maxillary sinus region.

Author contribution statement

Yutao Zhou: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Rui Jiang and Jindi Zeng: Performed the experiments; Analyzed and interpreted the data.

Yu Chen and Jing Ren: Contributed reagents, materials, analysis tools or data.

Songling Chen: Conceived and designed the experiments; Ermin Nie: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e20305.

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