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Genome-wide gene expression profiles of dental follicle stem cells

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Abstract

Objective. Dental stem cells (SCs) will be increasingly used for bone regeneration in the future. Recently, dental follicle cells (DFCs) from retained human third molars have been isolated and characterized as osteogenic progenitors. Although these results are promising for regenerative dentistry, molecular processes during osteogenic differentiation are not yet well understood. ***Materials and methods.*** This study compared DFCs before and during osteogenic differentiation. ALP activity was measured and cells were stained with alizarin red. Real-time RT-PCRs for osteogenic markers were done. The genome-wide expression profile was evaluated using a microarray. ***Results.*** DFCs showed strong mineralization and increased expression of osteogenic marker genes during osteogenic differentiation. A microarray analysis showed regulated genes before and in the process of osteogenic differentiation (day 7). Several regulated genes in DFCs were associated with skeletal development. Bioinformatic analysis revealed a number of factors associated with dental follicle osteogenic differentiation. Osteogenic differentiation affected expression levels of the transcriptional regulators FOXC2 and ZNF219. ***Conclusion.*** In conclusion, the results yielded new objectives for further studies on transcription factors like FOXC2 or ETV1 and their role in dental SCs during osteogenic differentiation.

Key Words: *Dental SCs, osteogenic differentiation, dental neural crest-derived progenitor cells, microarray analysis*

Introduction

Stem cell (SC)-based methods increasingly represent a real alternative to autogenous, allogenous and synthetic bone transplants and substitutes in bone regeneration. As a viable source for adult osteogenic SCs, orofacial tissues from the oral cavity, teeth or the jaw bone have increasingly become a focus of research [1,2]. Dental SCs isolated from the tooth pulp as well as from periodontal tissues were differentiated into cell types, such as odontoblasts, periodontal fibroblasts, osteoblasts, adipocytes, myoblasts, chondrocytes or neurons [3]. The excellent osteogenic differentiation potential of SCs can be partly explained by their developmental origin. Similar to any other skeletal element of the skull and most tooth structures, dental SCs are neural crest (NC)-derived and express several neuroectodermal and other developmental markers [4].

SC populations can be derived from the dental follicle ('dental sac') located in the developmental matrix of the tooth-supporting apparatus [5]. Precursor cells of the dental follicle (DFCs) isolated from the coronal part of the follicle express SC markers, such as STRO-1, nestin and notch-1 [6,7]. These highly proliferative cells can be differentiated into osteoblasts both *in vitro* and *in vivo*; however, these cells differ from SCs with regard to their expression of osteogenic markers [6].

So far, only a few studies have investigated the gene profiles of dental SCs after osteogenic differentiation [8,9]. In this study, we investigated genome-wide gene expression profiles of DFCs before and during osteogenic differentiation under *in vitro* conditions. In contrast to previous studies, we used the same standardized, commercially available differentiation medium that we also used in a similar study with SCs derived from dental pulp. We compared the

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results of this study with those of our previous study and focused on identifying the transcription factors involved in the molecular mechanisms of osteogenesis in craniofacial-derived SCs. Additionally, we compared the gene expression profiles of DFCs with differentiated dental SCs derived from the dental pulp of human exfoliated deciduous teeth and from the apical papilla. The purpose of this study is to find similarities and differences in the gene expression changes in the process of differentiation of dental SCs originating from dental pulp and dental follicle as well as differences in the gene expression changes after using different media to stimulate osteogenic differentiation in DFCs.

Materials and methods

Cell culture

DFCs were previously isolated from retained third molars of juvenile patients after informed consent. Methods for cell isolation were described by Morsczeck et al. [6]. Briefly, the follicle tissue was separated from the extracted tooth and minced using a scalpel. Subsequently, the tissue was digested in a collagenase/hyaluronidase solution (both Sigma, St. Louis, MO, United States) at 37°C and 5% CO_2 for 40 min. After that, the solution is filtered through a 70 μm cell strainer and centrifuged at 500 rpm for 5 min. Supernatant was discarded and the cell pellet was re-suspended and seeded in cell culture flasks. All cells were cultivated in standard medium comprising of DMEM supplement with 10% fetal calf serum and a Pen/Strep solution (PAA, Pasching, Austria) in 5% CO_2 at 37°C. Medium was changed every 2 or 3 days. When reaching sub-confluency cells were washed with phosphate buffered saline (PBS) and detached using a 0.25% trypsin solution (Sigma), centrifuged at 500 rpm for 5 min, re-suspended in cell culture medium and seeded into a new flask at a density of 5000 cells per cm^2.

Osteogenic differentiation

For osteogenic differentiation, cells at passage 6 were cultivated in standard medium until sub-confluence. At that stage, the medium was changed and cells were cultivated in osteogenic differentiation medium (ODM, StemPro® Osteogenesis Differentiation Kit, Life Technologies, Carlsbad, CA, USA) for various periods of time. Cells cultivated in standard medium served as control. Medium was changed every 2–3 days.

For the quantitative evaluation of alkaline phosphatase (ALP) activity, we used the phosphatase assay kit (Jena Biosciences, Jena, Germany) after 7 days of differentiation. Briefly, cells were lysed using a 0.1% triton-x solution. Cell lysate was split and one half was mixed with an alkaline buffer and an ALP-substrate. The colorless p-nitrophenyl phosphate (pNPP) is dephosphorylated to p-nitrophenole by ALP. Subsequently, under alkaline conditions p-nitrophenole is deprotonated to p-nitrophenolate which has a strong absorption at 405 nm. Absorbance was measured using a plate reader (Tecan, Männedorf, Switzerland). The alkaline phosphatase activity of the sample was normalized to the DNA content measured with the Quant-iT™ PicoGreen® dsDNA Assay Kit.

After 28 days of cell differentiation, we stained the samples with alizarin red to analyze biomineralization. A 2% Alizarin Red S in PBS solution was prepared and the pH-value was adjusted to 4.3 using ammonium hydroxide. Cells were fixed with 70% ethanol and stained with the alizarin red solution for 20 min. Subsequently, cells were washed thoroughly.

Real-time reverse transcription polymerase chain reaction (RT)-PCR

DFCs were cultivated in osteogenic differentiation medium and standard medium (days 0, 3, 7 and 21). For RNA isolation, cells were processed according to the manual of the RNA isolation kit RNeasy Mini Kit (Qiagen, Hilden, Germany). We used the QuantiTect Reverse Transcription Kit (Qiagen) for reverse transcription of total RNA into cDNA and conducted the reaction with 500 ng RNA as stated in the manual. The LightCycler® FastStart DNA MasterPLUS SYBR Green I was used for real-time RT-PCRs. Detailed information about primers is given in Table I. Samples were measured in triplicate and the gene expression of a housekeeper gene, GAPDH, was used for normalization. For relative quantification of gene expression, we applied the $\Delta\Delta C_t$ calculation method. The gene expression of DFCs on day 0 was used for calibration (relative gene expression = 1).

Microarray analysis

The total RNAs (1 μg/sample) were quality-controlled with the RNA 6000 Nano LabChip (Agilent

Table I. Sequences of primer pairs used in real-time RT-PCR.

Runx2 forward GTGCCTAGGCGCATTTCA
Runx2 reverse GCTCTTCTTACTGAGAGTGGAAGG
OCN forward CACTCCTCGCCCTATTGGC
OCN reverse CCCTCCTGCTTGGACACAAAG
ZBTB16 forward CACTCAAAGGGCTTCTCACC
ZBTB16 reverse CAAGAAGTTCAGCCTCAAGCA
Nestin forward TGCGGGCTACTGAAAAGTTC
Nestin reverse TGTAGGCCCTGTTTCTCCTG
Notch 1 forward GCACTGCGAGGTCAACAC
Notch 1 reverse AGGCACTTGGCACCATTC
NR4A3 forward GCAGCTGCAACAAAACACC
NR4A3 reverse CGTCCGCTCCTCCTACACT

Technologies, Santa Clara, CA). DNA microarray analyses were carried out with the Affymetrix Human Gene 1.1 ST array according to the Affymetrix standard protocol. We used the RNAs from undifferentiated DFCs and DFCs after 7 days of differentiation. The experiment was done in duplicate. Microarray hybridizations were done at the 'Center of Excellence for Fluorescent Bioanalytics' of the University of Regensburg (Germany). Data were analyzed with the NetAffx Analysis Center and the RMA algorithm [10]. A fold-change of more than 2 with a p-value less than 0.05 ($p < 0.05$) was considered significant. The database for Annotation, Visualization and Integrated Discovery (DAVID; http://niaid.abcc.ncifcrf.gov/) was used for annotations of significant regulated transcripts in differentiating DFCs [11–13].

Statistics

All statistics have been done using student's t-test (*p-value < 0.05; **p-value < 0.01; ***p-value < 0.001).

Results and discussion

For estimating the osteogenic differentiation potential, we tested ALP activity, calcium accumulation by alizarin red staining and expression of osteoblast markers. DFCs showed specific ALP activity after 7 days of differentiation (Figure 1A). ALP activity was more than twice as high as that in DMEM. DFCs formed clusters of mineralization after 28 days (Figure 1B). Moreover, the gene expression of the osteogenic markers OCN, RUNX2, ZBTB16 and NR4A3 was up-regulated during cell differentiation, but the expression of typical markers for undifferentiated DFCs, such as NESTIN and NOTCH1, was down-regulated (Figure 2).

Genome-wide gene expression profiles of DFCs were investigated in undifferentiated cells and after 7 days of differentiation. In total, 387 genes were up-regulated (Supplementary Table S1) and 139 genes were down-regulated (Supplementary Table S2) significantly (fold change > 2, p-value < 0.05) after 7 days of differentiation. Differentially expressed genes were analyzed by means of DAVID functional annotation clustering. This analysis tool shows the biological processes in which differentially expressed genes are over-represented. Genes up-regulated after osteogenic differentiation were over-represented in 203 biological processes (Supplementary Table S3) and genes down-regulated after osteogenic differentiation were over-represented in 219 biological processes (Supplementary Table S4). Not surprisingly, many processes in which up-regulated genes are over-represented are related to osteogenic differentiation (e.g. skeletal system development, regulation of ossification, bone development, calcium ion transport, amongst others). Regulated genes arranged into these processes are shown in Table II. Other noticeable groups comprise the negative regulation of cell death, angiogenesis, immune response and differentiation in general. This result shows that osteogenic processes are somehow involved in immune defense.

A close relation between immune response and cellular differentiation has been previously shown for DFCs [8]. Noticeable groups found for down-regulated genes are the positive regulation of cell proliferation, cell migration and neurogenesis. During differentiation, the capacity for proliferation and migration is impaired; thus, many down-regulated genes belong to these processes. Genes responsible for neural differentiation are also down-regulated after the induction of osteogenic differentiation. Interestingly, up-regulated genes are over-represented in the process of cartilage condensation, although osteogenic cells from the craniofacial region undergo intra-membranous ossification. In contrast to the endochondral ossification of long bone mesenchymal stem cells (MSCs), intra-membranous ossification occurs without cartilage formation. However, the three genes belonging to cartilage condensation (bone morphogenetic protein 1 (BMP1), receptor tyrosine kinase-like orphan receptor 2 (ROR2) and collagen, type XI, alpha 1 (COL11A1)) are also part of the process of skeletal system development.

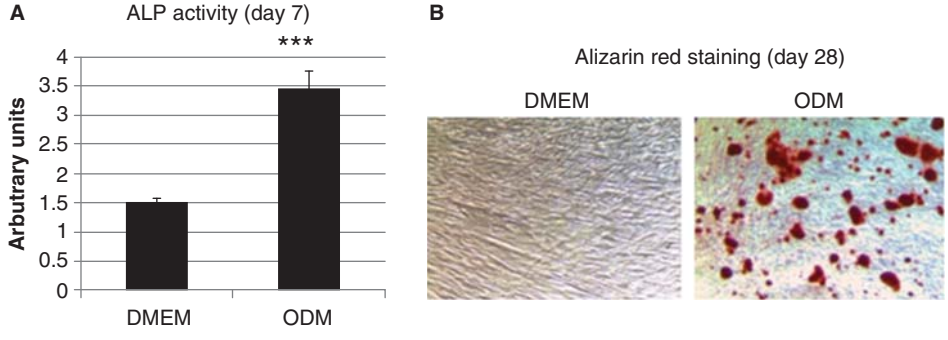

Figure 1. Osteogenic differentiation of DFCs. (A) Induction of ALP activity on day 7 of the differentiation (values are the means of three biological replicates plus SD). (B) Alizarin red staining after 28 days of differentiation (*** p-value < 0.001 differentiated cells compared to undifferentiated cells).

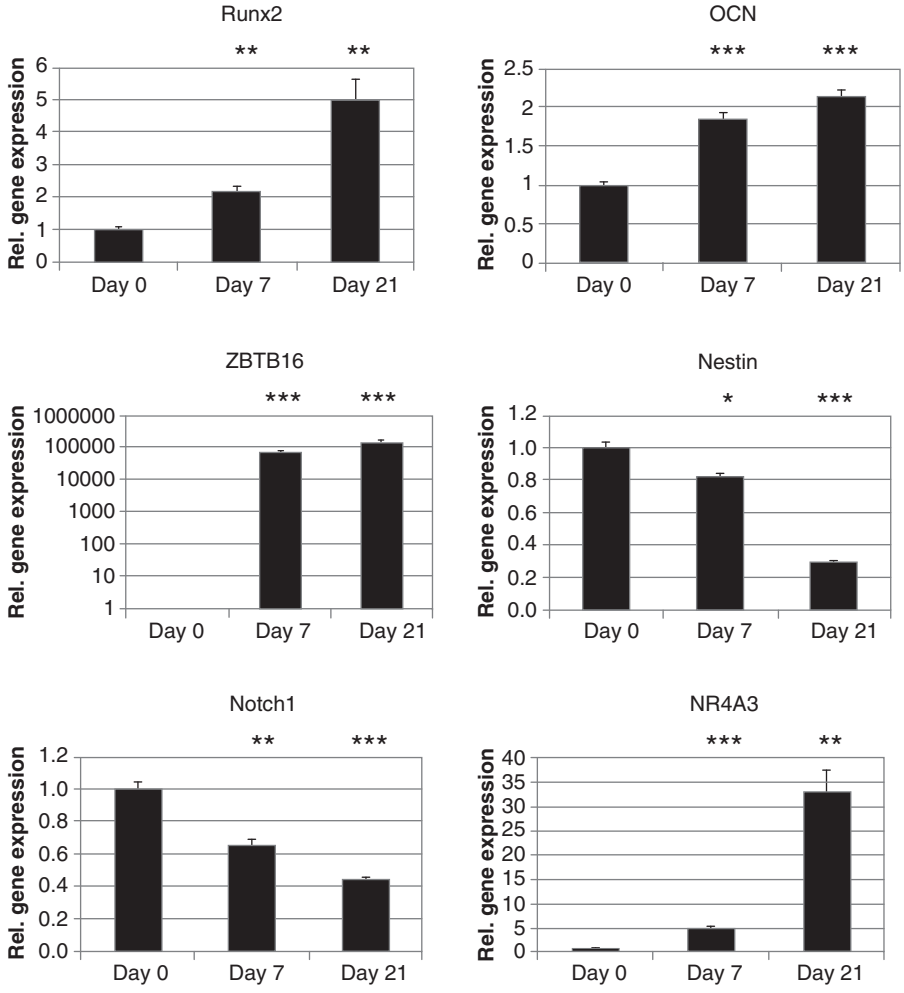

Figure 2. Gene expression (real-time RT-PCR analysis) of osteogenic differentiation markers and markers of undifferentiated DFCs after 7 and 21 days of osteogenic differentiation, respectively (values are the means of three biological replicates plus SD). Gene expression was calibrated on undifferentiated DFCs (day 0) (**p*-value < 0.05; ***p*-value < 0.01; ****p*-value < 0.001 differentiated cells compared to day 0).

Commonly up-regulated genes responsible for osteogenic processes affect, among others, ZBTB16 and frizzled-related proteins (FRZB). ZBTB16 promotes the osteoblastic differentiation of human MSCs as an upstream regulator of RUNX2, regulating limb and axial skeletal patterning [14]. Frizzled-related proteins are involved in the regulation of Wnt and BMP signaling [15].

Other dental SCs had similar gene expression profiles after osteogenic differentiation, indicating their common neuroectodermal origin [16]. Gene expression profiles of dNC-PCs and SHED were closely related [17] and more similar to each other than to DFCs. Figure 3 shows the correlation coefficient of the fold-change during differentiation (microarray data in the process of the differentiation) of the genome-wide gene expression of two dental stem cell types at any one time. The odontoblastic differentiation potency was achieved by detecting the expression of DSPP and DMP1 in SHED and dNC-PC cells, providing evidence for their excellent odontogenic potential [17]. In contrast, DFCs did not express any odontogenic marker genes under the same condition (reference 17 and data not shown). Regarding DSPP, this is not surprising because DSPP expression is restricted to dentin forming odontoblasts. DMP1, on the other hand, has been shown to be expressed in osteoblasts too [18–20]. Moreover, the expression of DMP1 has been shown for DFCs obtained from filial rats [21]. Another study, however, showed that DMP1 is expressed in neonatal murine follicles but not after differentiation [22]. In contrast to BM-MSCs (bone marrow mesenchymal stem cells), the expression of DMP1 in human DFCs is not evaluated well. Two observations might explain the lack of DMP1 expression in DFCs. First, several studies have shown that the transcription factor RUNX2 and the BMP pathway are important for DMP1-expression [23–25]. However, recent findings indicate that this pathway might be of minor importance for the differentiation of DFCs if stimulated with dexamethasone [1,26], resulting in a lower DMP1-expression. Second, it has been suggested that the members of the SIBLING (small integrin-binding ligand N-linked

Table II. Significantly up-regulated genes in DFCs after 7 days of osteogenic differentiation that are clustered into the biological processes that are related to osteogenic differentiation.

Gene symbol	Gene name	Fold change	*p*-value
ALPL	alkaline phosphatase, liver/bone/kidney	7.1349	0.0110
AXIN2	axin 2	2.4691	0.0046
BMP1	bone morphogenetic protein 1	2.4484	0.0064
BMP2	bone morphogenetic protein 2	2.0374	0.0190
COMP	cartilage oligomeric matrix protein	18.4299	0.0010
CMKLR1	chemokine-like receptor 1	6.1774	0.0004
CHRDL1	chordin-like 1	3.7094	0.0032
COL3A1	collagen, type III, alpha 1	3.8788	0.0019
COL11A1	collagen, type XI, alpha 1	3.7949	0.0056
FOXC2	forkhead box C2	4.0726	0.0083
FRZB	frizzled-related protein	10.9630	0.0012
IGFBP5	insulin-like growth factor binding protein 5	8.1281	0.0040
PBX1	pre-B-cell leukemia homeobox 1	2.0028	0.0040
PRELP	proline/arginine-rich end leucine-rich repeat protein	7.6290	0.0135
ROR2	receptor tyrosine kinase-like orphan receptor 2	3.7207	0.0045
STC1	stanniocalcin 1	4.7426	0.0005
TGFB3	transforming growth factor, beta 3	2.0662	0.0148
TGFBR2	transforming growth factor, beta receptor II	2.0841	0.0072
ZBTB16	zinc finger and BTB domain containing 16	11.4795	0.0083

glycoprotein)-family (osteopontin, bone sialoprotein, DMP1, DSPP, matrix extracellular phosphoglycoprotein) have overlapping functions [27,28]. Taken together, this might be an explanation for the observed mineralization in the absence of DMP1. Whether DMP1 is expressed in human DFCs after stimulation of the BMP pathway has to be evaluated.

However, with the culture conditions used in this study DFCs expressed neither DSPP nor DMP1. For DSPP, this is in accordance with previous studies on DFCs [29] and might be due to epigenetic regulations that repress the expression of both DSPP and DMP1 in DFCs but not in cells from the dental pulp [30]. However, it has been suggested that

	DFC	SHED	dNC-PC
DFC		0.72	0.71
SHED	0.72		0.78
dNC-PC	0.71	0.78	

Figure 3. Correlation coefficient of the genome-wide gene expression of DFCs, SHED and dNC-PCs. Correlation coefficient was calculated using the fold-change values assessed in a microarray after 7 days of osteogenic differentiation.

DFCs might be an heterogeneous cell population with cells belonging to the osteoblast/cementoblast lineage and cells belonging to a non-mineralizing progenitor lineage [29]. Anyway, these findings come from a study where clonally expanded immortalized lineages descending from single cells were used, whereas this study was performed with primary cells originating from the whole follicle tissue. However, genes related to cementoblastic differentiation were not found in differentiating DFCs. The lack of cementoblastic marker genes may be explained by the influence of Wnt signaling that inhibits cementoblast differentiation by regulating the expression of selective transcription factors [31]. Indeed, Wnt pathway-related genes were up-regulated in dNC-PCs (FRZB, TCF7) and DFCs (AXIN2, FRZB, TCF7L1). Therefore, cell culture conditions applied in this study obviously lacked the appropriate stimulus for cementoblastic differentiation. This is in accordance with a study that compared undifferentiated DFCs with alveolar bone osteoblasts, cementoblasts and periodontal ligament cells. Regarding the gene expression it was shown that DFCs are more similar to periodontal ligament cells and alveolar bone osteoblasts than to cementoblasts [32].

When investigating transcription factors, we found that FOXC2, ZNF219, NRF2F1 and ETV1 were up-regulated in cells of periodontal (DFCs) as well as of pulpal origin (dNC-PCs and SHED) [17]. FOXC2 is involved in a number of different developmental

processes including angiogenesis [33]. ZNF219 plays an important role in chondrocyte differentiation and is a transcription factor for Sox9 [34]. Nr2f1 was found to be involved in eye development, particularly in the early stages of optic vesicle development [35], in specifying neural SCs during central nervous system development into neurons of the temporal lobe [36] and in osteoblastogenesis of human MSCs [37]. Interestingly, genes responsible for urogenital and kidney development were also up-regulated. FOXC2, ROBO2 and SLIT2, for instance, are expressed in the metanephric mesenchyme inducing ureteric bud [38]. Their expression in the cell populations investigated in this study may indicate that these proteins may have a similar inducing role as the cells of the dental mesenchyme in the earlier stages of tooth development. Further studies should specify the detailed role of these factors in the osteogenic differentiation of dental SCs.

The ability of dental follicle cells to form hard tissue has been demonstrated, both in this study as well as in previous studies [8,39]. This ability correlates with genes that are over-represented and only regulated in DFCs associated with skeletal development. Osteogenic and chondrogenic genes that are more than 5-fold up-regulated are COMP, FRZB, ALP, PRELP, IGFBP-5, TGFβR2 or Axin2. Among them, the IGF-binding protein IGFBP-5 represents an important pro-osteogenic factor that promotes osteoblast differentiation and bone matrix production [40]. The detection of genes involved in chondrogenesis, such as COMP or PRELP [41], may be contradictory to the membranous osteogenetic fate of dental SCs. However, skeletal markers typical for chondral development can be found in cranial NC-derived cells [42]. The importance of the osteogenic differentiation medium is shown in comparison with these findings and the findings by Saugspier et al. [9] and Morsczeck et al. [8], who found that IGFBP-5, PRELP and COMP were also regulated in DFCs. This regulation indicates the robust role of these genes in osteogenic differentiation processes, even under different conditions. Interestingly, COMP was regulated after BMP2 stimulation for 7 days, but neither after dexamethasone (Dex) stimulation for 7 days [9] nor 28 days [8]. This might be explained by the differentiation kit (StemPro®) we have used in the present study. However, the exact formulation is not disclosed by the manufacturers.

However, many genes are regulated after both 7 and 28 days stimulation with Dex as well as after 7 days with StemPro®. Discussing all these genes would go beyond the scope of this study, but there are many genes regulated after all three treatments that are associated with differentiation. Among these are DPT, FRZB, GPM6B, IGFBP-2, IGFBP-5, LIF, NGF, SEMA3D, SHC3, TGFBI, TIMP4 and TRPA1. These genes might have important roles during the whole differentiation process. Although it is not surprising that many genes are regulated at both time points evaluated and with both stimulation media it is interesting to compare these with other genes that show a different regulation pattern.

Some genes are regulated after both Dex and StemPro treatment after 7 days but not after 28 days of Dex treatment. Among these are the down-regulated CADM1, KIT, MEST, STAC, TNC, TRIB3, VLDLR and the up-regulated COL7A1, CYSLTR1, Dkk1, ETV1, GPR77, PHKA1 and PLXNA2. Other genes are regulated after both 7 and 28 days of Dex stimulation but not after StemPro treatment. Among these are the down-regulated ITGA8, PHGDH and PSAT1 and the up-reguated FGF2 and GPRC5B. Taken together, this shows that the regulation of some genes is important for the onset of differentiation, but can be neglected in later time points and that other genes are regulated only if dexamethasone is the only inducer of differentiation.

However, the fact that many genes, regardless of the biological processes they are associated with, are regulated after all three treatments (APOD, BDKRB1, CD14, CORIN, DIO2, EFEMP1, ENTPD1, FAM107A, INMT, MAOA, PDE1C, PIP, PTGS1, RAB27B, SPON1, UGCG to mention a few) and the finding that only very few genes were regulated oppositely underlines the possible importance these genes have during the differentiation of DFCs.

In conclusion, this study revealed the genome wide gene expression changes after using a standardized, commercially available differentiation medium in DFCs during osteogenic differentiation for the first time. In our previous study, we evaluated the gene expression profiles of SHED and dNC-PCs [17] using the same medium. Gene expression profiles of these dental SCs were mainly related to developmental process responses including ossification, bone and skeletal system development, and to bacterial and lipopolysaccharide (LPS) response. By comparing the present study with our previous studies we could show differences and similarities between the gene expression changes during osteogenic differentiation regarding the time point, the induction medium and the origin of the dental SCs. This data might help to find interesting candidates to further evaluate differences and similarities in the differentiation process. However, transcription factors like FOXC2 or ETV1 might be interesting candidates for the evaluation of their role in osteogenic differentiation of all stem cells from ectomesenchymal origin.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online

Supplementary Table S1, S2, S3 and S4.