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MIKI TAKETOMI SAITO

ISOLAMENTO E CARACTERIZAÇÃO DE POPULAÇÕES HOMOGÊNEAS DE CÉLULAS MESENQUIMAIS INDIFERENCIADAS DO LIGAMENTO PERIODONTAL DE HUMANOS QUE APRESENTAM ALTO POTENCIAL OSTEOBLÁSTICO/CEMENTOBLÁSTICO

ISOLATION AND CHARACTERIZATION OF HIGHLY OSTEOBLAST/CEMENTOBLAST HOMOGENEOUS MESENCHYMAL STEM CELL POPULATIONS FROM HUMAN PERIODONTAL LIGAMENT

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Clínica Odontológica, na Área de Periodontia.

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Dental Clinics, in Periodontics area.

Orientadora: Profa. Dra. Karina Gonzales Silvério Ruiz

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PROFª. DRª. KARINA GONZALES SILVÉRIO RUIZ

PROF. DR. MÁRCIO MATEUS BELOTI

PROFª. DRª. ANA CLÁUDIA BRAGA AMORAS ALVES

PROF. DR. FRANCISCO HUMBERTO NOCITI JÚNIOR

PROFª. DRª. CRISTIANE RIBEIRO SALMON

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" Que eu continue com vontade de viver,
mesmo sabendo que a vida é, em muitos momentos, uma lição difícil de ser aprendida.
Que eu permaneça com vontade de ter grandes amigos,
mesmo sabendo que, com as voltas do mundo, eles vão indo embora de nossas vidas.
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que sinaliza o que de mais autêntico eu possuo.
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mesmo em meio à turbulência dos interesses.
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criada por alguém bem superior a todos nós!
E que as grandes mudanças não ocorrem por grandes feitos de alguns e, sim, nas pequenas parcelas cotidianas de todos nós!"

Chico Xavier

RESUMO

O periodonto é composto por populações heterogêneas de células, compreendendo células relacionadas a tecidos não mineralizados, ligamento periodontal e gengiva, e outros dois tecidos mineralizados, cemento e osso alveolar. Quando o periodonto é destruído na periodontite, a regeneração dos tecidos perdidos é bastante difícil de ser alcançada. Recentemente, abordagens regenerativas baseadas no conhecimento biológico da formação tecidual têm sido investigadas. Contudo, o entendimento do comprometimento das células do ligamento periodontal para formar os diferentes tecidos ainda não foi completamente elucidado. Para melhor compreender os mecanismos envolvidos na diferenciação das células periodontais, neste estudo, nós isolamos clones derivados de uma única célula e avaliamos o potencial de diferenciação em fenótipo osteoblástico/cementoblástico (O/C) (clones C-O) ou fenótipo fibroblástico (clones C-F) e analizamos o perfil transcricional dos clones celulares cultivados em meio padrão (DMEM - sem meio de indução O/C - e sob indução O/C (OM), usando tecnologia de sequenciamento de nova geração (RNA-seq). O objetivo deste estudo foi esclarecer o transcriptoma diferencial das células do periodonto com maior propensão da diferenciar em fenótipo O/C. Os clones C-O apresentaram maior número de genes diferencialmente expressos (DEG) com aumento da regulação do que os clones C-F, tanto quando cultivados em DMEM ou em OM. Além disso, os clones C-O mostraram maior enriquecimento dos processos biológicos que os clones C-F em ambas as condições de cultivo. A análise das vias revelou que as vias de sinalização da Caderina e Wnt estavam aumentadas nos clones C-O cultivados em DMEM, enquanto que as vias angiogênese e CCKR estavam aumentadas nos clones C-O quando cultivadas em OM. Nós experamos que o melhor entendimento da regulação molecular das células do periodonto possar ser potencialmente utilizada para promover melhores protocolos de terapias periodontais regenerativas no futuro.

Palavras-chave: calcificação; expressão gênica; ligamento periodontal; transcriptoma.

ABSTRACT

The periodontium is composed by heterogeneous cell populations, comprising cells related to non-mineralized tissues, the periodontal ligament and gingiva, and other two mineralized tissues, cementum and alveolar bone. When the periodontium is destructed in periodontitis, the regeneration of lost tissues is very difficult to be achieved. Recently, regenerative approaches based on biological knowledge of tissue formation have been investigated. However, the understanding of commitment of periodontal ligament cells to form different tissues still not been fully elucidated. In order to better comprehend the characteristics of periodontal-derived cells committed to distinct differentiation capacities, in this study, we isolated single-cell-derived clones and evaluated their potential to differentiation into osteo/cementoblastic (O/C) phenotype (C-O clones) or fibroblastic phenotype (C-F clones) *in vitro*, and analyzed the transcriptomic profile of the groups of clones in standard medium cultivation (DMEM - without O/C induction medium) and under O/C induction (OM), using next-generation sequencing technology (RNA-seq). The aim of this study was to clarify the differential transcriptome profile of periodontal cells more prone to differentiate into O/C phenotype. The C-O clones showed higher number of up-regulated differentially expressed genes (DEG) than C-F clones, when cultivated in DMEM or in OM. Also, C-O clones showed higher enrichment of biological processes than C-F clones in both cultivation conditions. Pathway analysis revealed that Cadherin and Wnt signaling pathways were up-regulated in C-O clones cultivated in DMEM, whereas angiogenesis, and CCKR signaling pathways were up-regulated in C-O clones cultivated in OM. We expect that better understating of molecular regulation of periodontium cells can be potentially used to promote better protocols for periodontal regenerative therapies in the future.

Keywords: calcification; gene expression profiling; periodontal ligament; transcriptome.

SUMÁRIO

1 INTRODUÇÃO

O periodonto corresponde ao aparato de proteção e inserção que circunda o dente e é composto por gengiva, cemento, ligamento periodontal e osso alveolar (Elangovan *et al.*, 2009). A função do periodonto é ancorar o dente ao osso alveolar, de forma a permitir a função mastigatória concomitantemente à manutenção da integridade dos tecidos ao redor do dente. A periodontite é uma doença inflamatória iniciada pelo biofilme bacteriano, a qual resulta em destruição da inserção conjuntiva e de osso alveolar, podendo levar à perda dentária (Elangovan *et al.*, 2009). O principal objetivo da terapia periodontal é controlar a inflamação induzida pelas bactérias periodontopatogênicas a fim de restabelecer a saúde periodontal, permitindo a preservação do dente e do sistema mastigatório como um todo. Uma vez que o aspecto infeccioso-inflamatório da doença esteja controlado, a correção de defeitos anatômicos e a regeneração dos tecidos periodontais perdidos também devem ser objetivos da terapia periodontal (Elangovan *et al.*, 2009; Ivanovski, 2009).

Desde o início dos anos de 1980, várias técnicas têm sido propostas com o objetivo de regenerar o periodonto, tais como regeneração tecidual guiada (RTG), utilização de enxertos e substitutos ósseos, condicionamento da superfície radicular, uso de proteínas derivadas do esmalte e fatores de crescimento (Bosshardt, 2008; Sculean *et al.*, 2008; Ivanovski, 2009). Contudo, como o periodonto é uma estrutura bastante complexa, não sendo composto apenas por um tipo tecidual, mas por tecidos mineralizados (osso alveolar e cemento) e não mineralizados (gengiva e ligamento periodontal), os quais estão intimamente relacionados, a regeneração do periodonto tem mostrado-se extremamente difícil de ser atingida. Estudos utilizando tais técnicas regenerativas têm demonstrado ampla variabilidade dos resultados e baixa previsibilidade de regeneração (Aichelmann-Reidy & Reynolds, 2008; Ivanovski, 2009). Além disso, as mesmas apresentam indicação clínica limitada, restringindo-se a defeitos periodontais infra-ósseos e lesões de furca Grau II de Hamp (Ivanovski, 2009; Avila-Ortiz *et al.*, 2015; Kao *et al.*, 2015).

Novas abordagens regenerativas tem sido propostas com embasamento em fundamentações do entendimento biológico da regeneração dos tecidos, com o objetivo de alcançar resultados clínicos melhores e mais previsíveis (Ivanovski *et al.*, 2014; Lin *et al.*, 2015). Contudo, o entendimento dos mecanismos regulatórios que coordenam a diferenciação das células que compõe o periodonto ainda não é completamente compreendido. Neste contexto, o presente estudo teve como objetivo isolar clones do ligamento periodontal e caracterizar o perfil transcricional dos clones com alto potencial para diferenciação osteoblástica/cementoblástica (O/C) em meio sem e com estímulo à diferenciação.

2 ARTIGOS

2.1. Artigo 1: Differential transcriptome profile of periodontal ligament cell clones

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Keywords (MeSH terms): cell differentiation; clone cells; gene expression profiling; high throughput RNA sequencing; molecular biology; tooth calcification.

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Abstract

Periodontal ligament (PDL) is constituted by heterogeneous cell populations and the mechanisms that regulate cell commitment to osteo/cementoblastic (O/C) or fibroblastic phenotypes in PDL are not fully understood. The better understanding of subpopulations of PDL cells that form different tissues is important to propose regenerative approaches based on a sound biological rationale. In order to better comprehend the characteristics of periodontal-derived cells committed to distinct differentiation capacities, we isolated single-cell-derived clones and evaluated their potential to differentiation into osteo/cementoblastic (O/C) phenotype (C-O clones) or fibroblastic phenotype (C-F clones) *in vitro*, and evaluated the transcriptomic profile of the groups of clones in standard medium cultivation (without O/C induction medium), using next-generation sequencing technology (RNA-seq). The aim of this study was to clarify the differential transcriptome profile of PDL cells more prone to differentiate into O/C phenotype. Our result revealed a total of 235 differentially expressed genes (DEG), where C-O clones showed higher number of up-regulated genes (193), and 42 down-regulated genes. Up-regulated genes were related to the Cadherin and Wnt signaling pathways and many biological processes, including "anatomical structure development" and "cell adhesion". Both transcriptome and qRT-PCR showed up-regulation of *WNT2, WNT16* and *WIF1* in C-O clones. This was the first study to make a comprehensive assessment of the transcriptome of human PDL progenitor cell clones with high O/C differentiation commitment, and revealed that expression of transcripts related biological process "anatomical structure development", Cadherin and Wnt signaling pathways, *WNT2, WNT16* and *WIF1*, are important to distinguish PDL cells with higher potential to commitment to O/C phenotype.

Introduction

Regenerative approaches based on biological rationale of periodontal tissues formation have been proposed to achieve more predictable clinical outcomes (Ivanovski et al. 2014). However, a profound comprehension of molecular mechanisms involved in periodontal-derived cell homeostasis, that is crucial for these emerging approaches, still not well elucidated (Fujii et al. 2008; Han et al. 2015; Yamada et al. 2016). Although we know that PDL is constituted by heterogeneous cell populations (Fujii et al. 2008; Saito et al. 2014), the molecular profile of cells committed to osteo/cementoblastic (O/C) or fibroblastic phenotypes in PDL are not fully understood (Fujii et al. 2008; Han et al. 2015).

Recently, emerging methods using high throughput deep sequencing technology, such as RNA-seq, has broadened our view of extent and complexity of transcriptome (Kebschull et al. 2017). The RNA-seq analysis allows to detect and quantify a large range of transcripts and their splice-forms without the need for a priori target specification, leading to an unbiased and systematic approach to get insights into biological pathways and molecular mechanisms important for cell regulation in a hypothesis-neutral environment (Ayturk et al. 2013; Kebschull et al. 2017; Twine et al. 2014). In this study, we purified PDL cell clones with potential to commitment to O/C phenotype (C-O clones) or potential to committment to fibroblastic phenotype (C-F clones), and employed RNA-seq to describe the differential transcriptional profile between them. The understanding of molecular regulation of different cells of periodontium can help to promote a microenvironment more favorable to formation of mineralizing and non-mineralizing tissues of periodontium and therefore lead to a more predictable outcome for future regenerative approaches.

Material and Methods

Cell culture, sorting, flow cytometric analysis, and cell cloning

The design and procedures of this study was approved by Institutional Review Board of Piracicaba Dental School – University of Campinas (#053/2013). Human completely erupted third molars were collected from healthy subjects (18-25 years old) after they had signed a written informed consent. PDL tissue isolation, culture of PDL-derived cells and flow cytometric analysis were performed as previously described (Silverio et al. 2010), using mouse anti-human monoclonal antibodies against CD105-allophycocianin (eBioscience, USA), CD146-allophycocianin (BioLegend, USA), CD166-phycoerythrin (BD, USA), CD34-fluorescein isothicyanate (BD, USA), CD45-peridinin chlorophyll (BD, USA), Stro-1 Alexa Fluor 647 (BioLegend, USA), or isotype-matched control IgGs /IgM for 40 min at 4°C. The

$CD105^+$ enriched cell subset was used for cell cloning by plating 500 cells in 100-mm culture dishes and obtaining single cell-derived colonies (clones) using colony rings as previously described (Saito et al. 2014).

In vitro biomineralization assay

To evaluate the ability to form mineralized matrix *in vitro,* unsorted PDL cell population and $CD105^+$ enriched population, and each cell clone were seeded ($2x10^5$cells/well) in 24-well plates with standard medium (Control), that was composed by Dulbecco's modified Eagle medium high glucose (DMEM) supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 mg/mL) (Gibco, USA) and incubated for 24 hours. After this period, cells were cultivated in fresh standard medium, or osteogenic medium (OM), composed by standard medium supplemented with 50 mg/mL ascorbic acid (Sigma-Aldrich, China) 10 mM b-glycerophosphate (Sigma-Aldrich, USA), and 10^{-8}M dexamethasone (Sigma-Aldrich, USA). At the end of the 28-day induction period, the Alizarin Red staining (AR-S) assay was performed as previously described (Zhao et al. 2015). The cell clones that were able to form mineralized matrix *in vitro* were classified as clones of oste/cementoblastic (O/C) phenotype (C-O), whereas the cell clones that were not able to form mineralized matrix *in vitro* were named as clones of fibroblastic phenotype (C-F).

Cell Metabolic Activity Assay

For the metabolic analysis, cell clones were seeded (5 x 10^3 cells/well) in a 96-well plate (Corning Costar, USA) using standard medium, and incubated in a humidified incubator at 37°C and 5% CO_2 for 24 h to allow cell adhesion to the discs. After this period, the medium was changed for DMEM supplemented with 2% FBS, penicillin (100 U/ml) and streptomycin (100 mg/mL) and this time point was considered the time 0h for the metabolic assay. The medium was changed on days 3 and 7, and the metabolic activity of the cell on the experimental groups were evaluated at days 1, 3, 7 and 10, as previously described (Marques et al. 2016).

RNA isolation, RNA-seq, and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Each cell clone was seeded and RNA extraction was performed as previously described (Saito et al. 2014). RNA isolated from two C-O clones and two C-F clones cultivated in standard medium during 14 days were submitted to RNA-seq, and each clone was considered a biological replicate for C-O and C-F group. RNA-seq was performed using Illumina TruSeq

RNA Sample Preparation kit v2 (Illumina, USA), according to the manufacturer's instruction. For qRT-PCR, single-stranded complementary DNA (cDNA) was synthesized from 1 µg total DNA-free RNA using Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, USA), following the manufacturer's recommendations. qRT-PCR was performed using the samples of cDNA and LightCycler 480 SYBR Green I master kit (Roche Applied Science, USA) on the LightCycler 480 II real-time PCR system (Roche Applied Science, USA) for primers sequences *WNT2, WNT2B, WNT16, WIF1, PCDHGA10, BMP4* and *GAPDH* (Appendix Table 1). Water (no template control) was used as negative control for all experiments. Relative quantification of reactions products was accomplished to *GAPDH* and calculated by the ΔCT-method.

Analysis of data

For RNA-seq data, quality of raw data was evaluated by *FastQC* (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Data was filtered by quality using *Perl* scripts, with quality cut over 20. The adapters were removed with Cutadapt (Martin 2011) and trim galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Filtered reads of RNA library were mapped against the human genome (GRCh38) using the pipeline *Tophat2-Cufflinks* (Trapnell et al. 2012). Number of reads aligned per genes and fragments per kilobase of transcript per million mapped reads (FPKM) were calculated by *RSEM* program (Li and Dewey 2011). Differentially expressed genes (DEG) between C-O and C-F clones were obtained by *DESeq* (Anders et al. 2013) and *EdgeR* packages (Robinson et al. 2010) (R/Bioconductor) with α=5% and $|\log2(\textit{fold change})| \geq 1$. The heatmap graphic was generated based on z-score values calculated from FPKMs (Fragments Per Kilobase Million) values of DEG using R package "pheatmap". DEG between C-O and C-F clones were submitted to functional annotation tool of DAVID program (Database for Annotation, Visualization and Integrated Discovery), version 6.8 (https://david.ncifcrf.gov/) (Sharan et al. 2003), in order to identify enriched terms of Gene Ontology (GO). GO analysis was generated by the DAVID software based on biological processes (GO_TERM_BP_2 database). Pathway overrepresentation analysis was performed in Panther Classification System (http://pantherdb.org/). We only considered the enriched GO terms and pathways generated by modified Fisher Exact test followed by the Bonferroni test and p value threshold of < 0.05.

For other experiments, the data were expressed as mean ± standard deviation (SD). t-test was used to analyze differences between two groups, and analysis of variance (ANOVA)

one-way or two-way were used to analyze difference among three or more groups. p values of <0.05 were considered statistically significant.

Results

The PDL CD105$^+$-enriched population exhibited high proportion of cells that expressed mesenchymal stem cell (MSC)-related markers (Appendix Figure 1A). Since we aimed to isolated clones with distinct O/C differentiation potentials from PDL CD105$^+$-enriched population, the biomineralization potential of this population was evaluated and compared to the total pool of cells obtained from PDL from third molars. The PDL CD105$^+$-enriched population displayed capacity of biomineralization similar to the unsorted population (PDL) (Appendix Figure 1B and 1C).

PDL clones present distinct hability to form mineralized matrix in vitro

A total of 46 cell clones were obtained from PDL CD105$^+$ mesenchymal progenitor population. According to the AR-S assay, two of the 46 clones demonstrated significant higher potential to form mineralized matrix *in vitro* under OM induction, namely G13 and G48, and were denominated as clones of O/C phenotype (C-O) (Figure 1A). The remaining 44 clones showed lower ability to form mineralized matrix *in vitro,* and were classified as clones of fibroblastic phenotype (C-F). Among the C-F group, two clones that rapidly expanded during the isolation and expansion periods were selected to represent the C-F counterpart, namely clones G16 and G23 (Figure 1A). The C-F and C-O groups were evaluated for their metabolic activity and did not show significant difference in each time point evaluated. Both clone groups showed increased metabolic activity at day 3 (Figure 1B).

C-O and C-F clones present different transcriptome profile

By the *FastQC* analysis, around 84% of the reads were at optimum quality and aligned to human genome (Appendix Table 2). Only the aligned portions to the genome were retained for subsequent analysis. As the heatmap shows, C-O clones presented a higher transcriptional activity (Figure 2). From a total of 235 DEG, from which 193 genes were significantly up-regulated in C-O group when compared to C-F, and 42 genes were significantly down-regulated in C-O group (Figure 2). The detailed lists of up- and downregulated genes are described in Appendix Table 3 and 4, respectively.

Functional classification shows distinct profile of DEG in C-O and C-F clones

To investigate the differences between C-O and C-F clones, DEG were analyzed for pathway overrepresentation. C-O clones showed upregulated genes related to 55 pathways (Appendix Table 5), from which Cadherin signaling pathway (P00012) and Wnt signaling pathway (P00057) were significantly enriched according to Panther Classification System (Table 1). Downregulated genes in C-O clones were related to 10 pathways, but no significantly overrepresented pathway was observed compared to *Homo sapiens* genome background (Appendix Table 6).

To understand the biological context of DEG, Gene Ontology (GO) analysis was used to map the biological process enrichment related to DEG. Genes up-regulated in C-O clones showed significant enrichment in 20 biological processes compared to *Homo sapiens* genome background (Figure 3A). Downregulated genes in C-O clones were related to significant enrichment of 9 biological processes (Figure 3B). Among the significant enriched biological processes in C-O clones compared to C-F clones, the biological process "anatomical structure development" (GO: 0048856) harbored genes related to Wnt pathway: *WNT2, WNT2B, WNT16,* and *WIF1* (Figure 3C), and the biological process "cell adhesion" (GO:0007155) presented the gene *PCDHGA10*, related to Cadherin pathway, and *BMP4* related to TGFβ/BMP pathway (Figure 3D).

WNT2, WNT16, and *WIF1 were validated to be upregulated by qRT-PCR*

The significantly upregulated genes *WNT2, WNT2B, WNT16, WIF1, PCDHGA10,* and *BMP4* observed in RNA-seq were selected to be validated by qRT-PCR because they were related to signaling mechanisms previously reported in the literature as related to osteoblastic differentiation, and were related to significantly overrepresented pathways Wnt and Cadherin, and significantly enriched biological processes anatomical structure development or cell adhesion. We validated RNA-seq results that C-O clones presented significantly higher expression of *WNT2, WNT16,* and *WIF1* (Figures 4A-4C*),* but, although the trend of higher expression in C-O clones could be observed, the genes *WNT2B, PCDHGA10,* and *BMP4* did not showed statistical significant difference (Figures 4D-4F).

Discussion

Although it has been reported that PDL contains heterogeneous cell subpopulations (Fujii et al. 2008), the molecular characteristics that distinguish these subpopulations still not well elucidated. In our previous study, we had characterized primary PDL cell clones from a PDL $CD105^{+}$ progenitor population and observed that this enriched population remains

heterogeneous. Further, this cell population was composed by PDL cell clones committed to O/C phenotype (C-O) or fibroblastic phenotype (C-F) clones (Saito et al., 2014). The isolation of cell clones allowed the analysis of highly purified cell populations, circumventing the heterogeneity of cell types that constitute the PDL (Barkana et al. 2007; Liu et al. 1997; Saito et al. 2014; Tomokiyo et al. 2008). Although CD105 has been considered as a maker of multi-lineage potential for bone MSCs (Rallapalli et al. 2009), our present study confirmed previous report (Saito et al. 2014) that CD105-enriched cell population from human PDL present subtypes with distinct O/C differentiation commitment.

Since it is known that the characteristics of a given cell is determined by the combination pattern of its expressed gene (Yamada et al. 2016), in this study, we expanded our investigation about PDL clones using a next-generation sequencing technology (RNa-seq) to explore the differential transcriptomic profile between C-O and C-F clones. The use of a label-free approach enables the most inclusive and unbiased description of genes, and it has been used to describe many types of cells, including osteoblasts (Ayturk et al. 2013; Twine et al. 2014) and MSC (De Luca et al. 2014; Jaager et al. 2012). In this context, this investigation aimed to identify the transcriptome profile associated to O/C compared to fibroblastic phenotypes in PDL by RNA-seq. Although research about a group of transcripts important to PDL have been previously described (Yamada et al. 2016), the investigation of high throughput analysis of human PDL clones with distinct differentiation capacity has not yet been explored. To our knowledge, this is the first study to analyze primary human PDL cell clones on comprehensive scale using RNA-seq.

This study revealed that, in a heterogeneous cells population derived from PDL, cell clones prone to differentiate into O/C phenotype (C-O clones) have a more complex transcriptional profile compared to clones related to fibroblastic phenotype (C-F clones). Furthermore, genes related to biological process described as “anatomical structure development”, and the Cadherin and Wnt signaling pathways (*WNT2, WNT16* and *WIF1*), were identified as significantly important to distinguish PDL cells with higher potential to commitment to O/C phenotype.

Wnt signaling has been reported to be important in the regulation of homeostasis of craniofacial tissue, including PDL and alveolar bone (Lim et al. 2015). It has been reported that the Wnt/β-catenin signaling pathway is important to direct mesenchymal progenitor cell differentiation toward osteoblastic phenotype, by inhibition of chondroblastic differentiation (Kook et al. 2016). According to some studies, when cells are in a more immature stage, the activation of Wnt/β-catenin signaling pathway may be important to direct cell fate toward

osteoblast phenotype, but after osteoblastic differentiation, there is a down-regulation of Wnt signaling, and continuous stimulation of Wnt pathway may inhibit mineralization (Eijken et al. 2008; Regard et al. 2012). It has been reported that up-regulation of *WNT2* in dental follicles cells in early time point may be related to promotion of commitment to O/C progeny, prior to acquisition of a more mature phenotype (Park et al. 2015). Further, *WNT16* has been reported to be related to regulation of cortical bone homeostasis (Gori et al. 2015), and to induce expression of osteoprotegerin, suggesting that *WNT16* expression can inhibit osteoclastogenesis (Kobayashi et al. 2015). Accordingly, we observed that periodontal ligament cells more prone to differentiate into O/C phenotype presented higher expression of *WNT2* and *WNT16,* demonstrating the importance of the expression of these genes to initial commitment toward O/C progeny in our study. Additionally, the gene *TNFRSF11B*, that encodes osteoprotegerin, was also significantly up-regulated in our C-O clones. In our study, *WIF1* was one of the most significantly upregulated gene in C-O clones compared to C-F clones. Although Panther analysis did not classified this gene as related to Wnt signaling pathway, the literature report this molecule as a Wnt antagonist (Jia et al. 2013). In a recent study comparing murine osteoblasts and cementoblasts, the inhibitor of Wnt pathway, *Wif1,* was reported to be more expressed in cementoblasts than osteoblasts and poorly expressed in PDL (Matthews et al. 2016). By this data, it is possible to suppose that C-O clones are more committed to differentiate toward a cementoblast phenotype, although our study has assessed the transcriptome profile from human cells.

It has also been declared that Wnt pathway alone may not enough for mature bone, and other signaling pathways, such as TGFβ/BMPs and Cadherin pathways may interact with Wnt pathway to control O/C differentiation (Castro et al. 2004; Romanos 2016). In our previous study, it was demonstrated that BMP2 requires the activation of canonical Wnt signaling at the early stage of differentiation of murine dental follicle cell line (SVF4 cells) along O/C phenotype (Silverio et al. 2012). On the other hand, at the time of follicle cell maturation, BMP2 promotes a negative Wnt-feedback loop by increasing expression of Wnt pathway inhibitors, including *Wif1, Dkk1* and *Sfrp4* (Silverio et al. 2012). The results of present study showed that the expression of *BMP4* was constitutively increased about six-fold in C-O clones compared to C-F clone cells. Similar findings were found in immortalized PDL cell clones, in which clones that possess high expression of *BMP4* showed intrinsic ability to form mineralized tissue in vitro, whereas another clone that did not expressed *BMP4* was unable to form mineral nodules (Tomokiyo et al. 2008). *BMP4*, another member of TGF/BMPs superfamily, plays an important role in the process of bone nodule formation as already described to *BMP2* (Chen et al. 2012;

Yamaguchi et al. 2008), and *BMP4* is considered one of the most predictive gene expression markers of in vivo bone formation potential according to a recent RNA-seq analysis (Twine et al. 2014). Also, it has been reported that *BMP4* also interacts with Wnt signaling pathway during tooth organogenesis (Jia et al. 2016; Jia et al. 2013), where higher *BMP4* levels showed to be important to overcome the inhibitory effects of Wnt antagonists, such as *WIF1* and *DKK2*, during tooth development beyond bud stage (Jia et al. 2013). Take this findings together, it is possible to speculate that *BMP4* expression in C-O clone cells might to be related to the capacity of these clones to acquire O/C phenotype through of functional interactions with Wnt signaling pathway. However, additional experiments must be performed to elucidate how Wnt-BMP interactions work to drive the O/C maturation process in these clone cells.

Cadherin superfamily was another significantly overrepresented signaling pathway in clones with potential to differentiate into O/C phenotype. Studies have reported that cross-talk between Cadherin and Wnt signaling regulates the mechanism underlining osteoblast differentiation (Castro et al. 2004; Hay et al. 2014; Marie et al. 2014). Some evidences revealed that Cadherins bind to β-catenin, hindering its translocation to the nucleus (Marie et al. 2014) and then, causing the reduction of canonical Wnt signaling. Additionally, Cadherins have also been reported to interact with Wnt co-receptor lipoprotein receptor-related protein 5 (*LRP5*) (Hay et al. 2014), which is important to regulate bone mass (Jacobsen et al. 2014). In agreement with these findings, our study showed that C-O clone cells presented nine upregulated genes common between Cadherin and Wnt pathways, suggesting an interaction of these two pathways in the regulation of osteoblast/cementoblast cell lineage commitment localized into periodontal dental ligament.

Here we provide a comprehensive assessment of the transcriptome of human PDL progenitor cell clones with high O/C differentiation potential using a next-generation sequencing technology (RNA-seq). This data shows potential genes and pathways associated to PDL cells with potential commitment into O/C phenotype. Together, these findings provide evidence that genes related to Wnt pathway are important for commitment of PDL cells toward O/C differentiation. However, subsequent studies are necessary to better understand the mechanisms of action and extent of this process of differentiation. We expect that better understating of molecular regulation of PDL cells committed to O/C phenotype can be potentially used to promote better protocols for periodontal regenerative therapies in the future.

Author contributions

M. T. Saito and K. G. Silvério contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; M. L. Albiero, L.S. Mofatto contributed to data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; F.H. Nociti Jr and K. Silvério contributed to conception, design, data analysis and interpretation, drafted and critically revised the manuscript; M. Z. Casati, E. A. Sallum, and G. A. G. Pereira contributed to data analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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Figures

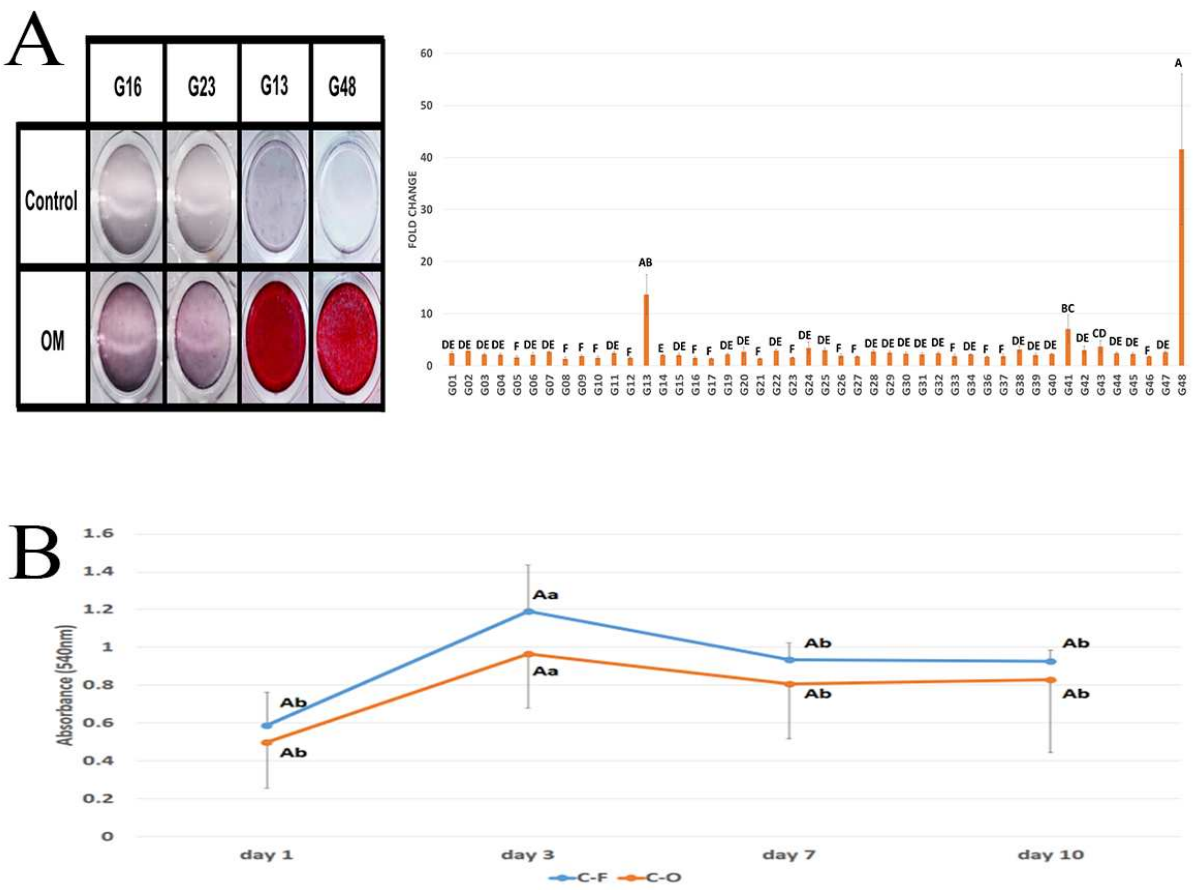

Figure 1. PDL CD105^{+}- derived cell clones. **A**: Alizarin Red Stain (AR-S) assay showed that G16 and G23 clones were not able to form mineralized deposits *in vitro*, whereas G13 and G48 clones displayed capacity of biomineralization *in vitro* when cultivated under osteogenic induction (OM). Clones G13 and G48 showed significant fold change of AR-S in OM/control medium when compared to other all clones, and were considered as clones with potential to differentiate into osteoblastic/cementoblastic phenotype (C-O). Clones G16 and G23 were selected as counterpart of fibroblastic phentopype (C-F) clones to be compared to C-O clones. Distinct letters represent significant difference (One way ANOVA/Tukey. P<0.05). **B**: Cell metabolic activity of between C-O and C-F clones did not show significant difference in each time point evaluated. Both clone types showed increased metabolic activity at day 3. Distinct uppercase letters represent significant difference (p<0.05) intragroup, and distinct lowercase letters represent significant difference (p<0.05) intergroup (Two-way Anova/Tukey test).

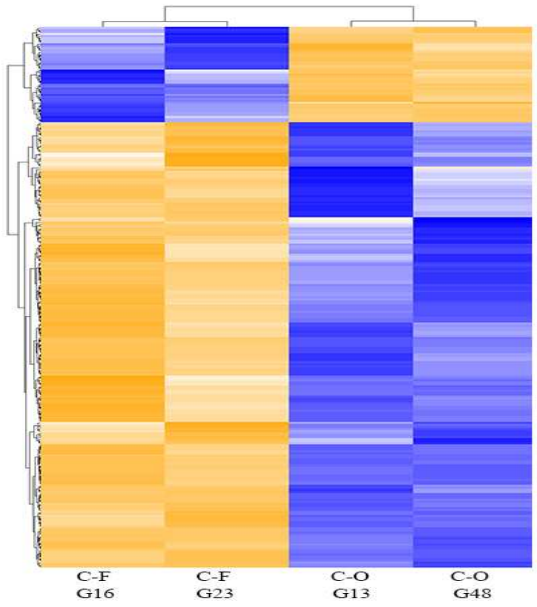

Figure 2. Heatmap of differentially expressed genes of C-O and C-F clones. A total of 235 DEG were observed between C-O and C-F clones. 193 genes were significantly up-regulated in C-O group, and 42 genes were significantly down-regulated in C-O group (up-regulated in C-F) (|log2(fold change)| ≥ 2; p <0.05).

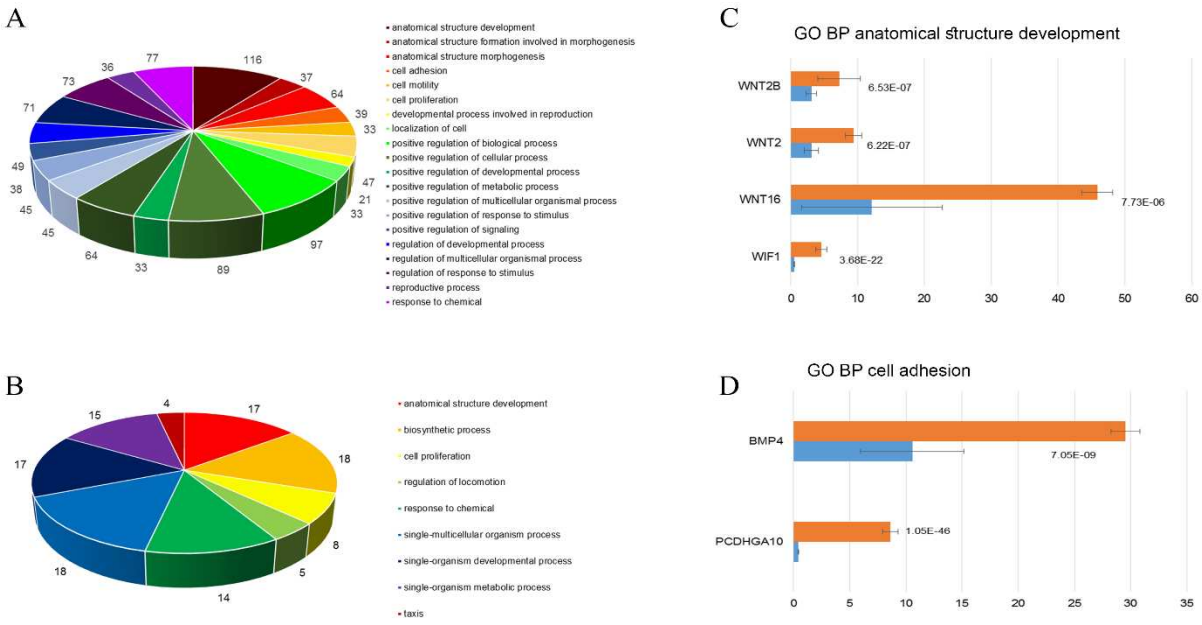

Figure 3. Gene Ontology (GO) of Biological Processes (BP) enrichment analysis of the list of differentially expressed genes between C-O and C-F clones. GO analysis was generated by the DAVID software based on biological processes (GO_TERM_BP_2 database). **A**: GO BP analysis from upregulated genes in C-O clones. **B**: GO BP analysis from downregulated genes in C-O clones. **C**: Barplot of FPKM of upregulated genes in C-O clones present GO BP anatomical structure development. **D**: Barplot of FPKM of upregulated genes in C-O clones present GO BP and cell adhesion. Mean ± standard deviation and p-value determined by negative binomial statistical test (DESEQ) in **C** and **D** barplots.

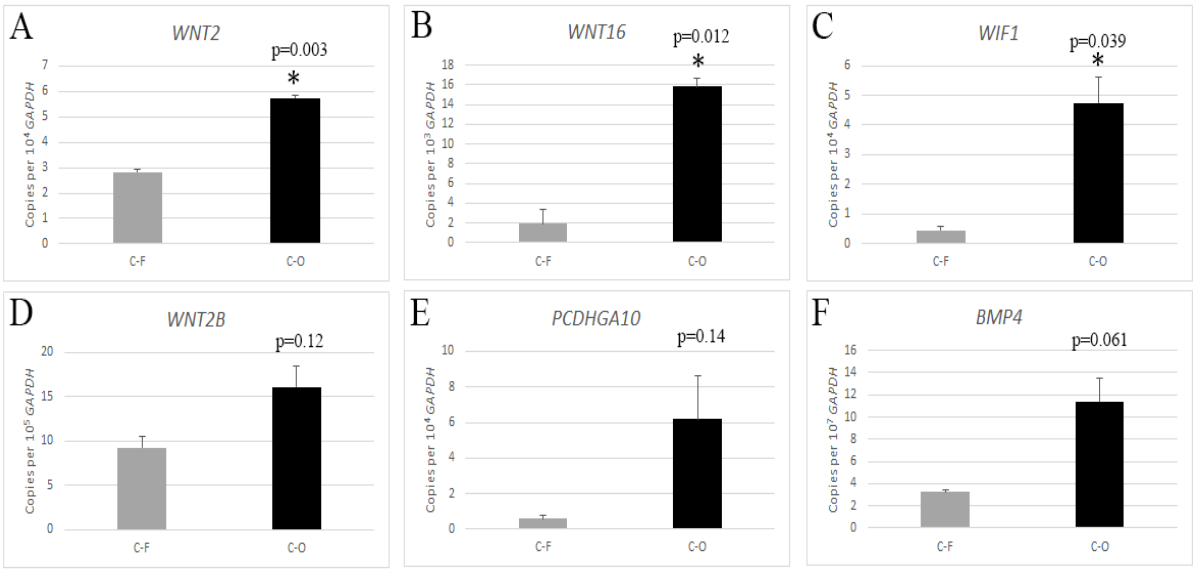

Figure 4. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) showed that the expression of *WNT2* (**A**), *WNT16* (**B**) and *WIF1* (**C**) was significantly higher in C-O clones than in C-F clones. The expression of *WNT2B* (**D**), *PCDHGA10* (**E**) and *BMP4* (**F**) was not statistically significantly up-regulaed in C-O clones (* represents $p < 0.05$, *t*-Test).

Table

Table 1. Significantly overrepresented pathways and respective upregulated genes in clones with potential to differentiate into osteoblastic/cementoblastic phenotype (C-O clones).

Pathway (code)	p value	Gene ID
Wnt signaling pathway (P00057)	5.70E-04	*ADSSL1; PCDH18; PCDHB2; PCDHGA6; PCDHGA10; PCDHGB2; PCDHGB4; PRKCD; PRKCZ; SFRP2; WNT16; WNT2; WNT2B*
Cadherin signaling pathway (P00012)	1.73E-03	*PCDH18; PCDHB2; PCDHB4; PCDHGA6; PCDHGA10; PCDHGB2; WNT16; WNT2; WNT2B*

Appendix: Differential Transcriptome profile of periodontal ligament cell clones

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Appendix Table 1. Specific primer sequence for quantitative Polymerase Chain Reaction (qPCR).

Gene ID	*Primers* (5'→ 3')
GAPDH	Forward: ACATCATCCCTGCCTCTAC; Reverse: CCACCTTCTTGATGTCATCATATTTG
WNT2	Forward: TTTGGCAGGGTCCTACTCC; Reverse: CCTGGTGATGGCAAATACAA
WNT2B	Forward: AACTTACATAATAACCGCTGTGGTC; Reverse: ACTCACGCCATGGCACTT
WNT16	Forward: CAATGAACCTACATAACAATGAAGC; Reverse: CAGCGGCAGTCTACTGACAT
WIF1	Forward: CCAGGGAGACCTCTGTTCAA; Reverse: TTGGGTTCATGGCAGGTT
PCDHGA10	Forward: ATTTGCCTGTGGGCACTC; Reverse: CACTTCTCCATTGGCACCTT
BMP4	Forward: CTGCAACCGTTCAGAGGTC; Reverse: TGCTCGGGATGGCACTAC

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Appendix Table 2. Summary of mapping results.

	R1		R2		Total	
Sample Name	# Total reads	# aligned	# Total reads	# aligned	# Total reads	# aligned
G13 (C-O)	22485809	18775954 (83.5%)	22485809	18836596 (83.8%)	44971618	37612550 (83.6%)
G48 (C-O)	22914459	19092937 (83.3%)	22914459	19145870 (83.6%)	45828918	38238807 (83.4%)
G16 (C-F)	26335319	22241223 (84.5%)	26335319	22332183 (84.8%)	52670638	44573406 (84.6%)
G23 (C-F)	26435828	22233598 (84.1%)	26435828	22329638 (84.5%)	52871656	44563236 (84.3%)

C-O: clone with potential to differentiate into osteo/cementoblastic phenotype; C-F: clone that shows fibroblastic phenotype

Appendix Table 3. Significantly upregulated genes of C-O clones (clones with potential to differentiate into osteo/cementoblastic phenotype) compared to C-F clones (clones that shows fibroblastic phenotype).

Gene ID	Gene_description	|log2FoldChange|	p value
PCDHGA10	protocadherin gamma subfamily A, 10 [Source:HGNC Symbol;Acc:HGNC:8697]	4.24549	1.05462E-46
CBLN2	cerebellin 2 precursor [Source:HGNC Symbol;Acc:HGNC:1544]	3.56478	1.52284E-35
PDPN	podoplanin [Source:HGNC Symbol;Acc:HGNC:29602]	3.95217	1.9629E-30
ITIH5	inter-alpha-trypsin inhibitor heavy chain family, member 5 [Source:HGNC Symbol;Acc:HGNC:21449]	3.13118	2.75103E-28
SDK1	sidekick cell adhesion molecule 1 [Source:HGNC Symbol;Acc:HGNC:19307]	3.22717	7.94864E-26
WIF1	WNT inhibitory factor 1 [Source:HGNC Symbol;Acc:HGNC:18081]	3.39957	3.67738E-22
CYP26B1	cytochrome P450, family 26, subfamily B, polypeptide 1 [Source:HGNC Symbol;Acc:HGNC:20581]	2.36631	2.43062E-21
CD4	CD4 molecule [Source:HGNC Symbol;Acc:HGNC:1678]	3.13156	2.85121E-19
PCDHGB4	protocadherin gamma subfamily B, 4 [Source:HGNC Symbol;Acc:HGNC:8711]	2.79096	5.56668E-19

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IL12RB2	interleukin 12 receptor, beta 2 [Source:HGNC Symbol;Acc:HGNC:5972]	4.57954	3.56492E-16
EPB41L3	erythrocyte membrane protein band 4.1-like 3 [Source:HGNC Symbol;Acc:HGNC:3380]	3.01442	5.69076E-15
CTAG2	cancertestis antigen 2 [Source:HGNC Symbol;Acc:HGNC:2492]	3.53231	7.41633E-15
ARHGAP28	Rho GTPase activating protein 28 [Source:HGNC Symbol;Acc:HGNC:25509]	2.99542	4.98714E-13
PTPRN	protein tyrosine phosphatase, receptor type, N [Source:HGNC Symbol;Acc:HGNC:9676]	1.76435	4.75154E-13
CYP7B1	cytochrome P450, family 7, subfamily B, polypeptide 1 [Source:HGNC Symbol;Acc:HGNC:2652]	3.23889	1.32703E-12
CCL11	chemokine (C-C motif) ligand 11 [Source:HGNC Symbol;Acc:HGNC:10610]	3.46256	1.72989E-12
GPRC5B	G protein-coupled receptor, class C, group 5, member B [Source:HGNC Symbol;Acc:HGNC:13308]	1.85847	2.47654E-12
HGF	hepatocyte growth factor (hepapoietin A; scatter factor) [Source:HGNC Symbol;Acc:HGNC:4893]	1.64454	3.9322E-12
RGS5	regulator of G-protein signaling 5 [Source:HGNC Symbol;Acc:HGNC:10001]	2.07866	1.00855E-11
RARRES2	retinoic acid receptor responder (tazarotene induced) 2 [Source:HGNC Symbol;Acc:HGNC:9868]	1.90944	1.78452E-11
PRKAR2B	protein kinase, cAMP-dependent, regulatory, type II, beta [Source:HGNC Symbol;Acc:HGNC:9392]	1.78616	3.79563E-11
STC1	stanniocalcin 1 [Source:HGNC Symbol;Acc:HGNC:11373]	1.56167	3.73425E-11
ATP8B4	ATPase, class I, type 8B, member 4 [Source:HGNC Symbol;Acc:HGNC:13536]	1.68163	4.77333E-11
RASSF2	Ras association (RalGDSAF-6) domain family member 2 [Source:HGNC Symbol;Acc:HGNC:9883]	1.55265	1.31829E-10
RGS2	regulator of G-protein signaling 2 [Source:HGNC Symbol;Acc:HGNC:9998]	1.91296	2.27637E-10
FER1L4	fer-1-like family member 4, pseudogene (functional) [Source:HGNC Symbol;Acc:HGNC:15801]	1.87366	2.54745E-10
CPE	carboxypeptidase E [Source:HGNC Symbol;Acc:HGNC:2303]	1.53984	3.20492E-10
RDH10	retinol dehydrogenase 10 (all-trans) [Source:HGNC Symbol;Acc:HGNC:19975]	1.48357	7.37919E-10
PCDHB2	protocadherin beta 2 [Source:HGNC Symbol;Acc:HGNC:8687]	2.35028	1.32862E-09
SLC40A1	solute carrier family 40 (iron-regulated transporter), member 1 [Source:HGNC Symbol;Acc:HGNC:10909]	1.89721	1.32381E-09
GPRASP1	G protein-coupled receptor associated sorting protein 1 [Source:HGNC Symbol;Acc:HGNC:24834]	1.58206	2.06756E-09
TMEFF2	transmembrane protein with EGF-like and two follistatin-like domains 2 [Source:HGNC Symbol;Acc:HGNC:11867]	2.58703	3.76804E-09
GPRC5C	G protein-coupled receptor, class C, group 5, member C [Source:HGNC Symbol;Acc:HGNC:13309]	5.00989	5.99626E-09

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ANGPTL4	angiopoietin-like 4 [Source:HGNC Symbol;Acc:HGNC:16039]	1.46281	6.57978E-09
ACSL4	acyl-CoA synthetase long-chain family member 4 [Source:HGNC Symbol;Acc:HGNC:3571]	1.36068	7.19252E-09
BMP4	bone morphogenetic protein 4 [Source:HGNC Symbol;Acc:HGNC:1071]	1.54228	7.05132E-09
ADRA2A	adrenoceptor alpha 2A [Source:HGNC Symbol;Acc:HGNC:281]	1.94034	1.41308E-08
MSX1	msh homeobox 1 [Source:HGNC Symbol;Acc:HGNC:7391]	1.36846	1.64356E-08
RGCC	regulator of cell cycle [Source:HGNC Symbol;Acc:HGNC:20369]	1.61296	3.14662E-08
CXCL3	chemokine (C-X-C motif) ligand 3 [Source:HGNC Symbol;Acc:HGNC:4604]	1.32072	3.40575E-08
HMOX1	heme oxygenase (decycling) 1 [Source:HGNC Symbol;Acc:HGNC:5013]	1.36216	3.87512E-08
JUP	junction plakoglobin [Source:HGNC Symbol;Acc:HGNC:6207]	3.50483	5.79384E-08
RASL12	RAS-like, family 12 [Source:HGNC Symbol;Acc:HGNC:30289]	1.99066	5.84738E-08
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1 [Source:HGNC Symbol;Acc:HGNC:29]	1.5986	6.86385E-08
PPFIA4	protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 4 [Source:HGNC Symbol;Acc:HGNC:9248]	1.47638	7.11774E-08
SNED1	sushi, nidogen and EGF-like domains 1 [Source:HGNC Symbol;Acc:HGNC:24696]	1.27218	7.33975E-08
SDPR	serum deprivation response [Source:HGNC Symbol;Acc:HGNC:10690]	1.34845	8.59599E-08
DPP4	dipeptidyl-peptidase 4 [Source:HGNC Symbol;Acc:HGNC:3009]	2.33549	8.89398E-08
ADAMTS9-AS2	ADAMTS9 antisense RNA 2 [Source:HGNC Symbol;Acc:HGNC:42435]	2.37547	1.13482E-07
CPZ	carboxypeptidase Z [Source:HGNC Symbol;Acc:HGNC:2333]	1.30417	1.65588E-07
KCNJ2	potassium inwardly-rectifying channel, subfamily J, member 2 [Source:HGNC Symbol;Acc:HGNC:6263]	1.31047	1.7474E-07
FHL1	four and a half LIM domains 1 [Source:HGNC Symbol;Acc:HGNC:3702]	1.22027	2.26562E-07
CLEC2B	C-type lectin domain family 2, member B [Source:HGNC Symbol;Acc:HGNC:2053]	1.45914	2.67557E-07
WSB1	WD repeat and SOCS box containing 1 [Source:HGNC Symbol;Acc:HGNC:19221]	1.21971	2.71196E-07
RP11-574K11.31	Bifunctional heparan sulfate N-deacetylaseN- sulfotransferase 2 {ECO:0000313|Ensembl:ENSP00000475031} [Source:UniProtKBTrEMBL;Acc:S4R438]	1.33573	3.11273E-07
ROR2	receptor tyrosine kinase-like orphan receptor 2 [Source:HGNC Symbol;Acc:HGNC:10257]	2.90473	3.78578E-07
MEG3	maternally expressed 3 (non-protein coding) [Source:HGNC Symbol;Acc:HGNC:14575]	1.18855	3.84665E-07
NABP1	nucleic acid binding protein 1 [Source:HGNC Symbol;Acc:HGNC:26232]	1.27097	3.97971E-07
RORB	RAR-related orphan receptor B [Source:HGNC Symbol;Acc:HGNC:10259]	1.38769	4.15514E-07
TNFRSF11B	tumor necrosis factor receptor superfamily, member 11b [Source:HGNC Symbol;Acc:HGNC:11909]	1.63493	6.18438E-07

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WNT2	wingless-type MMTV integration site family member 2 [Source:HGNC Symbol;Acc:HGNC:12780]	1.60302	6.22446E-07
WNT2B	wingless-type MMTV integration site family, member 2B [Source:HGNC Symbol;Acc:HGNC:12781]	1.24416	6.52856E-07
ABCA7	ATP-binding cassette, sub-family A (ABC1), member 7 [Source:HGNC Symbol;Acc:HGNC:37]	1.80062	7.49863E-07
TAC3	tachykinin 3 [Source:HGNC Symbol;Acc:HGNC:11521]	4.07224	7.51049E-07
COL27A1	collagen, type XXVII, alpha 1 [Source:HGNC Symbol;Acc:HGNC:22986]	1.20662	7.86327E-07
AREG	amphiregulin [Source:HGNC Symbol;Acc:HGNC:651]	1.69621	8.32209E-07
HMGN2P5	high mobility group nucleosomal binding domain 2 pseudogene 5 [Source:HGNC Symbol;Acc:HGNC:33568]	3.29067	9.62166E-07
PCDHGA9	protocadherin gamma subfamily A, 9 [Source:HGNC Symbol;Acc:HGNC:8707]	1.96426	9.95279E-07
RSPO1	R-spondin 1 [Source:HGNC Symbol;Acc:HGNC:21679]	2.33933	1.04161E-06
TMEM178B	transmembrane protein 178B [Source:HGNC Symbol;Acc:HGNC:44112]	1.5176	1.05343E-06
ADHFE1	alcohol dehydrogenase, iron containing, 1 [Source:HGNC Symbol;Acc:HGNC:16354]	1.66859	1.34897E-06
NEAT1	nuclear paraspeckle assembly transcript 1 (non- protein coding) [Source:HGNC Symbol;Acc:HGNC:30815]	1.11941	1.64333E-06
TMEM150C	transmembrane protein 150C [Source:HGNC Symbol;Acc:HGNC:37263]	1.63538	1.78918E-06
SLC27A6	solute carrier family 27 (fatty acid transporter), member 6 [Source:HGNC Symbol;Acc:HGNC:11000]	2.1816	1.9993E-06
MN1	meningioma (disrupted in balanced translocation) 1 [Source:HGNC Symbol;Acc:HGNC:7180]	1.34167	2.19509E-06
PCDHGA6	protocadherin gamma subfamily A, 6 [Source:HGNC Symbol;Acc:HGNC:8704]	1.85145	2.40947E-06
LAMA5	laminin, alpha 5 [Source:HGNC Symbol;Acc:HGNC:6485]	1.55834	2.44561E-06
ARHGAP6	Rho GTPase activating protein 6 [Source:HGNC Symbol;Acc:HGNC:676]	1.32046	2.52235E-06
CHL1	cell adhesion molecule L1-like [Source:HGNC Symbol;Acc:HGNC:1939]	4.08022	2.5772E-06
MST1	macrophage stimulating 1 (hepatocyte growth factor- like) [Source:HGNC Symbol;Acc:HGNC:7380]	1.37764	2.76513E-06
ATHL1	ATH1, acid trehalase-like 1 (yeast) [Source:HGNC Symbol;Acc:HGNC:26210]	1.36577	2.95143E-06
SHANK2	SH3 and multiple ankyrin repeat domains 2 [Source:HGNC Symbol;Acc:HGNC:14295]	1.24192	3.24887E-06
CLCA2	chloride channel accessory 2 [Source:HGNC Symbol;Acc:HGNC:2016]	1.13126	4.00996E-06
SPAG4	sperm associated antigen 4 [Source:HGNC Symbol;Acc:HGNC:11214]	1.4616	4.41202E-06
PRR5	proline rich 5 (renal) [Source:HGNC Symbol;Acc:HGNC:31682]	1.68234	5.02727E-06
PCDH18	protocadherin 18 [Source:HGNC Symbol;Acc:HGNC:14268]	1.09425	5.43378E-06
LINC01305	long intergenic non-protein coding RNA 1305 [Source:HGNC Symbol;Acc:HGNC:27690]	2.66685	6.958E-06

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UCP2	uncoupling protein 2 (mitochondrial, proton carrier) [Source:HGNC Symbol;Acc:HGNC:12518]	1.71815	7.1711E-06
WNT16	wingless-type MMTV integration site family, member 16 [Source:HGNC Symbol;Acc:HGNC:16267]	1.945	7.7253E-06
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin GH synthase and cyclooxygenase) [Source:HGNC Symbol;Acc:HGNC:9605]	1.04232	7.79974E-06
HSPA2	heat shock 70kDa protein 2 [Source:HGNC Symbol;Acc:HGNC:5235]	1.13304	8.69472E-06
HILPDA	hypoxia inducible lipid droplet-associated [Source:HGNC Symbol;Acc:HGNC:28859]	1.42496	8.76722E-06
APLP1	amyloid beta (A4) precursor-like protein 1 [Source:HGNC Symbol;Acc:HGNC:597]	1.16054	9.23808E-06
LINC00663	long intergenic non-protein coding RNA 663 [Source:HGNC Symbol;Acc:HGNC:28609]	2.06331	9.60987E-06
MIR210HG	MIR210 host gene (non-protein coding) [Source:HGNC Symbol;Acc:HGNC:39524]	1.26344	9.63018E-06
CYR61	cysteine-rich, angiogenic inducer, 61 [Source:HGNC Symbol;Acc:HGNC:2654]	1.02409	1.10586E-05
MMP12	matrix metallopeptidase 12 (macrophage elastase) [Source:HGNC Symbol;Acc:HGNC:7158]	1.3208	1.3066E-05
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10 [Source:HGNC Symbol;Acc:HGNC:11925]	1.87505	1.29981E-05
GALNTL6	polypeptide N-acetylgalactosaminyltransferase-like 6 [Source:HGNC Symbol;Acc:HGNC:33844]	1.42258	1.31939E-05
INHBE	inhibin, beta E [Source:HGNC Symbol;Acc:HGNC:24029]	1.92796	1.37312E-05
PTHLH	parathyroid hormone-like hormone [Source:HGNC Symbol;Acc:HGNC:9607]	1.04103	1.59472E-05
TBX1	T-box 1 [Source:HGNC Symbol;Acc:HGNC:11592]	2.82073	1.6372E-05
ABCA8	ATP-binding cassette, sub-family A (ABC1), member 8 [Source:HGNC Symbol;Acc:HGNC:38]	1.33572	1.75245E-05
GULP1	GULP, engulfment adaptor PTB domain containing 1 [Source:HGNC Symbol;Acc:HGNC:18649]	1.08536	1.81815E-05
ITGB8	integrin, beta 8 [Source:HGNC Symbol;Acc:HGNC:6163]	1.0853	2.13288E-05
RASL11A	RAS-like, family 11, member A [Source:HGNC Symbol;Acc:HGNC:23802]	1.58981	2.15851E-05
FILIP1L	filamin A interacting protein 1-like [Source:HGNC Symbol;Acc:HGNC:24589]	1.0124	2.1855E-05
LIMCH1	LIM and calponin homology domains 1 [Source:HGNC Symbol;Acc:HGNC:29191]	2.45246	2.31835E-05
MOCOS	molybdenum cofactor sulfurase [Source:HGNC Symbol;Acc:HGNC:18234]	1.13597	2.36632E-05
TLR2	toll-like receptor 2 [Source:HGNC Symbol;Acc:HGNC:11848]	1.50532	2.39797E-05
FENDRR	FOXF1 adjacent non-coding developmental regulatory RNA [Source:HGNC Symbol;Acc:HGNC:43894]	1.25891	2.55299E-05
MPP2	membrane protein, palmitoylated 2 (MAGUK p55 subfamily member 2) [Source:HGNC Symbol;Acc:HGNC:7220]	2.63995	2.74966E-05

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ABCC6	ATP-binding cassette, sub-family C (CFTRMRP), member 6 [Source:HGNC Symbol;Acc:HGNC:57]	1.43449	2.86218E-05
NEDD9	neural precursor cell expressed, developmentally down-regulated 9 [Source:HGNC Symbol;Acc:HGNC:7733]	1.23603	3.02056E-05
MIR210	microRNA 210 [Source:HGNC Symbol;Acc:HGNC:31587]	1.53024	3.19208E-05
SH3BGRL2	SH3 domain binding glutamate-rich protein like 2 [Source:HGNC Symbol;Acc:HGNC:15567]	1.29476	3.69352E-05
REC8	REC8 meiotic recombination protein [Source:HGNC Symbol;Acc:HGNC:16879]	1.31485	4.6937E-05
NR4A3	nuclear receptor subfamily 4, group A, member 3 [Source:HGNC Symbol;Acc:HGNC:7982]	1.01179	4.75244E-05
ADM2	adrenomedullin 2 [Source:HGNC Symbol;Acc:HGNC:28898]	1.29248	4.97492E-05
CHRNA7	cholinergic receptor, nicotinic, alpha 7 (neuronal) [Source:HGNC Symbol;Acc:HGNC:1960]	3.406	5.15205E-05
KBTBD11	kelch repeat and BTB (POZ) domain containing 11 [Source:HGNC Symbol;Acc:HGNC:29104]	1.37209	5.49048E-05
ARMC4	armadillo repeat containing 4 [Source:HGNC Symbol;Acc:HGNC:25583]	1.2362	6.00187E-05
CORO6	coronin 6 [Source:HGNC Symbol;Acc:HGNC:21356]	1.05161	6.18401E-05
ABCA6	ATP-binding cassette, sub-family A (ABC1), member 6 [Source:HGNC Symbol;Acc:HGNC:36]	1.06436	6.37063E-05
INMT	indolethylamine N-methyltransferase [Source:HGNC Symbol;Acc:HGNC:6069]	1.71313	6.76701E-05
ROBO3	roundabout, axon guidance receptor, homolog 3 (Drosophila) [Source:HGNC Symbol;Acc:HGNC:13433]	1.10175	8.53108E-05
COLEC10	collectin sub-family member 10 (C-type lectin) [Source:HGNC Symbol;Acc:HGNC:2220]	1.76357	9.96863E-05
ENTPD1	ectonucleoside triphosphate diphosphohydrolase 1 [Source:HGNC Symbol;Acc:HGNC:3363]	1.01319	0.000103996
BGN	biglycan [Source:HGNC Symbol;Acc:HGNC:1044]	2.38082	0.000105983
TNFRSF10C	tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain [Source:HGNC Symbol;Acc:HGNC:11906]	1.1611	0.000108966
SLC9A9	solute carrier family 9, subfamily A (NHE9, cation proton antiporter 9), member 9 [Source:HGNC Symbol;Acc:HGNC:20653]	1.03802	0.000113679
MASP1	mannan-binding lectin serine peptidase 1 (C4C2 activating component of Ra-reactive factor) [Source:HGNC Symbol;Acc:HGNC:6901]	2.01426	0.000116068
CPAMD8	C3 and PZP-like, alpha-2-macroglobulin domain containing 8 [Source:HGNC Symbol;Acc:HGNC:23228]	1.85271	0.000116631
ICAM5	intercellular adhesion molecule 5, telencephalin [Source:HGNC Symbol;Acc:HGNC:5348]	1.9301	0.000119378
SCN2B	sodium channel, voltage-gated, type II, beta subunit [Source:HGNC Symbol;Acc:HGNC:10589]	1.70566	0.000128748
NUDT10	nudix (nucleoside diphosphate linked moiety X)-type motif 10 [Source:HGNC Symbol;Acc:HGNC:17621]	2.05949	0.000141662
OLFML2A	olfactomedin-like 2A [Source:HGNC Symbol;Acc:HGNC:27270]	1.82282	0.000140823

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PCDHGB2	protocadherin gamma subfamily B, 2 [Source:HGNC Symbol;Acc:HGNC:8709]	2.38447	0.000152631
SP9	Sp9 transcription factor [Source:HGNC Symbol;Acc:HGNC:30690]	2.44408	0.000160934
ODF3B	outer dense fiber of sperm tails 3B [Source:HGNC Symbol;Acc:HGNC:34388]	1.61834	0.000169382
PIM1	Pim-1 proto-oncogene, serinethreonine kinase [Source:HGNC Symbol;Acc:HGNC:8986]	1.00963	0.000171383
MYO15B	myosin XVB pseudogene [Source:HGNC Symbol;Acc:HGNC:14083]	2.7617	0.000211859
PSAT1	phosphoserine aminotransferase 1 [Source:HGNC Symbol;Acc:HGNC:19129]	1.22993	0.000233103
ARHGEF19	Rho guanine nucleotide exchange factor (GEF) 19 [Source:HGNC Symbol;Acc:HGNC:26604]	1.01813	0.000265419
CMKLR1	chemokine-like receptor 1 [Source:HGNC Symbol;Acc:HGNC:2121]	3.8077	0.000275864
BNC1	basonuclin 1 [Source:HGNC Symbol;Acc:HGNC:1081]	1.4185	0.000301962
SLC2A1-AS1	SLC2A1 antisense RNA 1 [Source:HGNC Symbol;Acc:HGNC:44187]	1.68598	0.000311853
B3GALT4	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 4 [Source:HGNC Symbol;Acc:HGNC:919]	1.51923	0.000333388
FAM115C	family with sequence similarity 115, member C [Source:HGNC Symbol;Acc:HGNC:26878]	1.32815	0.000337956
ACSS1	acyl-CoA synthetase short-chain family member 1 [Source:HGNC Symbol;Acc:HGNC:16091]	1.36572	0.00036002
MX2	MX dynamin-like GTPase 2 [Source:HGNC Symbol;Acc:HGNC:7533]	1.35949	0.000369066
C1QL1	complement component 1, q subcomponent-like 1 [Source:HGNC Symbol;Acc:HGNC:24182]	1.41448	0.000389667
TNFSF15	tumor necrosis factor (ligand) superfamily, member 15 [Source:HGNC Symbol;Acc:HGNC:11931]	1.90156	0.00041443
FOXL1	forkhead box L1 [Source:HGNC Symbol;Acc:HGNC:3817]	1.56737	0.000434715
PLA2G6	phospholipase A2, group VI (cytosolic, calcium- independent) [Source:HGNC Symbol;Acc:HGNC:9039]	1.09882	0.000436934
ADSSL1	adenylosuccinate synthase like 1 [Source:HGNC Symbol;Acc:HGNC:20093]	1.18365	0.00045484
NIPSNAP3B	nipsnap homolog 3B (C. elegans) [Source:HGNC Symbol;Acc:HGNC:23641]	1.72535	0.000497826
PAPLN	papilin, proteoglycan-like sulfated glycoprotein [Source:HGNC Symbol;Acc:HGNC:19262]	1.51116	0.000494175
PPARG	peroxisome proliferator-activated receptor gamma [Source:HGNC Symbol;Acc:HGNC:9236]	1.04788	0.0004889
RGS4	regulator of G-protein signaling 4 [Source:HGNC Symbol;Acc:HGNC:10000]	2.19792	0.000491326
TM4SF1	transmembrane 4 L six family member 1 [Source:HGNC Symbol;Acc:HGNC:11853]	1.34867	0.00049749
MEG9	maternally expressed 9 (non-protein coding) [Source:HGNC Symbol;Acc:HGNC:43874]	1.13738	0.000525
NRCAM	neuronal cell adhesion molecule [Source:HGNC Symbol;Acc:HGNC:7994]	1.00905	0.000553536
PENK	proenkephalin [Source:HGNC Symbol;Acc:HGNC:8831]	2.75415	0.000548842
LBP	lipopolysaccharide binding protein [Source:HGNC Symbol;Acc:HGNC:6517]	1.85026	0.000555541

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KNDC1	kinase non-catalytic C-lobe domain (KIND) containing 1 [Source:HGNC Symbol;Acc:HGNC:29374]	1.8114	0.000597758
KCNJ2-AS1	KCNJ2 antisense RNA 1 (head to head) [Source:HGNC Symbol;Acc:HGNC:43720]	1.42512	0.000616035
SFRP2	secreted frizzled-related protein 2 [Source:HGNC Symbol;Acc:HGNC:10777]	1.21626	0.000619934
HSF4	heat shock transcription factor 4 [Source:HGNC Symbol;Acc:HGNC:5227]	1.13413	0.000661941
CD40	CD40 molecule, TNF receptor superfamily member 5 [Source:HGNC Symbol;Acc:HGNC:11919]	1.38645	0.000705633
MIR503HG	MIR503 host gene (non-protein coding) [Source:HGNC Symbol;Acc:HGNC:28258]	1.44105	0.000725127
ASMTL-AS1	ASMTL antisense RNA 1 [Source:HGNC Symbol;Acc:HGNC:25811]	1.1478	0.000738242
CLUHP3	clustered mitochondria (cluACLU1) homolog pseudogene 3 [Source:HGNC Symbol;Acc:HGNC:28447]	1.09777	0.000747621
SEPP1	selenoprotein P, plasma, 1 [Source:HGNC Symbol;Acc:HGNC:10751]	1.63958	0.000761785
PRKCZ	protein kinase C, zeta [Source:HGNC Symbol;Acc:HGNC:9412]	1.15858	0.000789937
EEPD1	endonucleaseexonucleasephosphatase family domain containing 1 [Source:HGNC Symbol;Acc:HGNC:22223]	1.14009	0.000807002
LINC00672	long intergenic non-protein coding RNA 672 [Source:HGNC Symbol;Acc:HGNC:44353]	1.53192	0.000834011
CMAHP	cytidine monophospho-N-acetylneuraminic acid hydroxylase, pseudogene [Source:HGNC Symbol;Acc:HGNC:2098]	1.17126	0.001014205
SIX2	SIX homeobox 2 [Source:HGNC Symbol;Acc:HGNC:10888]	1.22528	0.001017085
LPHN3	latrophilin 3 [Source:HGNC Symbol;Acc:HGNC:20974]	1.15322	0.001039395
OR7E155P	olfactory receptor, family 7, subfamily E, member 155 pseudogene [Source:HGNC Symbol;Acc:HGNC:31310]	2.48421	0.001047946
DNER	Delta notch-like EGF repeat containing [Source:HGNC Symbol;Acc:HGNC:24456]	2.10309	0.001052827
PDK4	pyruvate dehydrogenase kinase, isozyme 4 [Source:HGNC Symbol;Acc:HGNC:8812]	1.47279	0.001062613
LINC00958	long intergenic non-protein coding RNA 958 [Source:HGNC Symbol;Acc:HGNC:48671]	2.21984	0.001074528
DLL1	delta-like 1 (Drosophila) [Source:HGNC Symbol;Acc:HGNC:2908]	1.31061	0.001120041
MAP3K1	mitogen-activated protein kinase kinase kinase 1, E3 ubiquitin protein ligase [Source:HGNC Symbol;Acc:HGNC:6848]	1.04849	0.001185829
ENO3	enolase 3 (beta, muscle) [Source:HGNC Symbol;Acc:HGNC:3354]	1.04416	0.001240996
ARHGEF35	Rho guanine nucleotide exchange factor (GEF) 35 [Source:HGNC Symbol;Acc:HGNC:33846]	1.03407	0.001277462
MATN3	matrilin 3 [Source:HGNC Symbol;Acc:HGNC:6909]	1.10179	0.001354113
ZFPM2	zinc finger protein, FOG family member 2 [Source:HGNC Symbol;Acc:HGNC:16700]	1.07328	0.001356613
MYO3B	myosin IIIB [Source:HGNC Symbol;Acc:HGNC:15576]	1.27057	0.001409756

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Appendix Table 4. Significantly downregulated genes of C-O clones (clones with potential to differentiate into osteo/cementoblastic phenotype) compared to C-F (clones that shows fibroblastic phenotype).

Gene ID	Gene_description	|log2FoldChange|	p value
GABRB3	gamma-aminobutyric acid (GABA) A receptor, beta 3 [Source:HGNC Symbol;Acc:HGNC:4083]	2.578126	1.59258E-13
TAGLN	transgelin [Source:HGNC Symbol;Acc:HGNC:11553]	1.639792	3.93602E-12
INSIG1	insulin induced gene 1 [Source:HGNC Symbol;Acc:HGNC:6083]	1.657435	2.74927E-11
TM4SF20	transmembrane 4 L six family member 20 [Source:HGNC Symbol;Acc:HGNC:26230]	2.860736	2.39859E-10
SULF1	sulfatase 1 [Source:HGNC Symbol;Acc:HGNC:20391]	1.835271	2.53653E-10
TUBB3	tubulin, beta 3 class III [Source:HGNC Symbol;Acc:HGNC:20772]	1.509186	2.7384E-10
IRAK1	interleukin-1 receptor-associated kinase 1 [Source:HGNC Symbol;Acc:HGNC:6112]	1.481092	7.32617E-10
LINC01444	long intergenic non-protein coding RNA 1444 [Source:HGNC Symbol;Acc:HGNC:50769]	2.080967	1.84299E-09
LDLR	low density lipoprotein receptor [Source:HGNC Symbol;Acc:HGNC:6547]	1.698726	1.08937E-08
PHLDA2	pleckstrin homology-like domain, family A, member 2 [Source:HGNC Symbol;Acc:HGNC:12385]	1.405997	3.00783E-08
PMEPA1	prostate transmembrane protein, androgen induced 1 [Source:HGNC Symbol;Acc:HGNC:14107]	1.563129	7.72665E-08
MRPL2	mitochondrial ribosomal protein L2 [Source:HGNC Symbol;Acc:HGNC:14056]	1.998278	1.0772E-07
PLXNC1	plexin C1 [Source:HGNC Symbol;Acc:HGNC:9106]	1.259832	1.46288E-07
DHCR24	24-dehydrocholesterol reductase [Source:HGNC Symbol;Acc:HGNC:2859]	1.16178	8.26376E-07
TUBA1A	tubulin, alpha 1a [Source:HGNC Symbol;Acc:HGNC:20766]	1.129236	1.2827E-06
LAMA1	laminin, alpha 1 [Source:HGNC Symbol;Acc:HGNC:6481]	1.643878	1.8498E-06
KRT34	keratin 34 [Source:HGNC Symbol;Acc:HGNC:6452]	1.826842	5.46012E-06
COPRS	coordinator of PRMT5, differentiation stimulator [Source:HGNC Symbol;Acc:HGNC:28848]	1.058071	1.38316E-05
LRP8	low density lipoprotein receptor-related protein 8, apolipoprotein e receptor [Source:HGNC Symbol;Acc:HGNC:6700]	1.225934	1.46044E-05
CMSS1	cms1 ribosomal small subunit homolog (yeast) [Source:HGNC Symbol;Acc:HGNC:28666]	1.17734	1.90063E-05
FDPS	farnesyl diphosphate synthase [Source:HGNC Symbol;Acc:HGNC:3631]	1.02293	1.95916E-05
BHLHE41	basic helix-loop-helix family, member e41 [Source:HGNC Symbol;Acc:HGNC:16617]	1.161356	2.77057E-05
KDR	kinase insert domain receptor (a type III receptor tyrosine kinase) [Source:HGNC Symbol;Acc:HGNC:6307]	1.499663	2.92042E-05
CTPS1	CTP synthase 1 [Source:HGNC Symbol;Acc:HGNC:2519]	1.215288	3.41216E-05

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CCDC85A	coiled-coil domain containing 85A [Source:HGNC Symbol;Acc:HGNC:29400]	2.3642	5.15725E-05
RPL36A-HNRNPH2	RPL36A-HNRNPH2 readthrough [Source:HGNC Symbol;Acc:HGNC:48349]	2.969909	5.57432E-05
EBP	emopamil binding protein (sterol isomerase) [Source:HGNC Symbol;Acc:HGNC:3133]	1.086353	9.28651E-05
LINC01443	long intergenic non-protein coding RNA 1443 [Source:HGNC Symbol;Acc:HGNC:50768]	1.892397	0.000100212
SPECC1L-ADORA2A	SPECC1L-ADORA2A readthrough (NMD candidate) [Source:HGNC Symbol;Acc:HGNC:49185]	1.489043	0.000100812
ANKRD35	ankyrin repeat domain 35 [Source:HGNC Symbol;Acc:HGNC:26323]	2.082923	0.000125709
PRELP	Proline arginine-rich end leucine-rich repeat protein [Source:HGNC Symbol;Acc:HGNC:9357]	1.257404	0.000130688
FAM53B	family with sequence similarity 53, member B [Source:HGNC Symbol;Acc:HGNC:28968]	4.140408	0.000137422
LRRC8E	leucine rich repeat containing 8 family, member E [Source:HGNC Symbol;Acc:HGNC:26272]	1.132353	0.00014194
HSD3B7	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7 [Source:HGNC Symbol;Acc:HGNC:18324]	1.422454	0.000161714
NCR3LG1	natural killer cell cytotoxicity receptor 3 ligand 1 [Source:HGNC Symbol;Acc:HGNC:42400]	1.029262	0.000202593
CLCN5	chloride channel, voltage-sensitive 5 [Source:HGNC Symbol;Acc:HGNC:2023]	1.113708	0.00020509
ACAT2	acetyl-CoA acetyltransferase 2 [Source:HGNC Symbol;Acc:HGNC:94]	1.001669	0.000228977
DCTPP1	dCTP pyrophosphatase 1 [Source:HGNC Symbol;Acc:HGNC:28777]	1.005396	0.000252872
MARCH3	membrane-associated ring finger (C3HC4) 3, E3 ubiquitin protein ligase [Source:HGNC Symbol;Acc:HGNC:28728]	1.089764	0.000328179
FAM225A	family with sequence similarity 225, member A (non- protein coding) [Source:HGNC Symbol;Acc:HGNC:27855]	1.603495	0.000433013
IDI1	isopentenyl-diphosphate delta isomerase 1 [Source:HGNC Symbol;Acc:HGNC:5387]	1.055258	0.00059642
NME1	NMENM23 nucleoside diphosphate kinase 1 [Source:HGNC Symbol;Acc:HGNC:7849]	1.016969	0.00072965

Appendix Table 5. Pathways related to upregulated genes in C-O clones (clones with potential to differentiate into osteo/cementoblastic phenotype).

Pathway (code)	Fold Enrichment	*p* value	Gene ID
5HT1 type receptor mediated signaling pathway (P04373)	2.53	1.00E+00	*PRKAR2B*
5HT2 type receptor mediated signaling pathway (P04374)	3.48	1.00E+00	*PRKCD; PRKCZ*

Acetate utilization (P02722)	38.84	1.00E+00	*ACSS1*
Adrenaline and noradrenaline biosynthesis (P00001)	3.88	1.00E+00	*INMT*
Alpha adrenergic receptor signaling pathway (P00002)	9.32	1.00E+00	*ADRA2A; PRKCD*
Alzheimer disease-amyloid secretase pathway (P00003)	5.14	1.00E+00	*CHRNA7; PRKCD; PRKCZ*
Alzheimer disease-presenilin pathway (P00004)	4.7	7.24E-01	*JUP; MMP12; WNT16; WNT2; WNT2B;*
Angiogenesis (P00005)	4.63	1.42E-01	*DLL1; MAP3K1; PRKCD; PRKCZ; PRR5; WNT2; WNT2B*
Apoptosis signaling pathway (P00006)	4.78	6.77E-01	*HSPA2; MAP3K1; PRKCD; TNFRDF10C; TNFSF10;*
Axon guidance mediated by Slit/Robo (P00008)	4.48	1.00E+00	*ROBD3*
B cell activation (P00010)	1.62	1.00E+00	*PRKCD*
Beta1 adrenergic receptor signaling pathway (P04377)	2.53	1.00E+00	*PRKAR2B*
Beta2 adrenergic receptor signaling pathway (P04378)	2.53	1.00E+00	*PRKAR2B*
Cadherin signaling pathway (P00012)*	6.64	1.73E-03	*PCDH18; PCDHB2; PCDHB4; PCDHGA6; PCDHGA10; PCDHGB2; WNT16; WNT2; WNT2B*
CCKR signaling map (P06959)	3.37	1.00E+00	*CPE; PPARG; PRKCD; PTGS2; RGS2*
Cytoskeletal regulation by Rho GTPase (P00016)	2.81	1.00E+00	*MYO3B; PRR5*
De novo purine biosynthesis (P02738)	3.88	1.00E+00	*ADSSL1*
Dopamine receptor mediated signaling pathway (P05912)	3.95	1.00E+00	*EPB41L3; PRKAR2B*
EGF receptor signaling pathway (P00018)	4.19	1.00E+00	*AREG; MAP3K1; PRKCD; PRKCZ*
Endothelin signaling pathway (P00019)	5.42	1.00E+00	*PRKAR2B; PRKCD ; PRKCZ; PTGS2*
Enkephalin release (P05913)	6.66	1.00E+00	*PENK; PRKA2B*
FGF signaling pathway (P00021)	2.82	1.00E+00	*MAP3K1; PRKCD; PRKCZ*
GABA-B receptor II signaling (P05731)	3.07	1.00E+00	*PRKAR2B*
Gonadotropin-releasing hormone receptor pathway (P06664)	2.97	1.00E+00	*BMP4; MAP3K1; PLA2G6; PPARG; PRKCD; PRKCZ*
Hedgehog signaling pathway (P00025)	5.55	1.00E+00	*PRKAR2B*
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway (P00026)	3.55	1.00E+00	*ADRA2A; PRKAR2B; RGS2; RGS4; RGS5*
Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway (P00027)	4.74	7.00E-01	*RGS2; RGS4; RGS5; PRKCD; PRKCZ*
Histamine H1 receptor mediated signaling pathway (P04385)	5.3	1.00E+00	*PRKCD; PRKCZ*
Histamine H2 receptor mediated signaling pathway (P04386)	4.48	1.00E+00	*PRKA2B*

Inflammation mediated by chemokine and cytokine signaling pathway (P00031)	2.23	1.00E+00	*AGS4; CLL11; MYO3B; PRKCZ; PTGS2*
Integrin signaling pathway (P00034)	2.43	1.00E+00	*LAMA5; COL27A1; ITGB8; MAP3K1*
Interleukin signaling pathway (P00036)	1.19	1.00E+00	*IL12RB2*
Ionotropic glutamate receptor pathway (P00037)	2.33	1.00E+00	*SHANK2*
Metabotropic glutamate receptor group II pathway (P00040)	2.43	1.00E+00	*PRKAR2B*
Metabotropic glutamate receptor group III pathway (P00039)	1.69	1.00E+00	*PRKAR2B*
Muscarinic acetylcholine receptor 1 and 3 signaling pathway (P00042)	3.88	1.00E+00	*PRKCD; PRKCZ*
Muscarinic acetylcholine receptor 2 and 4 signaling pathway (P00043)	1.82	1.00E+00	*PRKAR2B*
Nicotine degradation (P05914)	10.59	1.00E+00	*INMT*
Nicotinic acetylcholine receptor signaling pathway (P00044)	2.31	1.00E+00	*CHRAN7; MYO3B*
Notch signaling pathway (P00045)	2.65	1.00E+00	*DLL1*
Opioid proenkephalin pathway (P05915)	3.43	1.00E+00	*PENK*
Oxytocin receptor mediated signaling pathway (P04391)	4.02	1.00E+00	*PRKCZ; SDK1*
Parkinson disease (P00049)	1.17	1.00E+00	*HSPA2*
PDGF signaling pathway (P00047)	1.56	1.00E+00	*ARHGAP6; PRR5*
Pyridoxal-5-phosphate biosynthesis (P02759)	58.26	1.00E+00	*PSAT1*
Ras Pathway (P04393)	1.53	1.00E+00	*MAP3K1*
Serine glycine biosynthesis (P02776)	23.3	1.00E+00	*PSAT1*
T cell activation (P00053)	1.21	1.00E+00	*MAP3K1*
TGF-beta signaling pathway (P00052)	2.26	1.00E+00	*BMP4; INHBE*
Thyrotropin-releasing hormone receptor signaling pathway (P04394)	3.88	1.00E+00	*PRKCD; PRKCZ*
Toll receptor signaling pathway (P00054)	5.83	1.00E+00	*MAP3K1; TLR2; PTGS2*
Transcription regulation by bZIP transcription factor (P00055)	2.08	1.00E+00	*PRKAR2B*
Vasopressin synthesis (P04395)	8.96	1.00E+00	*CPE*
VEGF signaling pathway (P00056)	4.85	1.00E+00	*PRKCD; PRKCZ; PRR5*
Vitamin B6 metabolism (P02787)	29.13	1.00E+00	*PSAT1*

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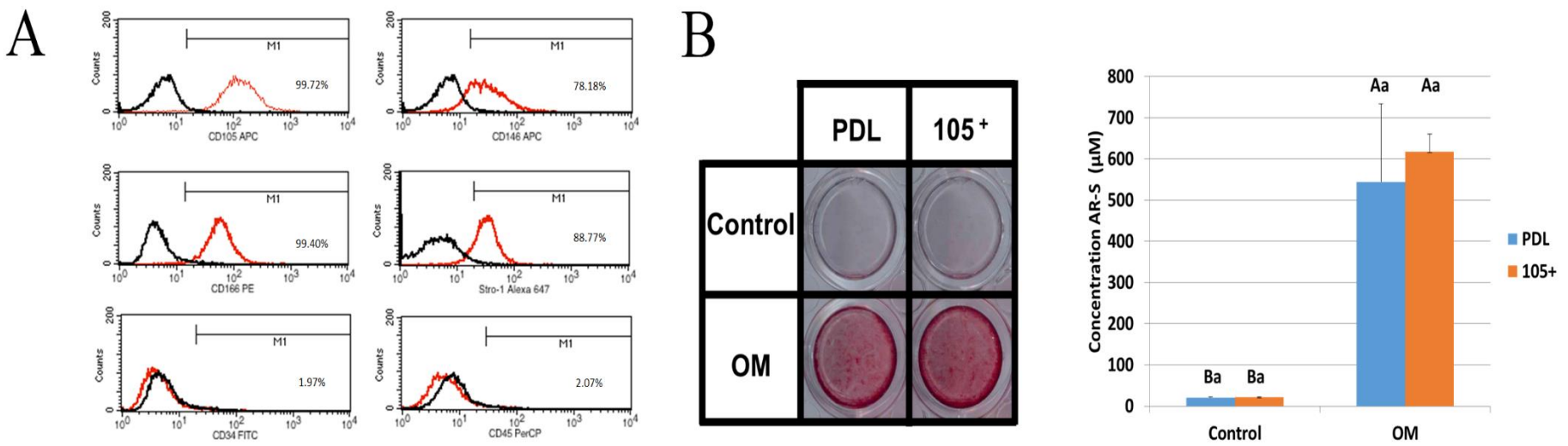
Wnt signaling pathway (P00057) *	4.87	5.70E-04	*ADSSL1; PCDH18; PCDHB2; PCDHGA6; PCDHGA10; PCDHGB2; PCDHGB4; PRKCD; PRKCZ; SFRP2; WNT16; WNT2; WNT2B*

Pathways in bold were pathways that presented both upregulated as downregulated genes in C-O clones (See Appendix Table 6).
*: represent significantly overrepresented pathways compared to *Homo sapiens* genome background (See Table 1).

Appendix Table 6. Pathways related to downregulated genes in C-O clones (clones with potential to differentiate into osteo/cementoblastic phenotype).

Pathway (code)	Fold Enrichment	*p* value	Gene ID
Androgen/estrogene/progesterone biosynthesis (P02727)	76.82	5.14E-02	*HDS3B7; SOAT2*
Angiogenesis (P00005)	2.84	1.00E+00	*KDR*
Cholesterol biosynthesis (P00014)	76.82	5.14E-02	*FDPS; IDI1*
Cytoskeletal regulation by Rho GTPase (P00016)	6.02	1.00E+00	*TUBB3*
De novo purine biosynthesis (P02738)	16.64	1.00E+00	*NME1*
De novo pyrimidine deoxyribonucleotide biosynthesis (P02739)	38.41	1.00E+00	*NME1*
De novo pyrimidine ribonucleotides biosythesis (P02740)	71.33	5.96E-02	*CTS1; NME1*
Gonadotropin-releasing hormone receptor pathway (P06664)	2.12	1.00E+00	*TUBA1A*
Huntington disease (P00029)	3.59	1.00E+00	*TUBB3*
Integrin signalling pathway (P00034)	2.6	1.00E+00	*LAMA1*
Toll receptor signaling pathway (P00054)	8.32	1.00E+00	*IRAK1*
VEGF signaling pathway (P00056)	6.94	1.00E+00	*KDR*

Pathways in bold were pathways that presented both upregulated as downregulated genes in C-O clones (See Appendix Table 5).



Appendix Figure 1. Periodontal ligament derived (PDL) CD105^{+}-enriched population characterization. **A**: Flow cytometric analysis of PDL CD105^{+}-enriched population. The relative levels of cell surface expression was analyzed using CD105, CD146, CD166, STRO-1, CD34 and CD45 antibodies (red histograms) and their isotype-matched antibodies as control (black histograms). Note that the purification process by magnetic cell sorting was efficient, since 99.85% of cells were positive for CD105. Cells also expressed other mesenchymal cell-related surface markers, such as CD166, STRO-1 and CD146, and did not expressed hematopoietic related markers CD34 and CD45. **B**: Alizarin Red Stain (AR-S) assay demonstrated that PDL CD105^{+}-enriched population (105+) showed similar potential of biomineralization as the unsorted population (PDL) when cultivated under osteogenic induction (OM). Quantification AR-S demonstrated that both PDL CD105^{+}-enriched population and unsorted PDL presented significant increase of AR-S when cultivated in OM comparing control medium, but no statistical significant difference between PDL CD105^{+}-enriched population and unsorted PDL was observed in OM. Distinct uppercase letters represent significant difference ($p<0.05$) intragroup, and distinct lowercase letters represent significant difference ($p<0.05$) intergroup (Two-way Anova/Tukey test).

2.2. Artigo 2: Transcriptomic profile of human periodontal ligament cell clones in osteoblastic/cementoblastic (O/C) differentiation

Short title: RNA-seq of periodontal cell clones in O/C differetiation

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Abstract

The periodontium is composed by heterogeneous cell populations, comprising cells related to the periodontal ligament and gingiva, non-mineralized tissues, and other two mineralized tissues, cementum and alveolar bone. When the periodontium is destructed in periodontitis, the regeneration of lost tissues is very difficult to obtain. Recently, regenerative approaches based on biological knowledge of tissue formation have been investigated. However, the understanding of commitment of periodontal ligament cells to form different tissues still not been fully elucidated. To better define the gene expression in differentiating periodontal cells, here we compared the transcriptomic profile of primary cell clones committed to osteoblastic/cementoblastic (O/C) differentiation (C-O clones) and clones committed to fibroblastic phenotype (C-F clones) using next-generation sequencing technology (RNA-seq) when cultivated under O/C differentiation induction condition (OM) *in vitro*. Some genes commonly reported in many biological processes and pathways were investigated in immortalized cell clones with correlated phenotypes during the process of differentiation induction to validate the RNA-seq. The C-O clones showed higher number of up-regulated differentially expressed genes (DEG) than C-F clones. The up-regulated genes in C-O clones were related to many biological processes, including "mesenchymal cell differentiation", "regulation of osteoblast differentiation", "regulation of ossification", "mesenchyme development", and "vasculature development". The "angiogenesis" and "CCKR signaling" pathways were significantly overrepresented in C-O clones compared to C-F clones. The genes *BMP4, WNT2, WNT5A* and *WIF1* showed to be differentially expressed between periodontal ligament clones with distinct differentiation commitment potential, both in primary as in immortalized cell clones. The up-regulation of these genes involved to those biological processes and pathways are related to higher commitment to O/C differentiation in cells of periodontal ligament.

Keywords: periodontal ligament cells, differentiation, mineralization, high-throughput transcriptome sequencing, differential gene expression, pathway analysis.

Introduction

Periodontitis is a polimicrobial infection-induced inflammatory disease in the periodontium characterized by connective attachment loss and alveolar bone destruction (Armitage, 1999). Epidemiological studies show that periodontitis still a prevalent disease world-wide (Dye, 2012), and that this periodontal disease may lead to functionally comprised dentition and affect the quality of life of many subjects (Meusel *et al.*, 2015; Mourao *et al.*, 2015).

For the last decades, many attempts have been made in order to regenerate the tissues destroyed by periodontitis, including bone replacement grafts, guided tissue regeneration, enamel matrix derivative, and combination of them (Susin & Wikesjo, 2013; Avila-Ortiz *et al.*, 2015; Kao *et al.*, 2015). However, these current clinical approaches have not shown complete and predictable regeneration of periodontal tissues, i.e., formation of cementum, periodontal ligament (PDL), and alveolar bone in a similar pattern as previously to the periodontal disease occurrence (Susin & Wikesjo, 2013; Avila-Ortiz *et al.*, 2015; Kao *et al.*, 2015; Lin *et al.*, 2015). Therefore, emerging regenerative approaches based on a sound biological rationale have been proposed to achieve improved clinical outcomes (Macneil & Somerman, 1999; Ivanovski *et al.*, 2014; Lin *et al.*, 2015).

The periodontium is a complex structure composed by mineralized (cementum and alveolar bone) and non-mineralized tissues (PDL and gingiva) in an intimate relationship. Consequently, the regeneration of the periodontium demands a well-coordinated process of cell differentiation to form these tissues. Although we know that PDL is constituted by heterogeneous cell populations, comprising fibroblastic and mineralized tissue-forming progenitor cells (Liu *et al.*, 1997; Seo *et al.*, 2004; Barkana *et al.*, 2007; Fujii *et al.*, 2008; Saito *et al.*, 2014), the mechanisms that regulate the differentiation process are not fully understood (Fujii *et al.*, 2008; Han *et al.*, 2015). Here we purified PDL cell clones committed to O/C phenotype (C-O clones) and clones committed to fibroblastic phenotype (C-F clones), and explored their transcriptome, using a next-generation sequencing technology (RNA-seq) , when cultivated under osteogenic induction factors. The present study aimed to describe the transcriptional profile involved in O/C differentiation of PDL cell clones. To our knowledge, this is the first study to analyze O/C differentiation in human PDL cell clones on comprehensive scale using high-throughput transcriptome sequencing.

Material and Methods

Cell culture and induction of O/C differentiation

The procedures of this study was approved by Institutional Review Board of Piracicaba Dental School – University of Campinas (#053/2013) and informed consent was obtained from patients. Culture of PDL-derived cells and magnetic-activated cell sorting (MACS) to obtain a $CD105^{+}$ enriched population were performed as previously described (Silverio *et al.*, 2010). Isolation of single cell–derived colonies (clones) from a CD105-enriched PDL progenitor cell population was performed by the ring-cloning technique as previously described (Saito et al., 2014).

Two primary cell clones that showed the potential to differentiate into O/C phenotype (G13 and G48 clones), named C-O clones, and two primary cell clones that did not showed this potential, defined as presenting fibroblastic phenotype (G16 and G23 clones), named C-F clones, by our previous studies were selected to have their RNA extracted after cultivation in O/C induction medium for 14 days and be analyzed by RNA-seq. One immortalized cell clone with characteristic of C-O clone (named 2-14), and one immortalized cell clone with characteristics of C-F clone (named 2-52) were used to validate the data by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

For *in vitro* biomineralization assay, each cell clone were seeded ($2x10^5$cells/well) in 24-well plates with Dulbecco's modified Eagle medium high glucose (Gibco, Cat #1199-065,USA) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, Cat #10500-064, USA), 1% Anti-Anti (Gibco, Cat #15240-062, USA) (DMEM) and incubated for 24 hours. After this period, cells were cultivated in osteogenic medium (OM) composed by DMEM supplemented with 50 mg/mL ascorbic acid (Sigma-Aldrich, Cat #A4544, China) 10 mM b-glycerophosphate (Sigma-Aldrich, Cat #G9422, USA), and 10^{-8} M dexamethasone (Sigma-Aldrich, Cat #D2915, USA) for 21 days (immortalized clones) or 28 days (primary clones) with media change twice per week. At the end of the induction period, the Alizarin Red staining (AR-S) assay was performed as previously described (Zhao *et al.*, 2015). Primary and immortalized cell clones were cultivated in control (DMEM) and O/C induction medium (OM), and confirmed their phenotype as C-O and C-F clones (Figure 1).

RNA extraction

For RNA isolation, each cell clone were seeded ($2x10^5$cells/well) in 6-well plates with DMEM and incubated for 24 hours. After this period, the medium was replaced by OM. Cells were cultured for 14 days, with media changes twice weekly. At the established period

(3, 7 or 14 days), cells were lyzed for total RNA extraction using TRIzol reagent (Invitrogen, Cat #15596-018, USA), as previously described (Saito *et al.*, 2014), followed by a phenol/chloroform extraction, and isopropanol precipitation. RNA samples were treated with Turbo DNA-free to remove genomic DNA (Ambion, Cat #1907, USA).

RNA deep sequencing (RNA-seq)

RNA-seq was performed using Illumina TruSeq RNA Sample Preparation kit v2 (Illumina, Cat# RS-122-2002, USA), according to the manufacturer's instruction. Briefly, 1 µg total DNA-free RNA samples obtained after cultivation of primary cell clones during 14 in OM were processed to purify mRNA molecules. mRNA was fragmented and copied into first strand cDNA, followed by second strand cDNA synthesis. cDNA fragments were submitted to end repair process, addition of single adenosine base and adapter ligation. Finally, processed cDNA were amplified by 15 PCR cycles to create de cDNA library. Library was read in HiSeq 2500 (v3) (Illumina, San Diego, CA, USA).

Analysis of RNA-seq data

Raw data quality was evaluated by *FastQC* (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and filtered reads of RNA library were mapped against the human genome (GRCh38) using the pipeline *Tophat2-Cufflinks* (Trapnell *et al*., 2012). Fragments per kilobase of transcript per million (FPKM) mapped reads were calculated by *RSEM* program (Li & Dewey, 2011). Differentially expressed genes (DEG) between C-O and C-F clones cultivated in OM were obtained by *DESeq* and *EdgeR* packages (R/Bioconductor)(Anders *et al.*, 2013) with α=5% and $|\log2(\textit{fold change})| \geq 1$. The heatmap graphic was generated based on z-score values calculated from FPKMs values of DEG between C-F versus C-O, using R package "pheatmap".

DEG between C-O versus C-F were evaluated for gene ontology (GO) overrepresentation analysis (http://amigo.geneontology.org) compared to *Homo sapiens* genome background (Ashburner *et al.*, 2000; Mao *et al.*, 2015), and pathway overrepresentation in Panther Classification System (http://pantherdb.org/), using Bonferroni correction for multiple tests to calculate p-value.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Single-stranded complementary DNA (cDNA) was synthesized from 0.5 µg total DNA-free RNA using ExScript RTase kit (Takara Biotechnology Co., P.R. China), following

the manufacturer's recommendations. qRT-PCR was performed using the samples of cDNA and SYBR Green II (Takara Biotechnology Co., P.R. China) on the Thermal Cycler Dice Real Time System Takara Biotechnology Co., P.R. China) under the following conditions: 95°C for 10 s (initial denaturation), then 40 cycles at [95°C for 5 s and 60°C for 30 s], followed by a dissociation program at 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s, using primers sequences *BMP4, WNT2, WNT5A, WIF1* and *GAPDH* (Table 1). Relative quantification of reactions products was accomplished to *GAPDH* and calculated by the ΔCT-method.

Statistical Analysis

The data were expressed as mean ± standard deviation (SD), two-way analysis of variance (ANOVA) were used to analyze difference among three or more groups.Transformation of data was used when necessary. P values of <0.05 were considered significant.

Results and discussion

The lack of characterization of different cell populations and markers of commited progenitors has hampered a faster development of tissue engineering approaches (Menicanin *et al.*, 2009). "–omics" has emerged as tool for comprehensively understand the biological events that occur in certain processes or diseases (Kebschull *et al.*, 2017). This is the first report of transcriptomic profile of purified cell clones from PDL during differentitation into O/C phenotype. The isolation of cell clones allowed the analysis of purified cell populations that constitute the PDL (Liu *et al.*, 1997; Barkana *et al.*, 2007; Tomokiyo *et al.*, 2008; Saito *et al.*, 2014). The RNA-seq analysis allows to describe the range of transcripts without the need for a priori target specification, leading to an unbiased approach to find novel genes important for cell commitment (Ayturk *et al.*, 2013; Twine *et al.*, 2014).

RNA collected from primary PDL cell clones were submitted to RNA-seq analysis. By the *FastQC* analysis, around 84% of the reads were at optimum quality and aligned to human genome (Appendix Table 1). Only the aligned portions to the genome were retained for subsequent analysis. The heatmap shows the total gene expression pattern of C-O clones compared to C-F clones when cultivated in OM for 14 days, illustrating that C-O clones present higher numbers of up-regulated genes (388) than down-regulated genes (84) when inducted to O/C differentiation (Figure 2) (list of up-regulated and down-regulated genes in Appendix Tables 2 and 3, respectively).

To understand the biological context of DEG, Gene Ontology (GO) analysis was used to map the biological process overrepresentation related to the up-regulated genes in C-O clones and C-F clones cultivated during 14 days in OM, GO analysis showed that there was 239 biological processes enriched compared to *Homo sapiens* genome (Appendix Table 4), whereas no overrepresented biological process was observed when down-regulated genes in C-O clones cultivated in OM were evaluated. Among the 239 biological processes overrepresented in C-O clones, we found out biological processes that are related to mesenchymal and osteoblastic differentiation and angiogenesis, such as "mesenchymal cell differentiation" (GO: 0048762), "regulation of osteoblast differentiation" (GO: 0045667), "regulation of ossification" (GO: 0030278), "mesenchyme development" (GO: 0060485), and "vasculature development" (GO: 0001944). These genes related to these biological processes are shown in Table 2.

To further investigate the differences between C-O and C-F clones, the DEG were analyzed for pathway overrepresentation. C-O clones exhibited a significantly enrichement of up-regulated genes in two pathways: "Angiogenesis" (P00005) and "CCKR signaling map" (P06959) (Table 3), and none significantly overrepresented pathway was detected in down-regulated genes of C-O clones. Although "Cadherin signaling pathway" (P00012), "Inflammation mediated by chemokine and cytokine signaling pathway" (P00031), and "Wnt signaling pathway" (P00057) were not considered statistically enriched pathways in C-O clones, they showed 10 or more genes related to these pathways (Appendix Table 5). Certain genes related to mineralized tissue formation (Yan *et al.*, 2009; Lan *et al.*, 2014), such as TGF-β/BMPs (*BMP2, BMP4*), Wnt family (*WNT2, WNT5A, WIF1*), and Angiogenesis (*PDGFRA, VEGFA, WNT5A*) were up-regulated in C-O clones, and the expression of these genes may favor the commitment to O/C differentiation of these C-O clones.

The up-regulation of genes related to Angiogenesis pathway in O/C differentiation of C-O clones is in accordance to previous report describing bone mesenchymal stem cell differentiation into osteoblastic phenotype (Granchi *et al.*, 2010), suggesting that expression of these genes are important to bone formation. The up-regulation of *VEGFA* was also recently reported in a RNA-seq analysis of genes related to osteoblast differentiation (Twine *et al.*, 2014). *ANGPT1* is also related to angiogenesis in angiopoetin-dependent pathway. Although the expression of genes related to this pathway have been reported in bone cells, the role in bone cell development is not clear (Granchi *et al.*, 2010). *AMIGO2* is another gene that was described to be up-regulated in our primary C-O clones compared to C-F clones (Appendix Table 2). Previous report had also shown that the expression of this gene was higher in

osteoblast differentiating cells (Granchi *et al.*, 2010). At the time of publication, Granchi *et al.* could not describe the function of *AMIGO2* in osteoblast differentiation, but a recent report described that *AMIGO2* interact with *PDK1* and they are related to cell survival and angiogenesis (Park *et al.*, 2015a). In fact, the gene *PDK1* was also up-regulated in our C-O clones (Appendix Table 2) and is related to many pathways, including the significant enriched "CCKR signaling pathway", as well as other pathways such as "Inflammation mediated by chemokine and cytokine signaling pathway" and "PDGF signaling pathway" (Appendix Table 5).

The gene *CPE* was up-regulated to primary C-O clones and is related to "CCKR signaling pathway". CPE was reported to interact with Wnt signaling pathway (Skalka *et al.*, 2013). Wnt signaling has been classically divided into canonical/β-catenin and non-canonical pathways (Ling *et al.*, 2009), but a cross-talk between these two Wnt pathways is important for osteoblastic differentiation (Baksh *et al.*, 2007). Although Wnt signaling pathway was not considered significantly enriched in C-O clones when cultivated in OM, it was noticed that some genes related to angiogenesis pathway (*SFRP1, WNT2, WNT2B* and *WNT5A)* were also present in Wnt signaling pathway. Therefore, we speculate that even though the up-regulation of these genes were important to commitment of C-O clones into O/C phenotype, the Wnt signaling pathway was not considered enriched by Panther Classification System because the number of genes expected to be up-regulated in *Homo sapiens* genome for Wnt pathway was higher (309 genes) than for the other pathways (176, and 173 for Angiogenesis, and CCKR signaling, respectively), therefore, the number of up-regulated genes was not sufficient to achieve statistical significance.

The expression of genes *WNT2, WIF1, BMP4,* and *WNT5A* were selected to be analyzed by qRT-PCR during the process of differentiation in immortalized periodontal cell clones that present similar distinct phenotypes of C-O and C-F clones as primary clones to validate the RNA-seq analysis. These genes had been commonly related to biological processes and pathways significantly overrepresented in C-O clones compared to C-F clones when cultivated under O/C differentiation induction, and had been previously shown to be up-regulated in C-O clones in cultivation without induction in our previous study (data not shown). Among the up-regulated genes in C-O clones cultivated in differentiating O/C medium induction, *WNT2*, *WIF1,* and *BMP4* were transcripts that were previously noticed to be also up-regulated in C-O clones compared to C-F clones when cultivated without stimulation in our previous study (data not shown). *WNT5A* was not observed in C-O clones cultivated without O/C induction, but it was significantly expressed in C-O clones compared to C-F clones in

differentiation induction, and it has demonstrated to influence PDL cells (Hasegawa *et al.*, 2015), therefore, it was also investigated during osteogenic induction of the immortalized clones that presented O/C phenotype (2-14) and fibroblastic phenotype (2-52).

WNT2 had a significant increase at day 14 of differentiation induction in 2-14 compared to 2-52 (Figure 3A). The gradual increase in expression of Wnt related genes, including *WNT2*, was also reported in osteoblast differentiation (Zhong *et al.*, 2012), and in human dental papilla cells treated with osteogenic medium associated with dental pulp cell-conditioned medium (Park *et al.*, 2015b).

WIF1 presented a different behavior comparing 2-14 and 2-52 by qRT-PCR to primary C-O and C-F clones analyzed by RNA-seq. *WIF1* was also detected to be up-regulated in primary C-O clones by RNA-seq, but no difference was detected between 2-14 and 2-52 by qRT-PCR at 14 days of induction, and in earlier time points, the expression of *WIF1* was higher in 2-52 than 2-14 (Figure 3B). The expression of *WIF1* has been reported in different types of cells in the literature. It has been reported to be expressed in $CD271^+$ mesenchymal stromal cells (Churchman *et al.*, 2012) and to be higher expressed in cementoblast compared to osteoblasts (Matthews *et al.*, 2016). *WIF1* is increased in late phases of osteoblast maturation, as a feedback loop that controls osteoblast maturation (Vaes *et al.*, 2005). Therefore, we can suggest that expression of *WIF1*can be both related to inhibition of commitment to osteoblastic phenotype, as well as downregulation of Wnt pathway after maturation of mineralizing phenotype in O/C cells.

BMP4 was up-regulated in C-O clone (2-14) compared to C-F clone (2-52) in all evaluated periods (Figure 3C). Previous study showed that expression of *BMP4* is higher in immortalized cell clones that present capacity to differentiate into O/C than clones that present less capacity (Tomokiyo *et al.*, 2008). *BMP4* was also shown to be one of the most predictive gene expression markers of *in vivo* bone formation potential according to a recent RNA-seq analysis (Twine *et al.*, 2014). *BMP4* has also been reported to necessary to act synergically with *VEGF* to enhance bone formation (Peng *et al.*, 2002).

WNT5A was up-regulated in 2-14 in all periods, but the expression was higher in the initial period of O/C induction and decreased through time (Figure 3D). Studies have shown that bone marrow commitment to osteoblast differentiation is regulated by a switch from Wnt canonical to non-canonical signaling (Baksh *et al.*, 2007), in which *WNT5A* is considered one of the main Wnt non-canonical molecules that regulate osteoblast differentiation (Baksh *et al.*, 2007; Hay *et al.*, 2014; Kobayashi *et al.*, 2015).

In conclusion, by the comparison of transcriptional profile between PDL cell clones that were able to differentiate into O/C phenotype (C-O clones) and clones that were not able to differentiate into O/C phenotype (fibroblastic committed) (C-F clones) using RNA-seq technology, we demonstrate that angiogenesis and CCKR signaling pathways, and genes related to Wnt signaling pathway are important to commitment of PDL cells to O/C phenotype and that the biological processes "mesenchymal cell differentiation", "regulation of osteoblast differentiation", "regulation of ossification", "mesenchyme development", and "vasculature development" are involved in this differentiation process. The genes *BMP4, WNT2, WNT5A* and *WIF1* showed to be differentially expressed between PDL clones with distinct differentiation commitment potential, both in primary cell clones or immortalized clones. The up-regulation of these genes involved to those biological processes and pathways are related to higher commitment to O/C differentiation in cells of periodontal ligament. We expect that the knowledge of these biological processes and pathways involved in regulation of PDL cells committed to O/C phenotype serve as basis for promoting new therapies for periodontal regeneration.

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Figures

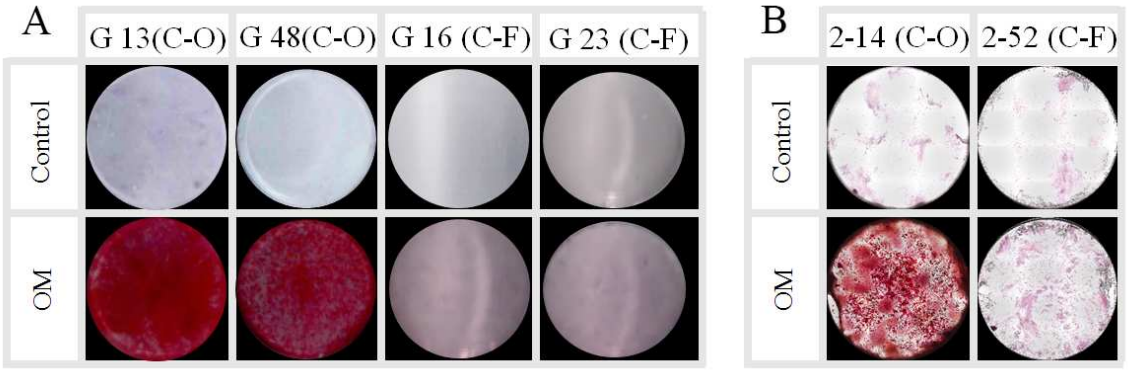

Figure 1. A. Alizarin Red Stain (AR-S) assay of primary periodontal ligament clones demonstrated that clones G13 and G48 were able to form minerals *in vitro,* and they were named as osteo/cementoblastic clones (C-O clones), whereas G16 and G23 were not able to form minerals *in vitro*, and were named as fibroblastic clones (C-F clones). **B.** AR-S assay of immortalized cell 2-14 and 2-52, showed two distincts phenotypes of C-O and C-F clones, respectively.

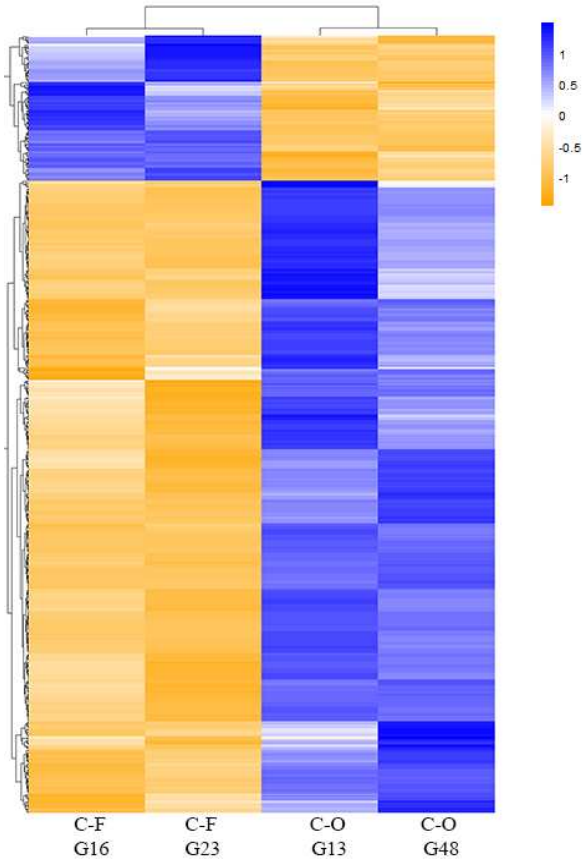

Figure 2. C-O clones showed a higher trancriptional activity when cultivated in osteoblastic/cementoblastic induction medium. C-O clones presented 388 genes up-regulated compared to C-F clones, and only 84 down-regulated genes.

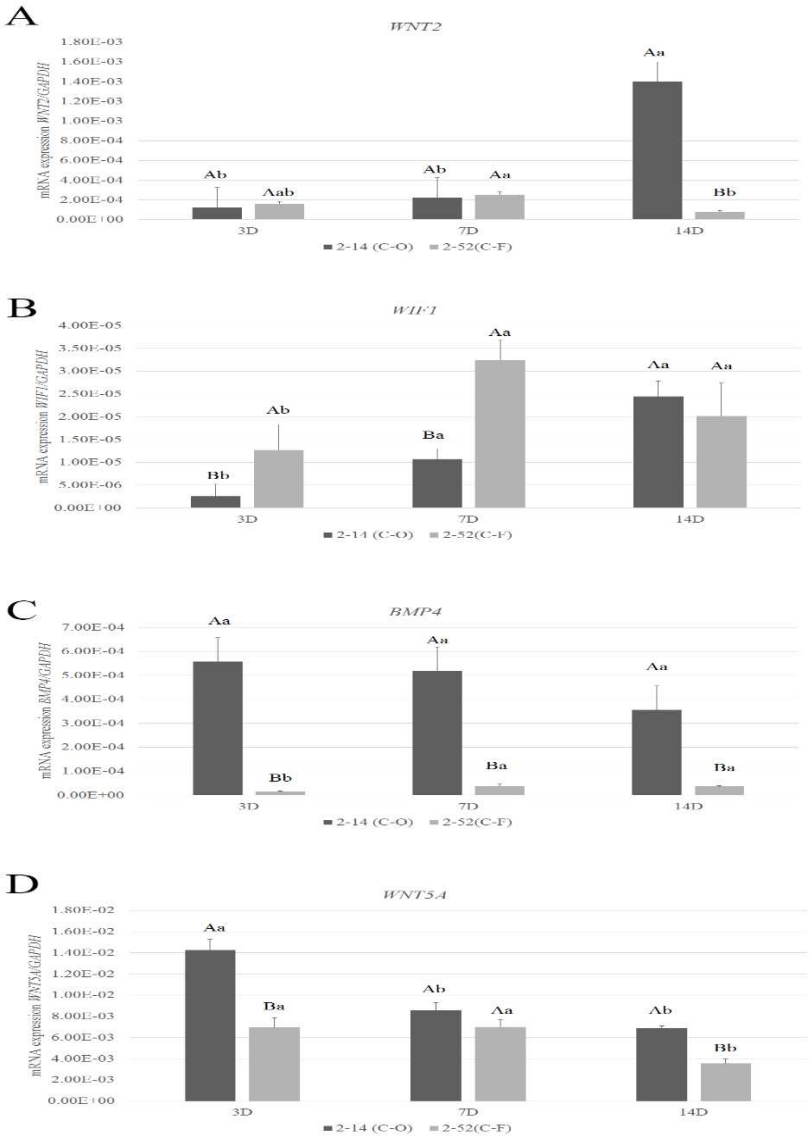

Figure 3. Gene expression analyzed by qRT-PCR in immortalized cell clones expressing distinct phenotypes: 2-14 present is a clone with potential to differentiate into osteo/cementoblastic phenotype (C-O), and 2-52 is a clone that express a fibroblastic phenotype (C-F). Different uppercase letters represent significant difference ($p<0.05$) in the same period (inter-group). Different lowercase letters represent significant difference ($p<0.05$) in the same clone and condition along the time (intra-group).

Tables

Table 1. Specific primer sequence for qRT-PCR.

Gene ID	*Primers* (5'→ 3')
BMP4	Forward: CTGCAACCGTTCAGAGGTC Reverse: TGCTCGGGATGGCACTAC
GAPDH	Forward: GTCAGTGGTGGACCTGACC Reverse: TGCTGTAGCCAAATTCGTTG
WIF1	Forward: CCAGGGAGACCTCTGTTCAA Reverse: TTGGGTTCATGGCAGGTT
WNT2	Forward: TTTGGCAGGGTCCTACTCC Reverse: CCTGGTGATGGCAAATACAA
WNT5A	Forward: CTGCAGCCAACTGGCAGGACT Reverse: CGCGGCTGCCTATCTGCATCA

Table 2. Overrepresented biological processes related mesenchymal differentiation, ossification and angiogenesis and respective up-regulated genes in C-O clones compared to C-F clones. (For additional overrepresented biological processes, see Appendix Table 4).

GO biological process	Fold Enrichment	p-value	Gene Symbol
mesenchymal cell differentiation (GO:0048762)	5.81	7.21E-04	BMP2;BMP4;FAM83D; EDNRB; EFNB1; HGF; LAMA5; MSX1; SEMA3B; SEMA4D; SEMA4G; SFRP1; TGFB2; WNT2; WNT5A
regulation of osteoblast differentiation (GO:0045667)	5.65	1.87E-02	AREG; BMP2; BMP4; CEBPD; FAM20C; HDAC4; HGF; IL6; PTCH1; RORB; SEMA4D; SFRP1
regulation of ossification (GO:0030278)	4.6	5.88E-03	AREG; BMP2; BMP4; CEBPD; ENPP1; FAM20C; HDAC4; HGF; IL6; PTCH1; RORB; SEMA4D; SFRP1;SOST; TGFB2; WNT5A
mesenchyme development (GO:0060485)	4.35	1.21E-02	BMP2; BMP4; EDNRB; EFNB1; FAM83D; FOXF1; HGF; LAMA5; MSX1; SEMA3B; SEMA4D; SEMA4G; SFRP1; TGFB2; WNT2; WNT5A
vasculature development (GO:0001944)	2.79	4.77E-02	ACKR3; ANGPT1; APOE; BMP4; DLL1; EPHB2; FOXF1; HMOX1; ITGB3; LAMA4; LAMA5; PDGFRA; PDPN; PGF; PLAU; PRDM1; PRICKLE1; PTGS2; RSPO3; SAT1; SOX4; TGFB2; THSD7A; VEGFA; WNT2

Table 3. Significantly overrepresented pathways related to up-regulated genes in C-O clones compared to C-F clones. (For additional pathways related to up-regulated genes in C-O clones compared to C-F clones, see Appendix Table 5).

PANTHER Pathways	Fold Enrichment	p-value	Gene Symbol
Angiogenesis (P00005)	4.96	4.09E-05	ANGPT1; BIRC5; DLL1; EFNB1; EPBH2; FOS; MAPK10; PDGFRA; PLD1; PRR5; RERG; SFRP1; VEGFA; WNT2; WNT2B; WNT5A
CCKR signaling map (P06959)	4.1	3.95E-03	CCK; CPE; CXCL1; FOS; IRS1; ITPR1; MAP2K6; MAPK10; MMP3; PDK1; PLAU; PTGS2; RGS2

Appendix: Transcriptomic profile of human periodontal ligament cell clones in osteoblastic/cementoblastic (O/C) differentiation

Appendix Table 1. Summary of mapping results for RNA sequencing.

	R1		R2		Total	
Sample Name	**# Total reads**	**# aligned**	**# Total reads**	**# aligned**	**# Total reads**	**# aligned**
G13 (C-O)	20209360	16988331 (84.1%)	20209360	17039626 (84.3%)	40418720	34027957 (84.2%)
G48 (C-O)	26359512	22089457 (83.8%)	26359512	22150373 (84.0%)	52719024	44239830 (83.9%)
G16 (C-F)	30410108	25617082 (84.2%)	30410108	25688051 (84.5%)	60820216	51305133 (84.4%)
G23 (C-F)	22090731	18685570 (84.6%)	22090731	18774559 (85.0%)	44181462	37460129 (84.8%)

C-O: clone that shows osteo/cementoblastic phenotype. C-F: clone that shows fibroblastic phenotype.

Appendix Table 2. Significantly upregulated genes of C-O clones (clones with potential to differentiate into osteo/cementoblastic phenotype) compared to C-F clones (clones that shows fibroblastic phenotype).

Gene Symbol	Gene_description	|log2FoldChange|	p value
TRPA1	transient receptor potential cation channel, subfamily A, member 1 [Source:HGNC Symbol;Acc:HGNC:497]	4.21649655	1.00426E-59
PDPN	podoplanin [Source:HGNC Symbol;Acc:HGNC:29602]	4.324237457	9.07393E-51
HGF	hepatocyte growth factor (hepapoietin A; scatter factor) [Source:HGNC Symbol;Acc:HGNC:4893]	3.66951968	8.54089E-51
CMKLR1	chemokinelike receptor 1 [Source:HGNC Symbol;Acc:HGNC:2121]	4.163665165	1.29748E-44
CSGALNACT1	chondroitin sulfate N acetylgalactosaminyltransferase 1 [Source:HGNC Symbol;Acc:HGNC:24290]	3.318636751	5.17146E-42
EPB41L3	erythrocyte membrane protein band 4.1like 3 [Source:HGNC Symbol;Acc:HGNC:3380]	3.305274542	1.44924E-40
ADAMTS3	ADAM metallopeptidase with thrombospondin type 1 motif, 3 [Source:HGNC Symbol;Acc:HGNC:219]	3.200155681	2.33735E-40
RGS18	regulator of Gprotein signaling 18 [Source:HGNC Symbol;Acc:HGNC:14261]	6.693539068	6.38254E-40
FGF7	fibroblast growth factor 7 [Source:HGNC Symbol;Acc:HGNC:3685]	3.17190694	7.92897E-40
NR4A2	nuclear receptor subfamily 4, group A, member 2 [Source:HGNC Symbol;Acc:HGNC:7981]	3.74913379	7.7205E-37
LHCGR	luteinizing hormonechoriogonadotropin receptor [Source:HGNC Symbol;Acc:HGNC:6585]	4.245053308	3.15217E-32
AREG	amphiregulin [Source:HGNC Symbol;Acc:HGNC:651]	3.618408623	8.71715E-31

MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase) [Source:HGNC Symbol;Acc:HGNC:7173]	3.054328931	8.73493E-28
COL4A5	collagen, type IV, alpha 5 [Source:HGNC Symbol;Acc:HGNC:2207]	5.490336479	2.15604E-27
TMEM56	transmembrane protein 56 [Source:HGNC Symbol;Acc:HGNC:26477]	4.769031722	1.35167E-26
ARHGAP28	Rho GTPase activating protein 28 [Source:HGNC Symbol;Acc:HGNC:25509]	4.083128281	1.43592E-26
SLC19A3	solute carrier family 19 (thiamine transporter), member 3 [Source:HGNC Symbol;Acc:HGNC:16266]	4.023590091	2.59961E-26
MMD	monocyte to macrophage differentiationassociated [Source:HGNC Symbol;Acc:HGNC:7153]	2.535636734	9.53298E-26
FAM198B	family with sequence similarity 198, member B [Source:HGNC Symbol;Acc:HGNC:25312]	2.882541658	8.33889E-25
PTGS2	prostaglandinendoperoxide synthase 2 (prostaglandin GH synthase and cyclooxygenase) [Source:HGNC Symbol;Acc:HGNC:9605]	3.620794815	2.00243E-24
SLC16A4	solute carrier family 16, member 4 [Source:HGNC Symbol;Acc:HGNC:10925]	3.480164431	2.63153E-24
CCL7	chemokine (CC motif) ligand 7 [Source:HGNC Symbol;Acc:HGNC:10634]	4.371943978	1.98785E-23
STC1	stanniocalcin 1 [Source:HGNC Symbol;Acc:HGNC:11373]	3.018735604	2.27611E-23
TGFB2	transforming growth factor, beta 2 [Source:HGNC Symbol;Acc:HGNC:11768]	2.214200564	7.38027E-23
CD4	CD4 molecule [Source:HGNC Symbol;Acc:HGNC:1678]	3.163178679	2.15262E-22
JUP	junction plakoglobin [Source:HGNC Symbol;Acc:HGNC:6207]	3.419476403	3.53123E-22
CXCL6	chemokine (CXC motif) ligand 6 [Source:HGNC Symbol;Acc:HGNC:10643]	2.569295411	4.05242E-21
GNLY	granulysin [Source:HGNC Symbol;Acc:HGNC:4414]	5.190535331	7.16559E-21
SLC1A3	solute carrier family 1 (glial high affinity glutamate transporter), member 3 [Source:HGNC Symbol;Acc:HGNC:10941]	2.420942616	7.87067E-21
CPE	carboxypeptidase E [Source:HGNC Symbol;Acc:HGNC:2303]	2.346388261	1.05747E-20
PTPN22	protein tyrosine phosphatase, nonreceptor type 22 (lymphoid) [Source:HGNC Symbol;Acc:HGNC:9652]	2.969498831	9.45935E-20
SAA1	serum amyloid A1 [Source:HGNC Symbol;Acc:HGNC:10513]	2.453562632	3.45584E-19
SMOX	spermine oxidase [Source:HGNC Symbol;Acc:HGNC:15862]	2.121291769	5.73511E-19
ITPRIP	inositol 1,4,5trisphosphate receptor interacting protein [Source:HGNC Symbol;Acc:HGNC:29370]	2.00799316	7.07928E-19
PAPPA	pregnancyassociated plasma protein A, pappalysin 1 [Source:HGNC Symbol;Acc:HGNC:8602]	1.897929577	2.32977E-18
PGF	placental growth factor [Source:HGNC Symbol;Acc:HGNC:8893]	3.307071944	3.22074E-18
HMOX1	heme oxygenase (decycling) 1 [Source:HGNC Symbol;Acc:HGNC:5013]	2.028589502	4.00601E-18
ACSL4	acylCoA synthetase longchain family member 4 [Source:HGNC Symbol;Acc:HGNC:3571]	2.748965521	8.22625E-18
TMEM176B	transmembrane protein 176B [Source:HGNC Symbol;Acc:HGNC:29596]	5.934031412	8.73013E-18
CSF3	colony stimulating factor 3 (granulocyte) [Source:HGNC Symbol;Acc:HGNC:2438]	3.266833782	9.6259E-18
FIBIN	fin bud initiation factor homolog (zebrafish) [Source:HGNC Symbol;Acc:HGNC:33747]	2.190914109	1.44041E-17
RRAD	Rasrelated associated with diabetes [Source:HGNC Symbol;Acc:HGNC:10446]	2.165021051	1.55056E-17

PCDHGB4	protocadherin gamma subfamily B, 4 [Source:HGNC Symbol;Acc:HGNC:8711]	2.461232656	1.86373E-17
MSX1	msh homeobox 1 [Source:HGNC Symbol;Acc:HGNC:7391]	1.898931149	4.56856E-17
ISYNA1	inositol3phosphate synthase 1 [Source:HGNC Symbol;Acc:HGNC:29821]	2.342154833	5.45923E-17
SDPR	serum deprivation response [Source:HGNC Symbol;Acc:HGNC:10690]	2.171792735	5.40222E-17
ACSL1	acylCoA synthetase longchain family member 1 [Source:HGNC Symbol;Acc:HGNC:3569]	1.893307986	6.55049E-17
STOM	stomatin [Source:HGNC Symbol;Acc:HGNC:3383]	2.008867676	1.07826E-16
MAP2K6	mitogenactivated protein kinase kinase 6 [Source:HGNC Symbol;Acc:HGNC:6846]	3.185003939	1.14815E-16
KIF20A	kinesin family member 20A [Source:HGNC Symbol;Acc:HGNC:9787]	2.140542708	1.23745E-16
TNFRSF11B	tumor necrosis factor receptor superfamily, member 11b [Source:HGNC Symbol;Acc:HGNC:11909]	1.873706737	1.4462E-16
CHRM2	cholinergic receptor, muscarinic 2 [Source:HGNC Symbol;Acc:HGNC:1951]	2.297458874	1.78018E-16
FLRT2	fibronectin leucine rich transmembrane protein 2 [Source:HGNC Symbol;Acc:HGNC:3761]	1.962986885	3.7985E-16
TIMP3	TIMP metallopeptidase inhibitor 3 [Source:HGNC Symbol;Acc:HGNC:11822]	2.288169091	4.28681E-16
RGS2	regulator of Gprotein signaling 2 [Source:HGNC Symbol;Acc:HGNC:9998]	4.735975619	4.80133E-16
VWA5A	von Willebrand factor A domain containing 5A [Source:HGNC Symbol;Acc:HGNC:6658]	1.899373513	1.56438E-15
SAA2	serum amyloid A2 [Source:HGNC Symbol;Acc:HGNC:10514]	3.263441845	2.97818E-15
PDE4D	phosphodiesterase 4D, cAMPspecific [Source:HGNC Symbol;Acc:HGNC:8783]	1.948567676	3.64869E-15
RGS4	regulator of Gprotein signaling 4 [Source:HGNC Symbol;Acc:HGNC:10000]	3.036383283	3.8428E-15
NR4A3	nuclear receptor subfamily 4, group A, member 3 [Source:HGNC Symbol;Acc:HGNC:7982]	2.252211504	7.30706E-15
SEPP1	selenoprotein P, plasma, 1 [Source:HGNC Symbol;Acc:HGNC:10751]	2.20515054	7.81288E-15
SLC30A1	solute carrier family 30 (zinc transporter), member 1 [Source:HGNC Symbol;Acc:HGNC:11012]	1.765628193	8.3085E-15
SOST	sclerostin [Source:HGNC Symbol;Acc:HGNC:13771]	4.430660028	1.4857E-14
TMEM176A	transmembrane protein 176A [Source:HGNC Symbol;Acc:HGNC:24930]	5.069130021	1.63279E-14
BDNF	brain derived neurotrophic factor [Source:HGNC Symbol;Acc:HGNC:1033]	1.847804442	2.05988E-14
CXCL3	chemokine (CXC motif) ligand 3 [Source:HGNC Symbol;Acc:HGNC:4604]	1.742513308	2.99741E-14
PDE4B	phosphodiesterase 4B, cAMPspecific [Source:HGNC Symbol;Acc:HGNC:8781]	1.708385286	7.0452E-14
MCTP2	multiple C2 domains, transmembrane 2 [Source:HGNC Symbol;Acc:HGNC:25636]	1.772164178	8.86118E-14
LPAR3	lysophosphatidic acid receptor 3 [Source:HGNC Symbol;Acc:HGNC:14298]	2.978249892	1.2615E-13
IGDCC4	immunoglobulin superfamily, DCC subclass, member 4 [Source:HGNC Symbol;Acc:HGNC:13770]	1.748262392	1.8247E-13
CDCP1	CUB domain containing protein 1 [Source:HGNC Symbol;Acc:HGNC:24357]	1.810342323	1.92947E-13
ARHGEF19	Rho guanine nucleotide exchange factor (GEF) 19 [Source:HGNC Symbol;Acc:HGNC:26604]	1.79837438	4.30573E-13
MMP8	matrix metallopeptidase 8 (neutrophil collagenase) [Source:HGNC Symbol;Acc:HGNC:7175]	2.52411215	4.41003E-13
TOP2A	topoisomerase (DNA) II alpha 170kDa [Source:HGNC Symbol;Acc:HGNC:11989]	1.634300664	5.38791E-13
MXRA5	matrixremodelling associated 5 [Source:HGNC Symbol;Acc:HGNC:7539]	1.871050754	5.67624E-13

CLCA2	chloride channel accessory 2 [Source:HGNC Symbol;Acc:HGNC:2016]	3.259014639	8.57007E-13
EYA4	EYA transcriptional coactivator and phosphatase 4 [Source:HGNC Symbol;Acc:HGNC:3522]	2.212032438	8.76573E-13
MIR503HG	MIR503 host gene (nonprotein coding) [Source:HGNC Symbol;Acc:HGNC:28258]	2.711206864	9.00472E-13
LAMA5	laminin, alpha 5 [Source:HGNC Symbol;Acc:HGNC:6485]	2.68100409	8.9872E-13
THSD7A	thrombospondin, type I, domain containing 7A [Source:HGNC Symbol;Acc:HGNC:22207]	2.841772773	1.29305E-12
VEGFA	vascular endothelial growth factor A [Source:HGNC Symbol;Acc:HGNC:12680]	1.552659519	1.83052E-12
RGCC	regulator of cell cycle [Source:HGNC Symbol;Acc:HGNC:20369]	1.684500949	2.4316E-12
PTCH1	patched 1 [Source:HGNC Symbol;Acc:HGNC:9585]	2.005248988	3.33122E-12
ARHGAP6	Rho GTPase activating protein 6 [Source:HGNC Symbol;Acc:HGNC:676]	1.98545695	3.83891E-12
SOCS2	suppressor of cytokine signaling 2 [Source:HGNC Symbol;Acc:HGNC:19382]	1.676574815	3.81419E-12
PTPRN	protein tyrosine phosphatase, receptor type, N [Source:HGNC Symbol;Acc:HGNC:9676]	3.447409141	4.11218E-12
ITPR1	inositol 1,4,5trisphosphate receptor, type 1 [Source:HGNC Symbol;Acc:HGNC:6180]	1.741698296	4.80942E-12
GALNT15	polypeptide Nacetylgalactosaminyltransferase 15 [Source:HGNC Symbol;Acc:HGNC:21531]	2.525662474	4.99756E-12
DPP4	dipeptidylpeptidase 4 [Source:HGNC Symbol;Acc:HGNC:3009]	3.949441641	5.32477E-12
TAC3	tachykinin 3 [Source:HGNC Symbol;Acc:HGNC:11521]	4.42383963	5.74089E-12
ASPM	asp (abnormal spindle) homolog, microcephaly associated (Drosophila) [Source:HGNC Symbol;Acc:HGNC:19048]	1.700495081	7.37928E-12
SLC12A8	solute carrier family 12, member 8 [Source:HGNC Symbol;Acc:HGNC:15595]	1.75035337	8.68104E-12
FAM20C	family with sequence similarity 20, member C [Source:HGNC Symbol;Acc:HGNC:22140]	1.458081035	1.08488E-11
TNFRSF21	tumor necrosis factor receptor superfamily, member 21 [Source:HGNC Symbol;Acc:HGNC:13469]	1.681113361	1.69523E-11
FOS	FBJ murine osteosarcoma viral oncogene homolog [Source:HGNC Symbol;Acc:HGNC:3796]	3.103318957	1.79732E-11
PRICKLE1	prickle homolog 1 (Drosophila) [Source:HGNC Symbol;Acc:HGNC:17019]	2.165648594	2.85314E-11
STAMBPL1	STAM binding proteinlike 1 [Source:HGNC Symbol;Acc:HGNC:24105]	1.581298796	4.47907E-11
UCN2	urocortin 2 [Source:HGNC Symbol;Acc:HGNC:18414]	1.744251996	5.10948E-11
CYGB	cytoglobin [Source:HGNC Symbol;Acc:HGNC:16505]	1.823961005	5.45157E-11
COL24A1	collagen, type XXIV, alpha 1 [Source:HGNC Symbol;Acc:HGNC:20821]	2.261294922	5.73269E-11
DENND2A	DENNMADD domain containing 2A [Source:HGNC Symbol;Acc:HGNC:22212]	2.139410589	5.75875E-11
PSD3	pleckstrin and Sec7 domain containing 3 [Source:HGNC Symbol;Acc:HGNC:19093]	1.707283735	5.92625E-11
ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA2 receptor) [Source:HGNC Symbol;Acc:HGNC:6137]	1.486573976	6.41165E-11
PTHLH	parathyroid hormonelike hormone [Source:HGNC Symbol;Acc:HGNC:9607]	3.404326849	7.66624E-11
PTPRU	protein tyrosine phosphatase, receptor type, U [Source:HGNC Symbol;Acc:HGNC:9683]	1.916804796	1.21008E-10
IL1R1	interleukin 1 receptor, type I [Source:HGNC Symbol;Acc:HGNC:5993]	1.855668092	1.22792E-10
ATP8B4	ATPase, class I, type 8B, member 4 [Source:HGNC Symbol;Acc:HGNC:13536]	4.971171415	1.4224E-10

ABCA1	ATPbinding cassette, subfamily A (ABC1), member 1 [Source:HGNC Symbol;Acc:HGNC:29]	1.477832629	1.62023E-10
GABARAPL1	GABA(A) receptorassociated protein like 1 [Source:HGNC Symbol;Acc:HGNC:4068]	1.490389882	1.96763E-10
SORBS2	sorbin and SH3 domain containing 2 [Source:HGNC Symbol;Acc:HGNC:24098]	1.793331848	1.99933E-10
CDC20	cell division cycle 20 [Source:HGNC Symbol;Acc:HGNC:1723]	1.629434875	2.19268E-10
GPRC5B	G proteincoupled receptor, class C, group 5, member B [Source:HGNC Symbol;Acc:HGNC:13308]	2.106186142	2.34158E-10
ADCY4	adenylate cyclase 4 [Source:HGNC Symbol;Acc:HGNC:235]	1.656931068	2.37539E-10
GPCPD1	glycerophosphocholine phosphodiesterase GDE1 homolog (S. cerevisiae) [Source:HGNC Symbol;Acc:HGNC:26957]	1.482577861	2.89792E-10
FILIP1L	filamin A interacting protein 1like [Source:HGNC Symbol;Acc:HGNC:24589]	1.476925205	2.98764E-10
F2RL2	coagulation factor II (thrombin) receptorlike 2 [Source:HGNC Symbol;Acc:HGNC:3539]	1.860601817	3.57435E-10
KLHL13	kelchlike family member 13 [Source:HGNC Symbol;Acc:HGNC:22931]	1.705466466	4.55878E-10
FAM83D	family with sequence similarity 83, member D [Source:HGNC Symbol;Acc:HGNC:16122]	1.786297671	5.21611E-10
CEMIP	cell migration inducing protein, hyaluronan binding [Source:HGNC Symbol;Acc:HGNC:29213]	1.903902854	5.7319E-10
DLGAP5	discs, large (Drosophila) homologassociated protein 5 [Source:HGNC Symbol;Acc:HGNC:16864]	1.705830509	5.851E-10
SNCAIP	synuclein, alpha interacting protein [Source:HGNC Symbol;Acc:HGNC:11139]	1.91755483	7.61386E-10
PTGS1	Prostaglandin endoperoxide synthase 1 (prostaglandin GH synthase and cyclooxygenase) [Source:HGNC Symbol;Acc:HGNC:9604]	2.043696928	8.45086E-10
LINC00958	long intergenic non protein coding RNA 958 [Source:HGNC Symbol;Acc:HGNC:48671]	2.919983058	8.76039E-10
PCDHGA10	protocadherin gamma subfamily A, 10 [Source:HGNC Symbol;Acc:HGNC:8697]	3.388749349	1.01781E-09
SFRP1	secreted frizzledrelated protein 1 [Source:HGNC Symbol;Acc:HGNC:10776]	1.705009476	1.30541E-09
COL7A1	collagen, type VII, alpha 1 [Source:HGNC Symbol;Acc:HGNC:2214]	1.348567895	1.3713E-09
BEX4	brain expressed, Xlinked 4 [Source:HGNC Symbol;Acc:HGNC:25475]	1.546952275	1.46354E-09
NEK2	NIMArelated kinase 2 [Source:HGNC Symbol;Acc:HGNC:7745]	2.104326295	1.99235E-09
GNA14	guanine nucleotide binding protein (G protein), alpha 14 [Source:HGNC Symbol;Acc:HGNC:4382]	2.035774327	2.30309E-09
AVPI1	arginine vasopressininduced 1 [Source:HGNC Symbol;Acc:HGNC:30898]	1.453807607	2.50823E-09
IQGAP3	IQ motif containing GTPase activating protein 3 [Source:HGNC Symbol;Acc:HGNC:20669]	1.572280749	2.75851E-09
PLAU	plasminogen activator, urokinase [Source:HGNC Symbol;Acc:HGNC:9052]	1.830372034	2.78833E-09
PCDH18	protocadherin 18 [Source:HGNC Symbol;Acc:HGNC:14268]	2.122606247	2.89371E-09
CLEC2B	Ctype lectin domain family 2, member B [Source:HGNC Symbol;Acc:HGNC:2053]	1.791336775	3.02486E-09
ABCC3	ATPbinding cassette, subfamily C (CFTRMRP), member 3 [Source:HGNC Symbol;Acc:HGNC:54]	2.787034468	3.16498E-09
RARRES2	retinoic acid receptor responder (tazarotene induced) 2 [Source:HGNC Symbol;Acc:HGNC:9868]	3.285761806	3.827E-09
FBXO32	Fbox protein 32 [Source:HGNC Symbol;Acc:HGNC:16731]	1.315865	3.98873E-09

SLC2A10	solute carrier family 2 (facilitated glucose transporter), member 10 [Source:HGNC Symbol;Acc:HGNC:13444]	1.303207083	3.96644E-09
NAMPTP1	nicotinamide phosphoribosyltransferase pseudogene 1 [Source:HGNC Symbol;Acc:HGNC:17633]	1.73429841	4.40552E-09
KIF14	kinesin family member 14 [Source:HGNC Symbol;Acc:HGNC:19181]	1.775793858	4.91223E-09
ENC1	ectodermalneural cortex 1 (with BTB domain) [Source:HGNC Symbol;Acc:HGNC:3345]	1.348325772	4.90009E-09
PRKAR2B	protein kinase, cAMPdependent, regulatory, type II, beta [Source:HGNC Symbol;Acc:HGNC:9392]	1.725208207	6.87641E-09
LMO4	LIM domain only 4 [Source:HGNC Symbol;Acc:HGNC:6644]	1.360684149	7.63884E-09
ALDH2	aldehyde dehydrogenase 2 family (mitochondrial) [Source:HGNC Symbol;Acc:HGNC:404]	1.428064032	7.76486E-09
CENPF	centromere protein F, 350400kDa [Source:HGNC Symbol;Acc:HGNC:1857]	1.303436867	8.18009E-09
EDNRB	endothelin receptor type B [Source:HGNC Symbol;Acc:HGNC:3180]	3.171377182	8.27407E-09
CYFIP2	cytoplasmic FMR1 interacting protein 2 [Source:HGNC Symbol;Acc:HGNC:13760]	1.644783233	1.00764E-08
HMMR	hyaluronanmediated motility receptor (RHAMM) [Source:HGNC Symbol;Acc:HGNC:5012]	1.762205503	1.01712E-08
PDE7B	phosphodiesterase 7B [Source:HGNC Symbol;Acc:HGNC:8792]	1.283788247	1.19074E-08
HSPA2	heat shock 70kDa protein 2 [Source:HGNC Symbol;Acc:HGNC:5235]	1.242422195	1.57994E-08
TROAP	trophinin associated protein [Source:HGNC Symbol;Acc:HGNC:12327]	1.854611031	1.58991E-08
SOCS1	suppressor of cytokine signaling 1 [Source:HGNC Symbol;Acc:HGNC:19383]	1.669405258	2.02308E-08
NID2	nidogen 2 (osteonidogen) [Source:HGNC Symbol;Acc:HGNC:13389]	1.491401821	2.04943E-08
SLC26A6	solute carrier family 26 (anion exchanger), member 6 [Source:HGNC Symbol;Acc:HGNC:14472]	1.330668649	2.62253E-08
ANGPT1	angiopoietin 1 [Source:HGNC Symbol;Acc:HGNC:484]	1.224820703	3.09235E-08
LAMA4	laminin, alpha 4 [Source:HGNC Symbol;Acc:HGNC:6484]	1.25661684	3.18819E-08
DEPDC1	DEP domain containing 1 [Source:HGNC Symbol;Acc:HGNC:22949]	1.548820713	3.38718E-08
PDK4	pyruvate dehydrogenase kinase, isozyme 4 [Source:HGNC Symbol;Acc:HGNC:8812]	4.209496208	3.52732E-08
GK	glycerol kinase [Source:HGNC Symbol;Acc:HGNC:4289]	2.426186978	5.15383E-08
SLC40A1	solute carrier family 40 (ironregulated transporter), member 1 [Source:HGNC Symbol;Acc:HGNC:10909]	2.070115603	5.41181E-08
VCAN	versican [Source:HGNC Symbol;Acc:HGNC:2464]	1.507805788	5.56796E-08
PRDM1	PR domain containing 1, with ZNF domain [Source:HGNC Symbol;Acc:HGNC:9346]	1.456549483	5.68423E-08
PENK	proenkephalin [Source:HGNC Symbol;Acc:HGNC:8831]	2.901042995	6.27906E-08
C1orf132	chromosome 1 open reading frame 132 [Source:HGNC Symbol;Acc:HGNC:32018]	1.638821486	6.43999E-08
PMAIP1	phorbol12myristate13acetateinduced protein 1 [Source:HGNC Symbol;Acc:HGNC:9108]	1.293763067	6.48202E-08
MGC32805	uncharacterized LOC153163 [Source:EntrezGene;Acc:153163]	4.145424887	7.03216E-08
PLOD2	procollagenlysine, 2oxoglutarate 5dioxygenase 2 [Source:HGNC Symbol;Acc:HGNC:9082]	1.114855511	7.39363E-08
CDKN3	cyclindependent kinase inhibitor 3 [Source:HGNC Symbol;Acc:HGNC:1791]	1.627406493	7.55941E-08
CHMP1B	charged multivesicular body protein 1B [Source:HGNC Symbol;Acc:HGNC:24287]	1.225146836	7.54475E-08

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PC	pyruvate carboxylase [Source:HGNC Symbol;Acc:HGNC:8636]	1.579060712	8.45907E-08
PFKFB4	6phosphofructo2kinasefructose2,6biphosphatase 4 [Source:HGNC Symbol;Acc:HGNC:8875]	1.254976385	8.46036E-08
FENDRR	FOXF1 adjacent noncoding developmental regulatory RNA [Source:HGNC Symbol;Acc:HGNC:43894]	2.085773935	9.85871E-08
PRC1	protein regulator of cytokinesis 1 [Source:HGNC Symbol;Acc:HGNC:9341]	1.212973158	1.06201E-07
ZRANB1	zinc finger, RANbinding domain containing 1 [Source:HGNC Symbol;Acc:HGNC:18224]	1.217890951	1.09978E-07
ABCC6	ATPbinding cassette, subfamily C (CFTRMRP), member 6 [Source:HGNC Symbol;Acc:HGNC:57]	2.22466057	1.29247E-07
ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61) [Source:HGNC Symbol;Acc:HGNC:6156]	1.747713334	1.37456E-07
SAPCD2	suppressor APC domain containing 2 [Source:HGNC Symbol;Acc:HGNC:28055]	2.159575919	1.49697E-07
VNN3	vanin 3 [Source:HGNC Symbol;Acc:HGNC:16431]	2.414803419	1.61776E-07
NAMPT	nicotinamide phosphoribosyltransferase [Source:HGNC Symbol;Acc:HGNC:30092]	1.638393886	1.61926E-07
TNFRSF10C	tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain [Source:HGNC Symbol;Acc:HGNC:11906]	1.846491343	1.75702E-07
ENTPD1	ectonucleoside triphosphate diphosphohydrolase 1 [Source:HGNC Symbol;Acc:HGNC:3363]	1.199325313	1.95758E-07
P2RX6	purinergic receptor P2X, ligandgated ion channel, 6 [Source:HGNC Symbol;Acc:HGNC:8538]	1.953802673	2.001E-07
NUSAP1	nucleolar and spindle associated protein 1 [Source:HGNC Symbol;Acc:HGNC:18538]	1.35470499	2.00116E-07
PLEKHA5	pleckstrin homology domain containing, family A member 5 [Source:HGNC Symbol;Acc:HGNC:30036]	1.265172054	2.01038E-07
ACKR3	atypical chemokine receptor 3 [Source:HGNC Symbol;Acc:HGNC:23692]	2.772134841	2.12373E-07
KSR1	kinase suppressor of ras 1 [Source:HGNC Symbol;Acc:HGNC:6465]	1.556125065	2.1211E-07
ENPP1	ectonucleotide pyrophosphatasephosphodiesterase 1 [Source:HGNC Symbol;Acc:HGNC:3356]	1.274453751	2.14386E-07
AMIGO2	adhesion molecule with Iglike domain 2 [Source:HGNC Symbol;Acc:HGNC:24073]	1.594583433	2.16611E-07
CHST7	carbohydrate (Nacetylglucosamine 6O) sulfotransferase 7 [Source:HGNC Symbol;Acc:HGNC:13817]	1.208381034	2.17176E-07
CRISPLD1	cysteinerich secretory protein LCCL domain containing 1 [Source:HGNC Symbol;Acc:HGNC:18206]	1.804681389	3.42482E-07
BTAF1	BTAF1 RNA polymerase II, BTFIID transcription factorassociated, 170kDa [Source:HGNC Symbol;Acc:HGNC:17307]	1.182474561	4.07368E-07
SNCA	synuclein, alpha (non A4 component of amyloid precursor) [Source:HGNC Symbol;Acc:HGNC:11138]	1.733075528	4.14963E-07
SLC16A6	solute carrier family 16, member 6 [Source:HGNC Symbol;Acc:HGNC:10927]	1.356662138	4.15107E-07
SPAG5	sperm associated antigen 5 [Source:HGNC Symbol;Acc:HGNC:13452]	1.230413674	5.11459E-07
LRCH2	leucinerich repeats and calponin homology (CH) domain containing 2 [Source:HGNC Symbol;Acc:HGNC:29292]	1.226124286	5.72787E-07

BIRC5	baculoviral IAP repeat containing 5 [Source:HGNC Symbol;Acc:HGNC:593]	1.244783767	5.78731E-07
BUB1	BUB1 mitotic checkpoint serinethreonine kinase [Source:HGNC Symbol;Acc:HGNC:1148]	1.315121102	5.91082E-07
FOXF1	forkhead box F1 [Source:HGNC Symbol;Acc:HGNC:3809]	1.083640291	6.06579E-07
EPHB2	EPH receptor B2 [Source:HGNC Symbol;Acc:HGNC:3393]	1.247703175	6.1564E-07
MKI67	marker of proliferation Ki67 [Source:HGNC Symbol;Acc:HGNC:7107]	1.130632256	6.32318E-07
SEMA3B	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B [Source:HGNC Symbol;Acc:HGNC:10724]	1.677459794	6.39406E-07
WSB1	WD repeat and SOCS box containing 1 [Source:HGNC Symbol;Acc:HGNC:19221]	1.138059111	6.50806E-07
PLD1	phospholipase D1, phosphatidylcholinespecific [Source:HGNC Symbol;Acc:HGNC:9067]	1.339429882	6.54372E-07
BUB1B	BUB1 mitotic checkpoint serinethreonine kinase B [Source:HGNC Symbol;Acc:HGNC:1149]	1.305828303	6.64501E-07
PPFIA3	protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 3 [Source:HGNC Symbol;Acc:HGNC:9247]	1.616135243	6.84246E-07
AKR1B1	aldoketo reductase family 1, member B1 (aldose reductase) [Source:HGNC Symbol;Acc:HGNC:381]	1.114712549	6.79505E-07
PIM1	Pim1 protooncogene, serinethreonine kinase [Source:HGNC Symbol;Acc:HGNC:8986]	1.315021216	6.99665E-07
ABHD17C	abhydrolase domain containing 17C [Source:HGNC Symbol;Acc:HGNC:26925]	1.686965749	7.08784E-07
ZNF521	zinc finger protein 521 [Source:HGNC Symbol;Acc:HGNC:24605]	1.23303544	7.78081E-07
SAT1	spermidinespermine N1acetyltransferase 1 [Source:HGNC Symbol;Acc:HGNC:10540]	1.391489748	9.81456E-07
PPARGC1A	peroxisome proliferatoractivated receptor gamma, coactivator 1 alpha [Source:HGNC Symbol;Acc:HGNC:9237]	2.146042743	1.06433E-06
PLK1	pololike kinase 1 [Source:HGNC Symbol;Acc:HGNC:9077]	1.276224646	1.0642E-06
CXCL1	chemokine (CXC motif) ligand 1 (melanoma growth stimulating activity, alpha) [Source:HGNC Symbol;Acc:HGNC:4602]	1.090210899	1.08459E-06
KIF18A	kinesin family member 18A [Source:HGNC Symbol;Acc:HGNC:29441]	1.671986214	1.17962E-06
TSPAN9	tetraspanin 9 [Source:HGNC Symbol;Acc:HGNC:21640]	1.571531798	1.21257E-06
OSGIN2	oxidative stress induced growth inhibitor family member 2 [Source:HGNC Symbol;Acc:HGNC:1355]	1.140174953	1.30204E-06
STEAP2	STEAP family member 2, metalloreductase [Source:HGNC Symbol;Acc:HGNC:17885]	1.239967045	1.33509E-06
GLIS3	GLIS family zinc finger 3 [Source:HGNC Symbol;Acc:HGNC:28510]	1.640690031	1.34514E-06
SLC6A9	solute carrier family 6 (neurotransmitter transporter, glycine), member 9 [Source:HGNC Symbol;Acc:HGNC:11056]	1.446087744	1.39581E-06
CXCL5	chemokine (CXC motif) ligand 5 [Source:HGNC Symbol;Acc:HGNC:10642]	2.391736847	1.48224E-06
TNFRSF19	tumor necrosis factor receptor superfamily, member 19 [Source:HGNC Symbol;Acc:HGNC:11915]	1.117113321	1.49357E-06
TMEM178A	transmembrane protein 178A [Source:HGNC Symbol;Acc:HGNC:28517]	2.261399194	1.61319E-06

CHST2	carbohydrate (Nacetylglucosamine6O) sulfotransferase 2 [Source:HGNC Symbol;Acc:HGNC:1970]	1.063334701	1.77711E-06
APOE	apolipoprotein E [Source:HGNC Symbol;Acc:HGNC:613]	4.55925421	1.79822E-06
GTSE1	G2 and Sphase expressed 1 [Source:HGNC Symbol;Acc:HGNC:13698]	1.23489859	1.79855E-06
ELOVL3	ELOVL fatty acid elongase 3 [Source:HGNC Symbol;Acc:HGNC:18047]	2.031871352	1.80481E-06
ST5	suppression of tumorigenicity 5 [Source:HGNC Symbol;Acc:HGNC:11350]	1.098798492	1.8148E-06
THBS2	thrombospondin 2 [Source:HGNC Symbol;Acc:HGNC:11786]	2.012547127	1.91027E-06
DTWD1	DTW domain containing 1 [Source:HGNC Symbol;Acc:HGNC:30926]	1.296894075	2.00567E-06
ANTXR2	anthrax toxin receptor 2 [Source:HGNC Symbol;Acc:HGNC:21732]	1.061550083	2.04148E-06
TTK	TTK protein kinase [Source:HGNC Symbol;Acc:HGNC:12401]	1.48864736	2.1284E-06
IRS1	insulin receptor substrate 1 [Source:HGNC Symbol;Acc:HGNC:6125]	1.036704621	2.74635E-06
FAM64A	family with sequence similarity 64, member A [Source:HGNC Symbol;Acc:HGNC:25483]	1.523365644	3.14727E-06
RGL3	ral guanine nucleotide dissociation stimulator like 3 [Source:HGNC Symbol;Acc:HGNC:30282]	2.230697008	3.28117E-06
ARID5B	AT rich interactive domain 5B (MRF1like) [Source:HGNC Symbol;Acc:HGNC:17362]	1.187164127	3.30693E-06
TMEM71	transmembrane protein 71 [Source:HGNC Symbol;Acc:HGNC:26572]	2.141125486	3.55432E-06
CCNB2	cyclin B2 [Source:HGNC Symbol;Acc:HGNC:1580]	1.283573911	3.75697E-06
KIF2C	kinesin family member 2C [Source:HGNC Symbol;Acc:HGNC:6393]	1.394675	3.98097E-06
ALG9	ALG9, alpha1,2mannosyltransferase [Source:HGNC Symbol;Acc:HGNC:15672]	1.046759341	4.10882E-06
CEP55	centrosomal protein 55kDa [Source:HGNC Symbol;Acc:HGNC:1161]	1.159765686	4.43337E-06
HDAC4	histone deacetylase 4 [Source:HGNC Symbol;Acc:HGNC:14063]	1.128993548	4.50451E-06
SLC16A14	solute carrier family 16, member 14 [Source:HGNC Symbol;Acc:HGNC:26417]	2.144596915	4.5725E-06
GPRASP1	G protein coupled receptor associated sorting protein 1 [Source:HGNC Symbol;Acc:HGNC:24834]	1.363079304	5.20293E-06
SOX4	SRY (sex determining region Y)box 4 [Source:HGNC Symbol;Acc:HGNC:11200]	1.233324103	5.94291E-06
RNF112	ring finger protein 112 [Source:HGNC Symbol;Acc:HGNC:12968]	1.629687986	6.37066E-06
MT1F	metallothionein 1F [Source:HGNC Symbol;Acc:HGNC:7398]	3.477904877	6.75724E-06
PCDHGA6	protocadherin gamma subfamily A, 6 [Source:HGNC Symbol;Acc:HGNC:8704]	1.401290882	6.76093E-06
SLC6A6	solute carrier family 6 (neurotransmitter transporter), member 6 [Source:HGNC Symbol;Acc:HGNC:11052]	1.037570762	7.01114E-06
PTGES	prostaglandin E synthase [Source:HGNC Symbol;Acc:HGNC:9599]	1.00017664	7.08945E-06
WNT2	wingless type MMTV integration site family member 2 [Source:HGNC Symbol;Acc:HGNC:12780]	5.403137697	7.37858E-06
LPCAT3	lysophosphatidylcholine acyltransferase 3 [Source:HGNC Symbol;Acc:HGNC:30244]	1.038639857	8.0604E-06
RAPGEF6	Rap guanine nucleotide exchange factor (GEF) 6 [Source:HGNC Symbol;Acc:HGNC:20655]	1.174491113	8.27042E-06
CES4A	carboxylesterase 4A [Source:HGNC Symbol;Acc:HGNC:26741]	1.620285039	9.64275E-06

SPAG4	sperm associated antigen 4 [Source:HGNC Symbol;Acc:HGNC:11214]	1.84173251	9.87019E-06
MBP	myelin basic protein [Source:HGNC Symbol;Acc:HGNC:6925]	2.038148774	1.06561E-05
CFAP69	cilia and flagella associated protein 69 [Source:HGNC Symbol;Acc:HGNC:26107]	1.347983633	1.08398E-05
FBXO2	Fbox protein 2 [Source:HGNC Symbol;Acc:HGNC:13581]	1.453020508	1.13583E-05
SPTSSA	serine palmitoyltransferase, small subunit A [Source:HGNC Symbol;Acc:HGNC:20361]	1.072893752	1.13999E-05
CDK14	cyclindependent kinase 14 [Source:HGNC Symbol;Acc:HGNC:8883]	1.010285808	1.15073E-05
RNF157	ring finger protein 157 [Source:HGNC Symbol;Acc:HGNC:29402]	1.042848633	1.15963E-05
SOWAHD	sosondowah ankyrin repeat domain family member D [Source:HGNC Symbol;Acc:HGNC:32960]	2.316567373	1.19645E-05
PDGFRA	platelet derived growth factor receptor, alpha polypeptide [Source:HGNC Symbol;Acc:HGNC:8803]	1.378949526	1.21615E-05
PF4V1	platelet factor 4 variant 1 [Source:HGNC Symbol;Acc:HGNC:8862]	4.306482632	1.33314E-05
WNT5A	winglesstype MMTV integration site family, member 5A [Source:HGNC Symbol;Acc:HGNC:12784]	1.024172763	1.39901E-05
DIRAS3	DIRAS family, GTPbinding RASlike 3 [Source:HGNC Symbol;Acc:HGNC:687]	2.30478155	1.4319E-05
WNT2B	wingless type MMTV integration site family, member 2B [Source:HGNC Symbol;Acc:HGNC:12781]	1.867516939	1.54326E-05
FAT4	FAT atypical cadherin 4 [Source:HGNC Symbol;Acc:HGNC:23109]	1.172949761	1.56339E-05
UBE2C	ubiquitinconjugating enzyme E2C [Source:HGNC Symbol;Acc:HGNC:15937]	1.237569994	1.63214E-05
PDK1	pyruvate dehydrogenase kinase, isozyme 1 [Source:HGNC Symbol;Acc:HGNC:8809]	1.148735215	1.64468E-05
PPFIA4	protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 4 [Source:HGNC Symbol;Acc:HGNC:9248]	1.680048063	1.76619E-05
AZIN2	antizyme inhibitor 2 [Source:HGNC Symbol;Acc:HGNC:29957]	1.419413791	1.77429E-05
CCNA2	cyclin A2 [Source:HGNC Symbol;Acc:HGNC:1578]	1.070014357	1.77018E-05
ANKDD1A	ankyrin repeat and death domain containing 1A [Source:HGNC Symbol;Acc:HGNC:28002]	1.278757932	1.81391E-05
LRRC61	leucine rich repeat containing 61 [Source:HGNC Symbol;Acc:HGNC:21704]	4.775020439	1.85561E-05
OR1J4	olfactory receptor, family 1, subfamily J, member 4 [Source:HGNC Symbol;Acc:HGNC:8211]	2.174744618	1.96131E-05
GBP5	guanylate binding protein 5 [Source:HGNC Symbol;Acc:HGNC:19895]	1.763714994	2.22009E-05
CELSR3	cadherin, EGF LAG sevenpass Gtype receptor 3 [Source:HGNC Symbol;Acc:HGNC:3230]	1.214953406	2.21869E-05
EBF4	early Bcell factor 4 [Source:HGNC Symbol;Acc:HGNC:29278]	1.972078765	2.33083E-05
MEDAG	mesenteric estrogendependent adipogenesis [Source:HGNC Symbol;Acc:HGNC:25926]	2.167338241	2.50592E-05
DLL1	deltalike 1 (Drosophila) [Source:HGNC Symbol;Acc:HGNC:2908]	1.650923139	2.64175E-05
EVI2B	ecotropic viral integration site 2B [Source:HGNC Symbol;Acc:HGNC:3500]	1.421886873	2.69616E-05
PELI2	pellino E3 ubiquitin protein ligase family member 2 [Source:HGNC Symbol;Acc:HGNC:8828]	1.16259072	2.92264E-05
SDC1	syndecan 1 [Source:HGNC Symbol;Acc:HGNC:10658]	1.320591098	2.95094E-05
PILRA	paired immunoglobinlike type 2 receptor alpha [Source:HGNC Symbol;Acc:HGNC:20396]	3.742543317	2.96256E-05

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BNC1	basonuclin 1 [Source:HGNC Symbol;Acc:HGNC:1081]	1.011140715	3.02685E-05
SOCS2AS1	SOCS2 antisense RNA 1 [Source:HGNC Symbol;Acc:HGNC:27054]	1.35195257	3.12045E-05
CCNB1	cyclin B1 [Source:HGNC Symbol;Acc:HGNC:1579]	1.08811855	3.54582E-05
PRR11	proline rich 11 [Source:HGNC Symbol;Acc:HGNC:25619]	1.054063519	3.73061E-05
ABTB2	ankyrin repeat and BTB (POZ) domain containing 2 [Source:HGNC Symbol;Acc:HGNC:23842]	1.342472674	3.86044E-05
KYNU	kynureninase [Source:HGNC Symbol;Acc:HGNC:6469]	1.991118689	4.12076E-05
LINCR0002	uncharacterized LincR0002 [Source:EntrezGene;Acc:103344926]	3.437729207	4.47948E-05
BMP4	bone morphogenetic protein 4 [Source:HGNC Symbol;Acc:HGNC:1071]	1.652112021	4.49006E-05
CEBPD	CCAATenhancer binding protein (CEBP), delta [Source:HGNC Symbol;Acc:HGNC:1835]	1.207564882	4.64113E-05
RAC2	rasrelated C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2) [Source:HGNC Symbol;Acc:HGNC:9802]	2.447864447	4.87689E-05
PAPLN	papilin, proteoglycan like sulfated glycoprotein [Source:HGNC Symbol;Acc:HGNC:19262]	1.263212775	5.43865E-05
ITGB4	integrin, beta 4 [Source:HGNC Symbol;Acc:HGNC:6158]	1.884599207	5.47162E-05
RORB	RAR related orphan receptor B [Source:HGNC Symbol;Acc:HGNC:10259]	2.331400274	5.52317E-05
MARCKSL1	MARCKS like 1 [Source:HGNC Symbol;Acc:HGNC:7142]	1.59779248	5.65053E-05
KCNIP3	Kv channel interacting protein 3, calsenilin [Source:HGNC Symbol;Acc:HGNC:15523]	1.71137176	5.77356E-05
GABRE	Gamma aminobutyric acid (GABA) A receptor, epsilon [Source:HGNC Symbol;Acc:HGNC:4085]	1.449332274	6.09851E-05
RSPO3	R spondin 3 [Source:HGNC Symbol;Acc:HGNC:20866]	2.068738511	6.44541E-05
TRIM16L	tripartite motif containing 16like [Source:HGNC Symbol;Acc:HGNC:32670]	1.150887382	6.83517E-05
SLITRK2	SLIT and NTRK like family, member 2 [Source:HGNC Symbol;Acc:HGNC:13449]	2.376086464	6.92329E-05
DEPDC7	DEP domain containing 7 [Source:HGNC Symbol;Acc:HGNC:29899]	1.063258941	6.9459E-05
PAQR5	progestin and adipoQ receptor family member V [Source:HGNC Symbol;Acc:HGNC:29645]	1.510773339	7.08969E-05
HNMT	histamine Nmethyltransferase [Source:HGNC Symbol;Acc:HGNC:5028]	1.003717959	7.10371E-05
NEAT1	nuclear paraspeckle assembly transcript 1 (non protein coding) [Source: HGNC Symbol;Acc:HGNC:30815]	1.275774158	7.35484E-05
S100P	S100 calcium binding protein P [Source: HGNC Symbol;Acc:HGNC:10504]	3.220309835	7.399E-05
EFNB1	ephrinB1 [Source: HGNC Symbol;Acc:HGNC:3226]	1.078362317	8.00175E-05
A2M	alpha2macroglobulin [Source: HGNC Symbol;Acc:HGNC:7]	4.678155598	8.11353E-05
LIPC	lipase, hepatic [Source:HGNC Symbol;Acc:HGNC:6619]	1.779447662	8.71711E-05
RNF175	ring finger protein 175 [Source: HGNC Symbol;Acc:HGNC:27735]	2.182041912	8.81028E-05
CKAP2L	cytoskeleton associated protein 2like [Source: HGNC Symbol;Acc:HGNC:26877]	1.13989413	9.78978E-05
ABCA10	ATP binding cassette, subfamily A (ABC1), member 10 [Source: HGNC Symbol;Acc:HGNC:30]	1.358625589	9.99564E-05
ST8SIA4	ST8 alpha-N-acetyl-neuraminide alpha2,8 sialyl-transferase 4 [Source: HGNC Symbol;Acc:HGNC:10871]	2.305450367	0.000100716

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RERG	RAS like, estrogen regulated, growth inhibitor [Source: HGNC Symbol;Acc:HGNC:15980]	2.280835625	0.000103906
STYK1	Serine threonine tyrosine kinase 1 [Source: HGNC Symbol;Acc:HGNC:18889]	1.606872728	0.00010525
C8orf4	chromosome 8 open reading frame 4 [Source: HGNC Symbol;Acc:HGNC:1357]	2.166181154	0.000108917
ZDHHC23	zinc finger, DHHC-type containing 23 [Source: HGNC Symbol;Acc:HGNC:28654]	1.091480908	0.000110659
MSI2	musashi RNA binding protein 2 [Source: HGNC Symbol;Acc:HGNC:18585]	1.347729636	0.000116432
PSRC1	Proline serine rich coiled coil 1 [Source :HGNC Symbol;Acc:HGNC:24472]	1.161852961	0.000121614
ROBO3	roundabout, axon guidance receptor, homolog 3 (Drosophila) [Source: HGNC Symbol;Acc:HGNC:13433]	1.266204307	0.000125843
HHIPL2	HHIP-like 2 [Source: HGNC Symbol;Acc:HGNC:25842]	2.487647866	0.000126516
ABCA8	ATP binding cassette, subfamily A (ABC1), member 8 [Source :HGNC Symbol;Acc:HGNC:38]	1.996876796	0.000127201
C1QL1	complement component 1, q sub-component-like 1 [Source: HGNC Symbol;Acc:HGNC:24182]	2.147034497	0.000133948
PRR5	proline rich 5 (renal) [Source: HGNC Symbol;Acc:HGNC:31682]	1.212140735	0.00013497
CYP26B1	cytochrome P450, family 26, subfamily B, polypeptide 1 [Source: HGNC Symbol;Acc:HGNC:20581]	2.106469377	0.000137015
GKAS1	GK antisense RNA 1 [Source: HGNC Symbol;Acc:HGNC:40255]	2.809517679	0.000154127
PCDHB2	protocadherin beta 2 [Source: HGNC Symbol;Acc:HGNC:8687]	1.931861094	0.000160349
ADAMTS9	ADAM metallopeptidase with thrombospondin type 1 motif, 9 [Source: HGNC Symbol;Acc:HGNC:13202]	1.04833192	0.00017249
WIF1	WNT inhibitory factor 1 [Source: HGNC Symbol;Acc:HGNC:18081]	2.598998601	0.000172855
SNAP25	Synapto-somalassociated protein, 25kDa [Source: HGNC Symbol;Acc:HGNC:11132]	1.310880292	0.000180367
IL11	interleukin 11 [Source: HGNC Symbol;Acc:HGNC:5966]	1.733467242	0.000180809
SPON1	spondin 1, extracellular matrix protein [Source: HGNC Symbol;Acc:HGNC:11252]	1.198452072	0.000184374
PBK	PDZ binding kinase [Source: HGNC Symbol;Acc:HGNC:18282]	1.018365077	0.000186442
MOCOS	molybdenum cofactor sulfurase [Source: HGNC Symbol;Acc:HGNC:18234]	1.449957648	0.000191886
IL1RN	interleukin 1 receptor antagonist [Source: HGNC Symbol;Acc:HGNC:6000]	3.756747596	0.000198771
RP11302B13.5	ADP-ribosylation factor 3 {ECO:0000313|Ensembl:ENSP00000438507} [Source:UniProtKBTrEMBL;Acc:F5H423]	1.521721905	0.000204409
PSD	pleckstrin and Sec7 domain containing [Source: HGNC Symbol;Acc:HGNC:9507]	1.37258198	0.000219233
SLC7A11	solute carrier family 7 (anionic amino acid transporter light chain, xc system), member 11 [Source: HGNC Symbol;Acc:HGNC:11059]	2.095707606	0.000236214
BDKRB2	bradykinin receptor B2 [Source: HGNC Symbol;Acc:HGNC:1030]	1.932771989	0.000251149
SELENBP1	selenium binding protein 1 [Source: HGNC Symbol;Acc:HGNC:10719]	1.123837394	0.000273975
CASC5	cancer susceptibility candidate 5 [Source: HGNC Symbol;Acc:HGNC:24054]	1.02450226	0.000283245
SEMA4G	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4G [Source: HGNC Symbol;Acc:HGNC:10735]	1.450019518	0.00028617

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CP	ceruloplasmin (ferroxidase) [Source: HGNC Symbol;Acc:HGNC:2295]	2.485725196	0.000333143
CCDC152	Coiled-coil domain containing 152 [Source: HGNC Symbol;Acc:HGNC:34438]	1.956060539	0.00033858
TSKU	tsukushi, small leucine rich proteoglycan [Source: HGNC Symbol;Acc:HGNC:28850]	1.109985687	0.000351772
HERC5	HECT and RLD domain containing E3 ubiquitin protein ligase 5 [Source: HGNC Symbol;Acc:HGNC:24368]	1.226108347	0.000359546
FAM20A	family with sequence similarity 20, member A [Source: HGNC Symbol;Acc:HGNC:23015]	2.618007506	0.000366003
IL6	interleukin 6 [Source: HGNC Symbol;Acc:HGNC:6018]	1.234029155	0.000365548
SSX2IP	synovial sarcoma, X breakpoint 2 interacting protein [Source: HGNC Symbol;Acc:HGNC:16509]	1.206118675	0.000373295
SHANK2	SH3 and multiple ankyrin repeat domains 2 [Source: HGNC Symbol;Acc:HGNC:14295]	1.136651874	0.000388737
LINC00472	long intergenic non-protein coding RNA 472 [Source: HGNC Symbol;Acc:HGNC:21380]	1.321400357	0.000419227
C3	complement component 3 [Source: HGNC Symbol;Acc:HGNC:1318]	1.331880192	0.00042987
GPX3	glutathione peroxidase 3 (plasma) [Source: HGNC Symbol;Acc:HGNC:4555]	1.548648466	0.000431408
BMP2	bone morphogenetic protein 2 [Source: HGNC Symbol;Acc:HGNC:1069]	1.248234873	0.000440233
SCG5	secreto-granin V (7B2 protein) [Source: HGNC Symbol;Acc:HGNC:10816]	1.147846041	0.000453189
CENPA	centromere protein A [Source: HGNC Symbol;Acc:HGNC:1851]	1.266025451	0.000518159
DLGAP1AS1	DLGAP1 antisense RNA 1 [Source: HGNC Symbol;Acc:HGNC:31676]	1.127883652	0.000519637
NOL4L	nucleolar protein 4like [Source: HGNC Symbol;Acc:HGNC:16106]	1.089428456	0.000526019
DNAJC22	Dna J (Hsp40) homolog, subfamily C, member 22 [Source: HGNC Symbol;Acc:HGNC:25802]	1.327149867	0.000527532
MAPK10	mitogenactivated protein kinase 10 [Source:HGNC Symbol;Acc:HGNC:6872]	1.237427446	0.000544489
TMEM158	transmembrane protein 158 (gene pseudogene) [Source:HGNC Symbol;Acc:HGNC:30293]	1.555254651	0.000548551
MIR210HG	MIR210 host gene (non-protein coding) [Source:HGNC Symbol;Acc:HGNC:39524]	1.735974419	0.000554445
DENND2D	DENNMADD domain containing 2D [Source:HGNC Symbol;Acc:HGNC:26192]	1.453501106	0.000560106
KCTD4	potassium channel tetramerization domain containing 4 [Source:HGNC Symbol;Acc:HGNC:23227]	1.420492726	0.000566299
PCDHGB2	protocadherin gamma subfamily B, 2 [Source:HGNC Symbol;Acc:HGNC:8709]	1.659461911	0.000582496
CCK	cholecystokinin [Source:HGNC Symbol;Acc:HGNC:1569]	2.589527604	0.000587844
NUF2	NUF2, NDC80 kinetochore complex component [Source:HGNC Symbol;Acc:HGNC:14621]	1.170872491	0.000587484
APOBEC3B	apolipoprotein B mRNA editing enzyme, catalytic polypeptidelike 3B [Source:HGNC Symbol;Acc:HGNC:17352]	1.124127398	0.000625331
CDX1	caudal type homeobox 1 [Source:HGNC Symbol;Acc:HGNC:1805]	2.056462272	0.000751578
SPIN2A	spindlin family, member 2A [Source:HGNC Symbol;Acc:HGNC:20694]	1.630582112	0.000752748
DUSP16	dual specificity phosphatase 16 [Source:HGNC Symbol;Acc:HGNC:17909]	1.2507682	0.000759462
SULF2	sulfatase 2 [Source:HGNC Symbol;Acc:HGNC:20392]	2.436767158	0.000784115
REC8	REC8 meiotic recombination protein [Source:HGNC Symbol;Acc:HGNC:16879]	1.262320225	0.000812165
CCDC69	Coiled coil domain containing 69 [Source:HGNC Symbol;Acc:HGNC:24487]	1.596606799	0.000829189

COL10A1	collagen, type X, alpha 1 [Source:HGNC Symbol;Acc:HGNC:2185]	1.65728122	0.000840403
CDCA3	cell division cycle associated 3 [Source:HGNC Symbol;Acc:HGNC:14624]	1.034419797	0.000923914
TEX11	testis expressed 11 [Source:HGNC Symbol;Acc:HGNC:11733]	1.213784013	0.001039721
SEMA4D	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4D [Source:HGNC Symbol;Acc:HGNC:10732]	1.216806783	0.001048128
ZNF608	zinc finger protein 608 [Source:HGNC Symbol;Acc:HGNC:29238]	1.49685264	0.001060637
TBX2AS1	TBX2 antisense RNA 1 [Source:HGNC Symbol;Acc:HGNC:50355]	1.439271965	0.001066534
AFAP1L2	actin filament associated protein 1like 2 [Source:HGNC Symbol;Acc:HGNC:25901]	5.072197481	0.001074663
HSF4	heat shock transcription factor 4 [Source:HGNC Symbol;Acc:HGNC:5227]	1.220391191	0.001156952
ELTD1	EGF, latrophilin and seven transmembrane domain containing 1 [Source:HGNC Symbol;Acc:HGNC:20822]	2.144126507	0.001171717
SLC26A10	solute carrier family 26, member 10 [Source:HGNC Symbol;Acc:HGNC:14470]	1.425133019	0.001184628
PIR	pirin (ironbinding nuclear protein) [Source:HGNC Symbol;Acc:HGNC:30048]	1.556707387	0.001212117
CPNE4	copine IV [Source:HGNC Symbol;Acc:HGNC:2317]	2.255603905	0.001218287

Appendix Table 3. Significantly downregulated genes of C-O clones (clones with potential to differentiate into osteo/cementoblastic phenotype) compared to C-F clones (clones that shows fibroblastic phenotype).

Gene Symbol	Gene_description	|log2FoldChange|	p value
LINC01085	long intergenic non-protein coding RNA 1085 [Source:HGNC Symbol;Acc:HGNC:27198]	3.964982	8.78E-21
RP11-385D13.1	Uncharacterized protein {ECO:0000313|Ensembl:ENSP00000402644} [Source:UniProtKBTrEMBL;Acc:H0Y626]	2.522707	5.47E-17
GADD45B	growth arrest and DNA-damage-inducible, beta [Source:HGNC Symbol;Acc:HGNC:4096]	1.798697	3.6E-14
APPL2	adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 2 [Source:HGNC Symbol;Acc:HGNC:18242]	1.713417	4.29E-14
TUBB3	tubulin, beta 3 class III [Source:HGNC Symbol;Acc:HGNC:20772]	1.708319	1.28E-13
NPAS1	neuronal PAS domain protein 1 [Source:HGNC Symbol;Acc:HGNC:7894]	3.459166	3.82E-13
OXTR	oxytocin receptor [Source:HGNC Symbol;Acc:HGNC:8529]	2.629509	7.59E-12
ARHGAP22	Rho GTPase activating protein 22 [Source:HGNC Symbol;Acc:HGNC:30320]	1.521047	7.02E-11
CDH2	cadherin 2, type 1, N-cadherin (neuronal) [Source:HGNC Symbol;Acc:HGNC:1759]	2.060399	2.03E-10
IPO4	importin 4 [Source:HGNC Symbol;Acc:HGNC:19426]	1.736697	4.32E-10

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TNFAIP3	tumor necrosis factor, alpha-induced protein 3 [Source:HGNC Symbol;Acc:HGNC:11896]	1.387013	5.07E-10
FAM180A	family with sequence similarity 180, member A [Source:HGNC Symbol;Acc:HGNC:33773]	1.404615	6.07E-10
GRIK2	glutamate receptor, ionotropic, kainate 2 [Source:HGNC Symbol;Acc:HGNC:4580]	1.518526	9.26E-10
FOSL1	FOS-like antigen 1 [Source:HGNC Symbol;Acc:HGNC:13718]	1.372762	1.91E-09
TUBA1C	tubulin, alpha 1c [Source:HGNC Symbol;Acc:HGNC:20768]	1.294287	5.75E-09
HECW2	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2 [Source:HGNC Symbol;Acc:HGNC:29853]	1.600211	3.73E-08
AFAP1L1	actin filament associated protein 1-like 1 [Source:HGNC Symbol;Acc:HGNC:26714]	1.431046	3.98E-08
TUBA1A	tubulin, alpha 1a [Source:HGNC Symbol;Acc:HGNC:20766]	1.176861	6.58E-08
BIRC3	baculoviral IAP repeat containing 3 [Source:HGNC Symbol;Acc:HGNC:591]	1.216271	6.71E-08
ANXA1	annexin A1 [Source:HGNC Symbol;Acc:HGNC:533]	1.173138	6.95E-08
ERCC6	excision repair cross-complementation group 6 [Source:HGNC Symbol;Acc:HGNC:3438]	2.063832	1.09E-07
HTRA1	HtrA serine peptidase 1 [Source:HGNC Symbol;Acc:HGNC:9476]	1.119954	1.73E-07
PRELP	prolinearginine-rich end leucine-rich repeat protein [Source:HGNC Symbol;Acc:HGNC:9357]	2.42967	1.97E-07
C6orf132	chromosome 6 open reading frame 132 [Source:HGNC Symbol;Acc:HGNC:21288]	1.360631	2.28E-07
AK4P1	adenylate kinase 4 pseudogene 1 [Source:HGNC Symbol;Acc:HGNC:364]	1.533566	2.59E-07
RCAN2	regulator of calcineurin 2 [Source:HGNC Symbol;Acc:HGNC:3041]	1.549079	2.8E-07
DSP	desmoplakin [Source:HGNC Symbol;Acc:HGNC:3052]	2.387321	3.98E-07
ADAM23	ADAM metallopeptidase domain 23 [Source:HGNC Symbol;Acc:HGNC:202]	1.994711	5.51E-07
FOXC2	forkhead box C2 (MFH-1, mesenchyme forkhead 1) [Source:HGNC Symbol;Acc:HGNC:3801]	1.325683	5.85E-07
TCEAL4	transcription elongation factor A (SII)-like 4 [Source:HGNC Symbol;Acc:HGNC:26121]	1.119773	6.5E-07
NAV2	neuron navigator 2 [Source:HGNC Symbol;Acc:HGNC:15997]	1.152368	6.69E-07
HMGA1	high mobility group AT-hook 1 [Source:HGNC Symbol;Acc:HGNC:5010]	1.092109	6.84E-07
CPNE7	copine VII [Source:HGNC Symbol;Acc:HGNC:2320]	1.131904	7.26E-07
ASPN	asporin [Source:HGNC Symbol;Acc:HGNC:14872]	1.670883	8.88E-07
AHNAK2	AHNAK nucleoprotein 2 [Source:HGNC Symbol;Acc:HGNC:20125]	1.522171	9.49E-07
ELN	elastin [Source:HGNC Symbol;Acc:HGNC:3327]	2.735783	1.34E-06
ARHGAP23	Rho GTPase activating protein 23 [Source:HGNC Symbol;Acc:HGNC:29293]	1.058018	1.88E-06
PTX3	pentraxin 3, long [Source:HGNC Symbol;Acc:HGNC:9692]	1.059165	1.91E-06
AK4	adenylate kinase 4 [Source:HGNC Symbol;Acc:HGNC:363]	1.179845	2.01E-06

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ALOX15B	arachidonate 15-lipoxygenase, type B [Source:HGNC Symbol;Acc:HGNC:434]	1.622412	2.54E-06
C11orf68	chromosome 11 open reading frame 68 [Source:HGNC Symbol;Acc:HGNC:28801]	1.064409	2.65E-06
PMP22	peripheral myelin protein 22 [Source:HGNC Symbol;Acc:HGNC:9118]	1.044198	3.36E-06
SCFD2	sec1 family domain containing 2 [Source:HGNC Symbol;Acc:HGNC:30676]	1.044562	4.06E-06
C6orf1	chromosome 6 open reading frame 1 [Source:HGNC Symbol;Acc:HGNC:1340]	1.071357	4.69E-06
MSMO1	methylsterol monooxygenase 1 [Source:HGNC Symbol;Acc:HGNC:10545]	1.027696	5.58E-06
BAIAP2	BAI1-associated protein 2 [Source:HGNC Symbol;Acc:HGNC:947]	1.17034	6.05E-06
MAPKAPK3	mitogen-activated protein kinase-activated protein kinase 3 [Source:HGNC Symbol;Acc:HGNC:6888]	1.004902	6.12E-06
TNFAIP8L3	tumor necrosis factor, alpha-induced protein 8-like 3 [Source:HGNC Symbol;Acc:HGNC:20620]	1.590004	6.53E-06
CYB5D1	cytochrome b5 domain containing 1 [Source:HGNC Symbol;Acc:HGNC:26516]	1.146515	6.68E-06
FGF1	fibroblast growth factor 1 (acidic) [Source:HGNC Symbol;Acc:HGNC:3665]	1.723254	7.12E-06
MET	MET proto-oncogene, receptor tyrosine kinase [Source:HGNC Symbol;Acc:HGNC:7029]	1.038341	7.86E-06
CBR3	carbonyl reductase 3 [Source:HGNC Symbol;Acc:HGNC:1549]	1.306181	7.99E-06
SUGCT	succinyl-CoA:glutarate-CoA transferase [Source:HGNC Symbol;Acc:HGNC:16001]	1.052222	8.13E-06
ALDH4A1	aldehyde dehydrogenase 4 family, member A1 [Source:HGNC Symbol;Acc:HGNC:406]	1.066567	8.42E-06
MBNL1-AS1	MBNL1 antisense RNA 1 [Source:HGNC Symbol;Acc:HGNC:44584]	1.019527	8.85E-06
ANK2	ankyrin 2, neuronal [Source:HGNC Symbol;Acc:HGNC:493]	1.070883	1.11E-05
DRAP1	DR1-associated protein 1 (negative cofactor 2 alpha) [Source:HGNC Symbol;Acc:HGNC:3019]	1.205896	1.13E-05
NEK10	NIMA-related kinase 10 [Source:HGNC Symbol;Acc:HGNC:18592]	2.214211	1.16E-05
PTRH1	peptidyl-tRNA hydrolase 1 homolog (S. cerevisiae) [Source:HGNC Symbol;Acc:HGNC:27039]	1.176916	1.19E-05
APCDD1L	adenomatosis polyposis coli down-regulated 1-like [Source:HGNC Symbol;Acc:HGNC:26892]	1.478632	1.36E-05
EVA1A	eva-1 homolog A (C. elegans) [Source:HGNC Symbol;Acc:HGNC:25816]	1.080478	2.67E-05
KCNQ5	potassium voltage-gated channel, KQT-like subfamily, member 5 [Source:HGNC Symbol;Acc:HGNC:6299]	2.200169	3.94E-05
COPS8	COP9 signalosome subunit 8 [Source:HGNC Symbol;Acc:HGNC:24335]	1.121761	4.09E-05
PPFIA2	protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 2 [Source:HGNC Symbol;Acc:HGNC:9246]	2.03639	4.08E-05
AP1S3	adaptor-related protein complex 1, sigma 3 subunit [Source:HGNC Symbol;Acc:HGNC:18971]	1.475281	4.93E-05
AXIN2	axin 2 [Source:HGNC Symbol;Acc:HGNC:904]	1.213789	6.17E-05

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ST6GALNAC5	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl- 1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 5 [Source:HGNC Symbol;Acc:HGNC:19342]	2.39866	6.37E-05
KRT19	keratin 19 [Source:HGNC Symbol;Acc:HGNC:6436]	2.984345	0.000103
PEG10	paternally expressed 10 [Source:HGNC Symbol;Acc:HGNC:14005]	1.130018	0.000118
NCAM2	neural cell adhesion molecule 2 [Source:HGNC Symbol;Acc:HGNC:7657]	1.713622	0.000183
MTSS1	metastasis suppressor 1 [Source:HGNC Symbol;Acc:HGNC:20443]	1.441807	0.000215
MMAB	methylmalonic aciduria (cobalamin deficiency) cblB type [Source:HGNC Symbol;Acc:HGNC:19331]	1.461812	0.000282
BMP6	bone morphogenetic protein 6 [Source:HGNC Symbol;Acc:HGNC:1073]	2.575909	0.000362
FAM53B	family with sequence similarity 53, member B [Source:HGNC Symbol;Acc:HGNC:28968]	1.891819	0.000381
C17orf61-PLSCR3	Uncharacterized protein {ECO:0000313|Ensembl:ENSP00000468219} [Source:UniProtKBTrEMBL;Acc:K7ERE1]	1.037653	0.000404
RGMB-AS1	RGMB antisense RNA 1 [Source:HGNC Symbol;Acc:HGNC:48666]	1.135076	0.000422
RAB4B-EGLN2	RAB4B-EGLN2 readthrough (NMD candidate) [Source:HGNC Symbol;Acc:HGNC:44465]	1.3314	0.000459
NPTX1	neuronal pentraxin I [Source:HGNC Symbol;Acc:HGNC:7952]	3.03133	0.000483
CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1 [Source:HGNC Symbol;Acc:HGNC:2594]	1.422893	0.000497
CNNM1	cyclin and CBS domain divalent metal cation transport mediator 1 [Source:HGNC Symbol;Acc:HGNC:102]	1.276371	0.000534
ERCC6-PGBD3	ERCC6-PGBD3 readthrough [Source:HGNC Symbol;Acc:HGNC:48347]	1.958482	0.000658
FAM149A	family with sequence similarity 149, member A [Source:HGNC Symbol;Acc:HGNC:24527]	1.390284	0.00067
C1orf233	chromosome 1 open reading frame 233 [Source:HGNC Symbol;Acc:HGNC:42951]	1.042885	0.000975
TUBB6	tubulin, beta 6 class V [Source:HGNC Symbol;Acc:HGNC:20776]	1.377469	0.001043

Appendix Table 4. Significantly overrepresented biological processes related to up-regulated genes in C-O clones compared to C-F clones.

GO biological process complete	Fold Enrichment	p-value
spindle assembly checkpoint (GO:0071173)	16.38	1.99E-02
spindle checkpoint (GO:0031577)	14.7	5.82E-03
negative regulation of mitotic nuclear division (GO:0045839)	11.95	2.27E-02
positive chemotaxis (GO:0050918)	11.81	4.77E-03
regulation of mitotic metaphase/anaphase transition (GO:0030071)	9.5	2.36E-02
regulation of metaphase/anaphase transition of cell cycle (GO:1902099)	9.3	2.76E-02

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metaphase plate congression (GO:0051310)	9.27	6.87E-03
regulation of mitotic sister chromatid separation (GO:0010965)	9.1	3.22E-02
regulation of morphogenesis of a branching structure (GO:0060688)	8.78	1.08E-02
positive regulation of leukocyte chemotaxis (GO:0002690)	8.35	8.57E-05
epithelial to mesenchymal transition (GO:0001837)	8.19	1.88E-02
mitotic sister chromatid segregation (GO:0000070)	8.19	8.18E-06
establishment of chromosome localization (GO:0051303)	8.01	1.76E-03
chromosome localization (GO:0050000)	7.9	2.01E-03
regulation of chromosome segregation (GO:0051983)	7.9	5.77E-04
sister chromatid segregation (GO:0000819)	6.98	6.95E-09
positive regulation of leukocyte migration (GO:0002687)	6.94	7.28E-05
regulation of leukocyte chemotaxis (GO:0002688)	6.89	7.76E-04
positive regulation of smooth muscle cell proliferation (GO:0048661)	6.5	3.93E-02
positive regulation of chemotaxis (GO:0050921)	6.02	1.27E-03
regulation of nuclear division (GO:0051783)	5.99	2.30E-05
regulation of mitotic nuclear division (GO:0007088)	5.85	6.59E-04
mesenchymal cell differentiation (GO:0048762)	5.81	7.21E-04
sister chromatid cohesion (GO:0007062)	5.75	1.57E-02
nuclear chromosome segregation (GO:0098813)	5.68	2.14E-08
regulation of leukocyte migration (GO:0002685)	5.66	1.44E-04
regulation of osteoblast differentiation (GO:0045667)	5.65	1.87E-02
response to estradiol (GO:0032355)	5.36	7.89E-04
cellular response to hypoxia (GO:0071456)	5.31	5.59E-03
regulation of chemotaxis (GO:0050920)	5.09	2.67E-04
cellular response to decreased oxygen levels (GO:0036294)	5.03	1.05E-02
response to glucocorticoid (GO:0051384)	4.99	8.54E-04
cell chemotaxis (GO:0060326)	4.88	2.71E-03
morphogenesis of a branching structure (GO:0001763)	4.82	7.41E-03
chromosome segregation (GO:0007059)	4.81	7.51E-07
mitotic nuclear division (GO:0007067)	4.75	6.69E-09
cellular response to oxygen levels (GO:0071453)	4.75	2.02E-02
morphogenesis of a branching epithelium (GO:0061138)	4.75	2.02E-02
regulation of ossification (GO:0030278)	4.6	5.88E-03
response to corticosteroid (GO:0031960)	4.6	2.67E-03
response to reactive oxygen species (GO:0000302)	4.51	3.48E-03

positive regulation of peptidyl-tyrosine phosphorylation (GO:0050731)	4.47	3.99E-02
positive regulation of cell migration (GO:0030335)	4.45	7.27E-09
mesenchyme development (GO:0060485)	4.35	1.21E-02
positive regulation of cell motility (GO:2000147)	4.3	1.89E-08
positive regulation of cellular component movement (GO:0051272)	4.3	8.99E-09
renal system development (GO:0072001)	4.28	1.74E-04
response to hypoxia (GO:0001666)	4.27	1.95E-05
nuclear division (GO:0000280)	4.24	6.20E-10
chemotaxis (GO:0006935)	4.17	4.77E-09
taxis (GO:0042330)	4.16	5.06E-09
positive regulation of locomotion (GO:0040017)	4.16	2.24E-08
response to decreased oxygen levels (GO:0036293)	4.14	3.56E-05
regulation of peptidyl-tyrosine phosphorylation (GO:0050730)	4.11	1.21E-02
response to oxygen levels (GO:0070482)	4.07	2.43E-05
carboxylic acid transport (GO:0046942)	4.03	1.94E-03
organelle fission (GO:0048285)	4	3.65E-09
organic acid transport (GO:0015849)	3.99	2.30E-03
urogenital system development (GO:0001655)	3.98	3.07E-04
response to lipopolysaccharide (GO:0032496)	3.95	1.75E-04
kidney development (GO:0001822)	3.93	5.62E-03
regulation of epithelial cell proliferation (GO:0050678)	3.88	9.35E-04
extracellular matrix organization (GO:0030198)	3.86	9.87E-04
extracellular structure organization (GO:0043062)	3.85	1.04E-03
regulation of organ morphogenesis (GO:2000027)	3.84	1.52E-02
regulation of cell migration (GO:0030334)	3.84	7.14E-12
response to molecule of bacterial origin (GO:0002237)	3.79	3.74E-04
response to steroid hormone (GO:0048545)	3.75	1.26E-04
response to oxidative stress (GO:0006979)	3.7	1.64E-04
activation of protein kinase activity (GO:0032147)	3.68	7.87E-03
regulation of cell motility (GO:2000145)	3.65	2.58E-11
positive regulation of response to external stimulus (GO:0032103)	3.61	3.49E-02
regulation of cellular component movement (GO:0051270)	3.61	2.80E-12
cell division (GO:0051301)	3.6	7.03E-06
regulation of protein serine/threonine kinase activity (GO:0071900)	3.55	1.76E-05
positive regulation of kinase activity (GO:0033674)	3.54	5.54E-06

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regulation of locomotion (GO:0040012)	3.5	3.55E-11
blood coagulation (GO:0007596)	3.49	3.05E-02
coagulation (GO:0050817)	3.47	3.36E-02
positive regulation of protein kinase activity (GO:0045860)	3.46	5.57E-05
negative regulation of protein phosphorylation (GO:0001933)	3.45	1.14E-03
hemostasis (GO:0007599)	3.44	3.86E-02
response to acid chemical (GO:0001101)	3.42	4.12E-03
regulation of protein kinase activity (GO:0045859)	3.38	8.92E-09
response to lipid (GO:0033993)	3.37	1.46E-11
response to organic cyclic compound (GO:0014070)	3.36	2.53E-12
regulation of kinase activity (GO:0043549)	3.33	2.67E-09
positive regulation of transferase activity (GO:0051347)	3.31	1.01E-06
negative regulation of phosphorylation (GO:0042326)	3.26	1.80E-03
cellular response to lipid (GO:0071396)	3.26	2.15E-04
regulation of cell cycle phase transition (GO:1901987)	3.25	4.88E-02
cellular response to organic cyclic compound (GO:0071407)	3.21	3.02E-04
gland development (GO:0048732)	3.2	2.64E-03
cellular response to organonitrogen compound (GO:0071417)	3.18	1.04E-03
positive regulation of protein phosphorylation (GO:0001934)	3.17	1.69E-09
positive regulation of cell proliferation (GO:0008284)	3.17	5.18E-09
inflammatory response (GO:0006954)	3.17	1.13E-03
wound healing (GO:0042060)	3.16	1.17E-03
positive regulation of phosphorylation (GO:0042327)	3.15	7.69E-10
response to drug (GO:0042493)	3.14	2.26E-03
tube development (GO:0035295)	3.1	1.33E-04
positive regulation of phosphorus metabolic process (GO:0010562)	3.1	5.62E-11
positive regulation of phosphate metabolic process (GO:0045937)	3.1	5.62E-11
response to hormone (GO:0009725)	3.09	6.49E-08
mitotic cell cycle process (GO:1903047)	3.09	8.95E-07
regulation of protein phosphorylation (GO:0001932)	3.05	9.75E-14
regulation of transferase activity (GO:0051338)	3.03	5.56E-09
regulation of body fluid levels (GO:0050878)	2.99	3.43E-03
response to wounding (GO:0009611)	2.99	4.98E-04
response to extracellular stimulus (GO:0009991)	2.99	5.82E-03
mitotic cell cycle (GO:0000278)	2.98	2.68E-06

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cell proliferation (GO:0008283)	2.96	3.43E-05
regulation of mitotic cell cycle (GO:0007346)	2.96	6.78E-03
regulation of phosphorylation (GO:0042325)	2.96	9.84E-14
cellular response to nitrogen compound (GO:1901699)	2.95	1.81E-03
regulation of cell cycle process (GO:0010564)	2.94	1.15E-03
response to nutrient levels (GO:0031667)	2.94	1.94E-02
cellular response to oxygen-containing compound (GO:1901701)	2.94	3.33E-07
cell migration (GO:0016477)	2.94	4.06E-06
response to inorganic substance (GO:0010035)	2.91	5.80E-03
cellular response to hormone stimulus (GO:0032870)	2.87	3.13E-03
negative regulation of phosphate metabolic process (GO:0045936)	2.83	6.56E-03
localization of cell (GO:0051674)	2.83	3.19E-06
cell motility (GO:0048870)	2.83	3.19E-06
negative regulation of phosphorus metabolic process (GO:0010563)	2.82	6.80E-03
positive regulation of protein modification process (GO:0031401)	2.81	5.83E-09
tissue morphogenesis (GO:0048729)	2.81	1.80E-02
locomotion (GO:0040011)	2.8	2.68E-08
vasculature development (GO:0001944)	2.79	4.77E-02
regulation of response to external stimulus (GO:0032101)	2.79	2.84E-05
regulation of phosphorus metabolic process (GO:0051174)	2.78	5.93E-14
transmembrane receptor protein tyrosine kinase signaling pathway (GO:0007169)	2.76	3.73E-02
negative regulation of protein modification process (GO:0031400)	2.71	1.54E-02
response to oxygen-containing compound (GO:1901700)	2.7	3.28E-11
regulation of phosphate metabolic process (GO:0019220)	2.7	1.30E-12
response to bacterium (GO:0009617)	2.68	4.19E-02
response to organonitrogen compound (GO:0010243)	2.67	1.51E-04
response to nitrogen compound (GO:1901698)	2.66	1.99E-05
regulation of cell proliferation (GO:0042127)	2.65	3.37E-11
regulation of cell cycle (GO:0051726)	2.64	7.45E-06
regulation of cell adhesion (GO:0030155)	2.64	7.82E-03
cell-cell signaling (GO:0007267)	2.62	5.36E-07
regulation of protein modification process (GO:0031399)	2.59	1.86E-11
regulation of MAPK cascade (GO:0043408)	2.58	8.21E-03
animal organ morphogenesis (GO:0009887)	2.52	6.92E-04
response to cytokine (GO:0034097)	2.51	7.08E-03

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cell cycle process (GO:0022402)	2.51	2.80E-05
response to endogenous stimulus (GO:0009719)	2.48	3.66E-08
response to external stimulus (GO:0009605)	2.46	4.53E-11
regulation of secretion (GO:0051046)	2.45	3.50E-02
enzyme linked receptor protein signaling pathway (GO:0007167)	2.45	3.60E-02
cellular response to endogenous stimulus (GO:0071495)	2.45	1.22E-04
anatomical structure formation involved in morphogenesis (GO:0048646)	2.44	6.48E-03
chemical homeostasis (GO:0048878)	2.43	8.88E-04
positive regulation of cellular protein metabolic process (GO:0032270)	2.41	6.22E-07
positive regulation of transport (GO:0051050)	2.39	1.99E-03
negative regulation of multicellular organismal process (GO:0051241)	2.37	9.21E-04
response to abiotic stimulus (GO:0009628)	2.35	4.10E-04
positive regulation of catalytic activity (GO:0043085)	2.35	4.30E-07
movement of cell or subcellular component (GO:0006928)	2.34	1.58E-05
circulatory system development (GO:0072359)	2.32	4.15E-02
positive regulation of protein metabolic process (GO:0051247)	2.31	2.61E-06
cell cycle (GO:0007049)	2.3	6.13E-05
regulation of anatomical structure morphogenesis (GO:0022603)	2.29	8.02E-03
tissue development (GO:0009888)	2.29	3.47E-06
positive regulation of intracellular signal transduction (GO:1902533)	2.24	3.60E-02
positive regulation of molecular function (GO:0044093)	2.21	3.79E-07
cellular response to organic substance (GO:0071310)	2.2	1.32E-07
regulation of multicellular organismal development (GO:2000026)	2.2	2.40E-06
positive regulation of developmental process (GO:0051094)	2.19	2.30E-03
cell surface receptor signaling pathway (GO:0007166)	2.19	9.40E-09
positive regulation of signal transduction (GO:0009967)	2.19	1.78E-04
positive regulation of cell communication (GO:0010647)	2.19	3.94E-05
positive regulation of multicellular organismal process (GO:0051240)	2.18	1.48E-04
positive regulation of signaling (GO:0023056)	2.18	4.66E-05
positive regulation of cellular component organization (GO:0051130)	2.18	3.90E-03
regulation of multicellular organismal process (GO:0051239)	2.17	2.58E-11
reproductive process (GO:0022414)	2.17	5.90E-04
response to organic substance (GO:0010033)	2.16	4.37E-11
reproduction (GO:0000003)	2.16	6.18E-04
positive regulation of response to stimulus (GO:0048584)	2.15	4.12E-07

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regulation of localization (GO:0032879)	2.14	8.36E-10
regulation of developmental process (GO:0050793)	2.12	3.63E-08
regulation of immune system process (GO:0002682)	2.11	7.78E-04
regulation of intracellular signal transduction (GO:1902531)	2.1	5.50E-05
cell development (GO:0048468)	2.1	9.09E-04
cell adhesion (GO:0007155)	2.1	6.34E-03
negative regulation of molecular function (GO:0044092)	2.1	4.07E-02
cellular response to chemical stimulus (GO:0070887)	2.1	2.89E-08
biological adhesion (GO:0022610)	2.09	6.92E-03
defense response (GO:0006952)	2.08	1.41E-02
regulation of cell differentiation (GO:0045595)	2.06	7.52E-04
regulation of cellular protein metabolic process (GO:0032268)	2.03	5.72E-07
anatomical structure morphogenesis (GO:0009653)	2.02	4.88E-05
regulation of cell communication (GO:0010646)	2.01	6.57E-10
regulation of catalytic activity (GO:0050790)	2.01	1.32E-06
regulation of signal transduction (GO:0009966)	1.99	3.52E-08
regulation of molecular function (GO:0065009)	1.98	1.55E-08
regulation of protein metabolic process (GO:0051246)	1.98	5.94E-07
regulation of signaling (GO:0023051)	1.98	1.94E-09
regulation of transport (GO:0051049)	1.95	1.28E-03
regulation of cellular component organization (GO:0051128)	1.93	3.06E-05
system development (GO:0048731)	1.93	9.92E-13
response to chemical (GO:0042221)	1.9	2.78E-11
response to stress (GO:0006950)	1.89	1.84E-08
regulation of response to stimulus (GO:0048583)	1.88	4.66E-10
animal organ development (GO:0048513)	1.87	2.32E-06
nervous system development (GO:0007399)	1.86	5.95E-04
cell differentiation (GO:0030154)	1.86	1.35E-07
immune system process (GO:0002376)	1.84	3.22E-03
multicellular organism development (GO:0007275)	1.83	1.76E-12
single-multicellular organism process (GO:0044707)	1.83	4.55E-16
cellular developmental process (GO:0048869)	1.83	2.71E-07
positive regulation of cellular metabolic process (GO:0031325)	1.81	4.67E-05
anatomical structure development (GO:0048856)	1.81	5.40E-13
positive regulation of cellular process (GO:0048522)	1.8	1.47E-11

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single-organism developmental process (GO:0044767)	1.77	8.50E-13
positive regulation of macromolecule metabolic process (GO:0010604)	1.75	3.53E-04
regulation of biological quality (GO:0065008)	1.75	3.48E-06
developmental process (GO:0032502)	1.74	4.95E-12
positive regulation of metabolic process (GO:0009893)	1.73	2.69E-04
negative regulation of biological process (GO:0048519)	1.72	7.09E-09
positive regulation of biological process (GO:0048518)	1.72	5.71E-11
signaling (GO:0023052)	1.72	9.15E-11
single organism signaling (GO:0044700)	1.71	1.94E-10
signal transduction (GO:0007165)	1.69	1.30E-08
cell communication (GO:0007154)	1.69	4.17E-10
multicellular organismal process (GO:0032501)	1.68	6.54E-14
negative regulation of cellular process (GO:0048523)	1.67	1.92E-06
cellular response to stimulus (GO:0051716)	1.55	4.80E-08
localization (GO:0051179)	1.55	4.69E-05
single-organism cellular process (GO:0044763)	1.52	6.09E-16
response to stimulus (GO:0050896)	1.47	1.63E-08
single-organism process (GO:0044699)	1.37	1.06E-16
regulation of cellular process (GO:0050794)	1.35	1.93E-08
regulation of biological process (GO:0050789)	1.32	1.97E-07
biological regulation (GO:0065007)	1.32	7.65E-09
cellular process (GO:0009987)	1.16	4.15E-03
biological_process (GO:0008150)	1.13	2.07E-05

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Appendix Table 5. Pathways related to up-regulated genes in C-O clones compared to C-F clones.

PANTHER Pathways	#genes	Fold Enrichment	p-value	Gene Symbol
Angiogenesis (P00005)	16	4.96	4.09E-05	ANGPT1; BIRC5; DLL1; EFNB1; EPBH2; FOS; MAPK10; PDGFRA;PLD1; PRR5; RERG; SFRP1; VEGFA; WNT2; WNT2B; WNT5A
CCKR signaling map (P06959)	13	4.1	3.95E-03	CCK; CPE; CXCL1; FOS; IRS1; ITPR1; MAP2K6; MAPK10; MMP3; PDK1; PLAU; PTGS2; RGS2
Cadherin signaling pathway (P00012)	10	3.46	1.25E-01	CELSR3; WNT2; WNT2B;WNT5A; PCDH18; PCDHGA6; PCDHGA10; PCDHGB2; PCDHGB4
Inflammation mediated by chemokine and cytokine signaling pathway (P00031)	12	2.51	5.72E-01	CCL7; GNA14; IL6; ITGA2; ITPR1; PDK1;PF4V1; PTGS1; PTGS2; RAC2; RGS4; RGS18
Plasminogen activating cascade (P00050)	3	9.1	7.33E-01	MMP3; MMP8; PLAU
Endothelin signaling pathway (P00019)	6	3.81	8.67E-01	ADCY4; EDNRB; GNA14; ITPR1; PTGS2; PRKAR2B
Wnt signaling pathway (P00057)	13	2.28	8.69E-01	CELSR3; GNA14; ITPR1; PCDH18; PCDHB2; PCDHGA6; PCDHGA10; PCDHGB2; PCDHGB4; SFRP1; WNT2; WNT2B; WNT5A
Alzheimer disease-amyloid secretase pathway (P00003)	2	1.61	1.00E+00	CHRM2; MAPK10
Alpha adrenergic receptor signaling pathway (P00002)	2	4.37	1.00E+00	ITPR1; SNAP25
Adrenaline and noradrenaline biosynthesis (P00001)	1	1.82	1.00E+00	SANP25
Triacylglycerol metabolism (P02782)	1	27.31	1.00E+00	LIPC
Synaptic vesicle trafficking (P05734)	1	1.88	1.00E+00	SNAP25
GABA-B receptor II signaling (P05731)	2	2.87	1.00E+00	ADCY4; PRKAR2B
Pyruvate metabolism (P02772)	1	4.96	1.00E+00	PC
p38 MAPK pathway (P05918)	3	3.9	1.00E+00	IL1R1; MAP2K6; RAC2
Opioid proopiomelanocortin pathway (P05917)	1	1.61	1.00E+00	SNAP25
Opioid prodynorphin pathway (P05916)	1	1.65	1.00E+00	SANP25
Opioid proenkephalin pathway (P05915)	2	3.21	1.00E+00	PENK; SNAP25
Enkephalin release (P05913)	2	3.12	1.00E+00	PENK; PRKAR2B
Dopamine receptor mediated signaling pathway (P05912)	3	2.78	1.00E+00	EPB41L3; PRKAR2B; SNAP25

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Angiotensin II-stimulated signaling through G proteins and beta-arrestin (P05911)	1	1.4	1.00E+00	ITPR1
Ornithine degradation (P02758)	1	13.65	1.00E+00	AZIN2
Ubiquitin proteasome pathway (P00060)	1	0.83	1.00E+00	UBE2C
p53 pathway (P00059)	4	2.48	1.00E+00	CCNB1; GTSE1; PDK1; PMAIP1
VEGF signaling pathway (P00056)	4	3.03	1.00E+00	PRR5; RAC2; RERG; VEGFA
Transcription regulation by bZIP transcription factor (P00055)	1	0.98	1.00E+00	PRKAR2B
Toll receptor signaling pathway (P00054)	2	1.82	1.00E+00	MAPK10; PTGS2
T cell activation (P00053)	4	2.28	1.00E+00	FOS; ITPR1; MAPK10; RAC2;
p53 pathway feedback loops 2 (P04398)	2	2.06	1.00E+00	CCNA2; PDK1
TGF-beta signaling pathway (P00052)	5	2.65	1.00E+00	BMP2; BMP4; MAPK10; RERG; TGFB2
Vasopressin synthesis (P04395)	1	4.2	1.00E+00	CPE
Thyrotropin-releasing hormone receptor signaling pathway (P04394)	2	1.82	1.00E+00	GNA14; SNAP25
Ras Pathway (P04393)	5	3.59	1.00E+00	MAPK10; MAP2K6; PDK1; PLD1; RAC2
Oxytocin receptor mediated signaling pathway (P04391)	2	1.88	1.00E+00	GNA14; SNAP25
Parkinson disease (P00049)	4	2.18	1.00E+00	MAPK10; HSPA2; SNCA; SNCAIP;
PI3 kinase pathway (P00048)	3	2.98	1.00E+00	GNA14; IRS1; PDK1
PDGF signaling pathway (P00047)	8	2.93	1.00E+00	ARHGAP6; FOS; ITPR1; MAPK10; PDK1; PRR5; RERG; PDGFRA
Oxidative stress response (P00046)	3	2.93	1.00E+00	DUSP16; MAPK10; MAP2K6;
Notch signaling pathway (P00045)	1	1.24	1.00E+00	DLL1
Nicotinic acetylcholine receptor signaling pathway (P00044)	1	0.54	1.00E+00	SNAP25
Muscarinic acetylcholine receptor 2 and 4 signaling pathway (P00043)	3	2.56	1.00E+00	CHRM2; PRKAR2B; SNAP25
Muscarinic acetylcholine receptor 1 and 3 signaling pathway (P00042)	3	2.73	1.00E+00	GNA; ITPR1; SNAP25
Metabotropic glutamate receptor group I pathway (P00041)	1	2.1	1.00E+00	ITPR1
Histamine H2 receptor mediated signaling pathway (P04386)	1	2.1	1.00E+00	PRKAR2B
Metabotropic glutamate receptor group II pathway (P00040)	3	3.41	1.00E+00	BDNF; PRKAR2B; SNAP25

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Histamine H1 receptor mediated signaling pathway (P04385)	2	2.48	1.00E+00	GNA14; ITPR1
Cortocotropin releasing factor receptor signaling pathway (P04380)	2	3.41	1.00E+00	GNA; SNAP25
Metabotropic glutamate receptor group III pathway (P00039)	3	2.37	1.00E+00	BDNF; PRKAR2B; SNAP25
JAK/STAT signaling pathway (P00038)	1	3.21	1.00E+00	SOCS1
Ionotropic glutamate receptor pathway (P00037)	3	3.28	1.00E+00	SHANK2; SLC1A3; SNAP25
Interleukin signaling pathway (P00036)	5	2.79	1.00E+00	FOS; IL6; IL11; IRS1; PDK1
Interferon-gamma signaling pathway (P00035)	2	3.64	1.00E+00	MAPK10; SOCS2
Integrin signalling pathway (P00034)	8	2.28	1.00E+00	COL4A5; COL10A1; ITGA2; ITGB3; ITGB4;LAMA5; MAPK10; RAC2
Beta3 adrenergic receptor signaling pathway (P04379)	1	1.95	1.00E+00	SNAP25
Insulin/IGF pathway-protein kinase B signaling cascade (P00033)	2	2.66	1.00E+00	IRS1; PDK1
Gonadotropin-releasing hormone receptor pathway (P06664)	8	1.86	1.00E+00	BMP2; BMP4; FOS; IRS1; ITPR1; KSR1; MAP2K6; TGFB2
Beta2 adrenergic receptor signaling pathway (P04378)	2	2.37	1.00E+00	SNAP25
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade (P00032)	2	3.31	1.00E+00	IRS1; PDK1
Beta1 adrenergic receptor signaling pathway (P04377)	2	2.37	1.00E+00	PRKAR2B; SNAP25
5HT4 type receptor mediated signaling pathway (P04376)	1	1.65	1.00E+00	SNAP25
5HT3 type receptor mediated signaling pathway (P04375)	1	3.21	1.00E+00	SNAP25
5HT2 type receptor mediated signaling pathway (P04374)	2	1.63	1.00E+00	GNA14; SNAP25
5HT1 type receptor mediated signaling pathway (P04373)	2	2.37	1.00E+00	PRKAR2B; SNAP25
5-Hydroxytryptamine degredation (P04372)	1	2.6	1.00E+00	ALDH2

Huntington disease (P00029)	4	1.57	1.00E+00	BDNF; CYFIP2; FOS; RAC2
Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway (P00027)	7	3.11	1.00E+00	BDKRB2; CHRM2; GNA14; ITPR1; RGS2; RGS4; RGS18
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway (P00026)	6	2	1.00E+00	ADCY4; CHRM2; PRKAR2B; RGS2; RGS4; RGS18
Hedgehog signaling pathway (P00025)	2	5.2	1.00E+00	PRKAR2B; PTCH1
FGF signaling pathway (P00021)	5	2.2	1.00E+00	FGF7; MAPK10; MAP2K6; RAC2; RERG
FAS signaling pathway (P00020)	1	1.61	1.00E+00	MAPK10
EGF receptor signaling pathway (P00018)	5	2.36	1.00E+00	AREG; MAPK10; MAP2K6; RAC2; RERG
DNA replication (P00017)	1	1.56	1.00E+00	TOP2A
Cytoskeletal regulation by Rho GTPase (P00016)	2	1.32	1.00E+00	PRR5; RAC2
Cell cycle (P00013)	1	2.48	1.00E+00	CCNB1
Blood coagulation (P00011)	4	4.65	1.00E+00	A2M; F2RL2; ITGB3; PLAU
B cell activation (P00010)	4	3.03	1.00E+00	FOS; ITPR1; MAPK10;RAC2
Axon guidance mediated by netrin (P00009)	1	1.56	1.00E+00	RAC2
Axon guidance mediated by Slit/Robo (P00008)	2	4.2	1.00E+00	RAC2; ROBO3
Axon guidance mediated by semaphorins (P00007)	2	4.75	1.00E+00	RAC2; SEMA4D
Apoptosis signaling pathway (P00006)	4	1.79	1.00E+00	FOS; HSPA2; MAPK10; TNFRSF10C
Alzheimer disease-presenilin pathway (P00004)	5	2.2	1.00E+00	JUP; MMP8; WNT2; WNT2B; WNT5A

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3 DISCUSSÃO

O presente estudo avaliou o transcriptoma de populações homogêneas (clones) do ligamento periodontal com alto potencial para diferenciação O/C comparado com clones sem este potencial, a fim de identificar genes que possam caracterizar populações do ligamento periodontal com maior potencial para diferenciação O/C, assim como determinar quais genes são mais expressos durante o processo de diferenciação O/C. A identificação deste perfil transcricional tem como objetivo o melhor entendimento da regulação gênica nestas populações a fim de servir como base para a proposição de novos marcadores, assim como moléculas-alvo para aplicação em procedimentos regenerativos mais previsíveis e com ampliação na indicação em Periodontia.

Identificamos que os clones C-O apresentaram maior expressão dos genes *WNT2, WNT16* e *WIF1*, relacionados às vias de sinalização Wnt/Caderina e ao processo biológico "desenvolvidmento de estrutura anatômica". Genes relacionados a estas vias já foram reportados na literatura por estarem envolvidos no maior comprometimento de células mesenquimais da medula óssea para a diferenciação osteoblástica (Granchi *et al.*, 2010).

Quando os clones C-O são estimulados à diferenciação O/C, observou-se maior quantidade de genes expressos do que quando cultivados em condições padrões (sem estímulo à diferenciação O/C). Processos biológicos incluindo "diferenciação celular mesenquimal", "regulação de diferenciação osteoblástica", "regulação de ossificação", "desenvolvimento mesenquimal" e "desenvolvimento vascular" foram significativamente mais aumentados. As vias consideradas significativamente elevadas foram as vias da angiogênese e CCKR. É sabido pela literaturatura que o aumento da formação de vasos sanguíneos é importante para a formação óssea e diferenciação das células mesenquimais em células osteoprogenitoras (Kaigler *et al.*, 2010), o que está de acordo com os dados encontrados neste estudo. Apesar de vários genes relacionados à via Wnt também terem sido significativamente mais expressos nos clones C-O durante o processo de diferenciação O/C, esta via não foi considerada signficativamente elevada durante o processo em virtude da proporção dos genes relacionados a esta via em relação ao número total de genes diferencialmente expressos ter sido menor do que a proporção de genes desta via na condição sem estímulo.

Além disso, a análise comparativa dos genes diferencialmente significativamente expressos entre as condições sem e com estímulo à diferenciação O/C foi realizada nos clones C-O e também não foi observado o aumento o enriquecimento da via Wnt após a diferenciação O/C (dados não mostrados). Nós observamos que, pelo fato de genes relacionados à via Wnt já estarem mais expressos mesmo em condições sem estímulo para a diferenciação nos clones

C-O, o envolvimento desta via não foi significativamente aumentado após a indução à diferenciação. Contudo, a maior expressão dos genes relacionados à via Wnt nos clones C-O comparados aos clones C-F, tanto quando cultivados sem estímulo, como quando cultivados com estímulo à diferenciação, demonstram que os genes relacionados à esta via parecem ser importantes para o maior compromentimento das células do ligamento periodontal para o perfil O/C. Adicionalmente, para a diferenciação dos clones C-O em células produtoras de matriz mineralizada *in vitro*, é necessário o estímulo com o meio indutor de diferenciação celular (OM), que é suplementado com β-glicerol fosfato, dexametasona e ácido ascórbico, o qual estimula o aumento da expresssão de outros genes, principalmente relacionados à via CCKR e angiogênese para promover o comprometimento com o fenótipo O/C dos clones C-O.

4 CONCLUSÃO

Pela análise do perfil transcricional entre os clones celulares derivados do ligamento periodontal humano com potencial para diferenciação osteo/cementoblástica e clones sem este potencial (perfil fibroblástico), nós concluímos que genes relacionados às vias de sinalização Wnt/Caderina e processo biológico "desenvolvidmento de estrutura anatômica" são constitutivamente mais expressos em clones com maior potencial para diferenciação osteo/cementoblástica mesmo na ausência de estímulo para diferenciação. Após a estimulação para diferenciação, genes relacionados à via de sinalização da angiogênese e CCKR, assim como processos biológicos relacionados à mineralização e diferenciação mesenquimal são significativamente mais expressos em clones com maior potencial para diferenciação, favorecendo a aquisição do fenótipo osteo/cementoblástico.

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ANEXO 1 - CERTIFICADO DE APROVAÇÃO DO COMITÊ DE ÉTICA EM PESQUISA

04/07/13 Comitê de Ética em Pesquisa - Certificado

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Isolamento e caracterização de populações homogêneas de células mesenquimais indiferenciadas do ligamento periodontal de humanos que apresentam alto potencial osteoblástico/cementoblástico**", protocolo nº 053/2013, dos pesquisadores Karina Gonzales Silverio Ruiz e Miki Taketomi Saito, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 03/07/2013.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project "**Isolation and characterization of homogeneous populations of human periodontal ligament stem cells that present high osteoblastic/cementoblastic potential**", register number 053/2013, of Karina Gonzales Silverio Ruiz and Miki Taketomi Saito, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 07/03/2013.

Prof. Dr. Felippe Bevilacqua Prado
Secretário
CEP/FOP/UNICAMP

Profa. Dra. Lívia Maria Andaló Tenuta
Coordenadora
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição
Notice: The title of the project appears as provided by the authors, without editing.

www.fop.unicamp.br/cep/sistema/certificado.php?Protocolo=053/2013&Id=18488&Passo=2&DataPar=2013-07-03 1/1