Upregulation of pro-inflammatory cytokines during dexamethasone-induced osteogenesis in human BMSCs

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INTRODUCTION: Osteogenesis is often recapitulated *in vitro* by inducing differentiation of human bone marrow mesenchymal stromal cells (hBMSCs), with the aim to study fundamental mechanisms and the effects of drugs, biomaterials and novel approaches to the regeneration and repair of bone tissue. Even though this model has been key in increasing our knowledge on bone cell formation, it suffers from several limitations: among others, the use of the synthetic glucocorticoid (GC) dexamethasone to induce cell differentiation. Dexamethasone is a commonly used anti-inflammatory drug that acts via binding to the GC receptor (GR). GR, when activated by dexamethasone, can control the expression of target genes either by transactivation or transrepression. Some recently developed GC, such as (+)-ZK216348, allow only for the transrepressional activity of the GR [1], providing a tool to understand GC effects on cells with more details on the activated pathways. The use of dexamethasone *in vitro* promotes differentiation up to a certain extent and mineral deposition in osteogenic cultures. However, we have previously showed that dexamethasone also promotes an adipocytic off-target differentiation, driven by the higher levels of *PPARG* which shows a dexamethasone dose-dependent behavior [2]. Therefore, with this study we aimed to study the effect of dexamethasone on osteogenic differentiation at a whole transcriptomic level, with a particular focus on other off-target genes and pathways.

METHODS: RNA and cDNA samples generated during a previous study [2] were used for RNA sequencing (RNAseq) and qPCR validation of sequencing results. Briefly, hBMSCs were induced to osteogenic differentiation for 7 days using either 0, 10, or 100 nM dexamethasone, or using same concentration of (+)-ZK216348, or vehicle control. Samples were collected for total RNA isolation and cDNA synthesis. Samples remaining from this study were stored at -80°C (RNA) or -20°C (cDNA) for future experiments. For the purposes of this project, the samples from n=3 donors were selected for RNAseq analysis. The selected RNA samples were subjected to a DNase treatment to remove contaminant genomic DNA.
Oxford Nanopore Technologies (ONT) library preparation was performed according to manufacturer instructions (Kit SQK-PCB109) and sequencing was performed with an ONT R9.4 flow cell. After accurate basecalling with ONT Guppy basecalling software and barcoding with ONT Guppy barcoding software, ONT Pychopper was used to assess RNA quality and process sequencing reads for further processing. Finally, the ONT long-reads pipeline for the identification of differentially expressed genes (DEGs) [3] was used for transcripts quantification. The snakemake computational pipeline included minimap2, salmon, edgeR, DEXSeq and stageR. The transcriptome ("Homo sapiens.GRCh38.cdna.all.fa.gz") and annotation ("Homo sapiens.GRCh38.95.gtf.gz") files were downloaded from the ftp.ensembl.org repository.
For validation of RNAseq results, the cDNA previously synthesized [2] (n=4 samples) was used for qPCR analysis of the selected target genes.

RESULTS: RNAseq analysis allowed the identification of the signature of dexamethasone transcriptional effects during osteogenic differentiation. Principal component analysis successfully captured the general effect of dexamethasone on the variability of gene expression. Hierarchical clustering of DEGs allowed the identification of functional groups of genes and key pathways regulated by the glucocorticoid. The comparison of results obtained from dexamethasone-, (+)-ZK216348-, or DMSO-treated samples led to the identification of DEGs regulated by either transactivation or transrepression of gene expression. Some genes were regulated by dexamethasone as expected in virtue of its anti-inflammatory properties, such as *MMP1* and *CXCL12* that were downregulated by dexamethasone and (+)-ZK216348 treatment, suggesting a direct repression of their transcription. On the contrary, several pro-inflammatory cytokines and chemokines were also upregulated. In particular, *IL18* was upregulated in a dose-dependent manner by dexamethasone (Figure 1), but not by (+)-ZK216348, suggesting its regulation via direct transactivation at *IL18* promoter level.

DISCUSSION: This work provides a deeper insight on the effect of dexamethasone on hBMSC differentiation, revealing further unexpectedly regulated pathways. Of note, the upregulation of several pro-inflammatory cytokines and chemokines including *IL18* was observed. Those findings should be further validated at the protein level, and functionally to elucidate their role during hBMSCs growth and differentiation.

SIGNIFICANCE/CLINICAL RELEVANCE: Improving current models of *in vitro* osteogenesis is pivotal to increase the translational potential of basic research findings. The production of pro-inflammatory cytokines and chemokines deserves further investigation, in order to understand the role of these molecules in the context of osteogenic differentiation and if they might contribute to the detrimental effect of long-term use of glucocorticoids on bone health.

REFERENCES: [1] *Proc. Natl. Acad. Sci. U. S. A.* **101**, 227-232 (2004). [2] *Int. J. Mol. Sci.* **22**, 4785 (2021). [3] *F1000Res* **7**, 952 (2018).

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IMAGES AND TABLES:

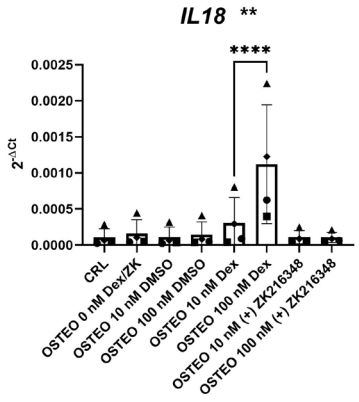

Figure 1: qPCR validation of the expression of *IL18* during the first week of osteogenic differentiation of hBMSCs. *IL18* was upregulated by dexamethasone only in a dose-dependent manner. Two-way ANOVA analysis with Tukey's multiple comparison test. **: $p<0.01$, OSTEO 10 nM Dex and OSTEO 100 nM Dex vs. all other groups; **** $p>0.0001$, OSTEO 10 nM Dex vs. OSTEO 100 nM Dex.