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Human amelogenin up-regulates osteogenic gene expression in human bone marrow stroma cells

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ABSTRACT

Extracts of enamel matrix proteins are used to regenerate periodontal tissues. Amelogenin, the most abundant enamel protein, plays an important role in the regeneration of these tissues. However, the molecular mechanisms by which amelogenin contributes to periodontal regeneration remain unknown. Using primary human bone marrow stroma cells (hBMSCs) transduced with lentivirus encoding human amelogenin (hAm), we performed genome-wide expression profiling to analyze the effects of hAm transduction on the regulation of genes involved in osteogenic differentiation. Our results revealed that BMP-2, BMP-6, OPN and VEGFC were up-regulated. These results suggest that hAm may be a key element in regulating hBMSCs osteogenic differentiation.

1. Introduction

The use of enamel matrix proteins (EMPs) for inducing periodontium regeneration of new cementum, bone and dentin has been studied in *in vivo* and *in vitro* conditions and in clinical trials [1,2]. These proteins, secreted by Hertwig's epithelial sheath, are reported to have chondrogenic, osteogenic and cementogenic properties [3]. Although there is evidence for a function of EMPs in periodontal regeneration, the active material in EMPs and the mechanisms involved in these processes are not well understood.

The majority of EMPs activities have been attributed to amelogenin as it is the most abundant enamel protein. Zeichner-David et al. [4] found that amelogenin modulated bone morphogenetic protein (BMP) expression and down-regulated the expression of type I collagen. The authors came to the conclusion that amelogenin might have some growth factor activity during periodontal tissue development and regeneration. Likewise, research by Saito et al. [5] indicates that amelogenin is the molecule involved in the activation of the extracellular signal-regulated kinase pathway when osteoblasts are stimulated with EMPs, suggesting its role as a signaling molecule. Notably, many studies suggest that amelogenin and its alternative/degradation spliced products may have cell-signaling effects and may be involved in the regeneration of periodontal tissues [6]. Finally, amelogenin appears to have a direct effect on cementoblast activity during development as well as in the regeneration of periodontal tissues [7].

Although it is well documented that amelogenin (Am) products may function as potential epithelial–mesenchymal signaling molecules during tooth development [8], far less is known about the exact mechanism of its action. To investigate the molecular mechanisms underlying hAm-dependent regulation of the osteogenic response, we performed whole-genome expression analysis in hAm gene transduced hBMSCs and control cells using cDNA microarray. Several genes involved in osteogenic differentiation were up-regulated by hAm, suggesting that hAm may be a key element in regulating hBMSCs osteogenic differentiation.

2. Materials and methods

2.1. Construction of the recombinant human amelogenin lentivirus (LV-hAm)

Reconstruction and expression of LV-hAm were performed according to previously described protocols [9]. Human amelogenin X cDNA (Gene Bank Accession No. M86932) was subcloned into the FUGW transfer vector (a transfer vector encoding enhanced green fluorescent protein, EGFP) of the lentivirus expression system. The recombinant plasmid was named FUAmW. Titers of the virus were estimated by flow cytometry (FCM) analysis (determined by the percentage of EGFP positive cells). The lentiviral vector preparations had an infectivity of 5×10^7–1×10^8 IU/mL.

Abbreviations: EMPs, enamel matrix proteins; hAm, human amelogenin; hBMSCs, human bone marrow stroma cells; BMP, bone morphogenetic protein; OPN, osteopontin; VEGFC, vascular endothelial growth factor C; EGFP, enhanced green fluorescent protein; FCM, flow cytometry; rhBMP-2, recombinant human bone morphogenetic protein-2; TRAP, tyrosine-rich amelogenin peptide.

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2.2. Culture of hBMSCs and lentivirus-mediated transduction of hAm

hBMSCs were isolated and cultured by methods reported previously [10]. Third-passage hBMSCs were infected with the generated recombinant lentiviruses, LV-hAm and LV-GFP. Control transduced hBMSCs (infected with LV-GFP) were harvested 72 h after transduction and analyzed by flow cytometry (FCM) to determine the expression of transgenes. The transduced cell colonies were selected with 800 μg/mL Zeocin (Invitrogen). The expression of human amelogenin in hBMSCs was detected by Reverse transcription-PCR.

2.3. Genome-wide expression profile analysis by Illumina BeadArray

Total RNA from LV-hAm infected hBMSCs and control cells (hBMSCs infected with LV-GFP) were amplified and labeled with the Illumina RNA Amplification kit (Ambion, Inc., Austin, TX) following the manufacturer's instructions. The resulting material (700 ng/array) was hybridized to a pilot version of the Illumina Sentrix Human-6 Expression BeadChip according to the manufacturer's instructions (Illumina, Inc., San Diego, CA). Three arrays were done per sample. Arrays were scanned with an Illumina Bead Array Reader confocal scanner according to the manufacturer's instructions. The data processing and analysis were performed using Illumina BeadStudio software.

2.4. Identification of differentially expressed genes and quantitative real-time PCR

The genes with statistically significant differential expression were determined according to the fold change in transcript level and Diffscore value between LV-hAm infected hBMSCs and control cells. The genes that exhibited at least 3-fold greater expressions in LV-hAm infected hBMSCs compared to the control were isolated as candidate genes for further study. The Diffscore represents the significance of the difference in gene expression between two samples. It is defined as a method of measuring significant differential expression for a gene in two samples by Illumina BeadStudio, with the following levels of significance:

Diffscore of >13 or <−13 correspond to p values of 0.05.
Diffscore of >20 or <−20 correspond to p values of 0.01.
Diffscore of >30 or <−30 correspond to p values of 0.001.

Hence the genes with Diffscore values (LV-hAm infected hBMSCs vs. the control cells) from −13 to 13 were eliminated. According to functional annotation of the genes by Illumina, we further selected the genes involved in osteogenic differentiation.

The transcript level of the four selected genes (BMP-2, BMP-6, OPN and VEGFC) and one housekeeping gene (β-actin) were confirmed by quantitative real-time PCR with the Applied Biosystems 7700 instrument. All PCR reactions were done in triplicate.

2.5. Statistical evaluation

All statistics were performed using Student's t test in Excel. p values <0.05 were considered to indicate statistical significance.

3. Results

3.1. Lentivirus-mediated transduction of hAm in hBMSCs

Third-passage hBMSCs were infected with LV-hAm or LV-GFP. After a 72-h incubation, the FCM assay was carried out to determine the expression of EGFP. As shown in Fig. 1A, GFP was expressed in the cells infected with LV-hAm. Reverse transcription-PCR assays were carried out to determine hAm mRNA expression in the hAm-transduced cells. LV-hAm infection resulted in expression of hAm in hBMSCs. hAm mRNA (540 bp) was expressed in the cells infected with LV-hAm, whereas transduction with LV-GFP had no effect on hAm mRNA expression (Fig. 1B). As a control, the expression of β-actin (220 bp) was measured. The results indicate that LV-hAm efficiently expressed hAm mRNA in hBMSCs.

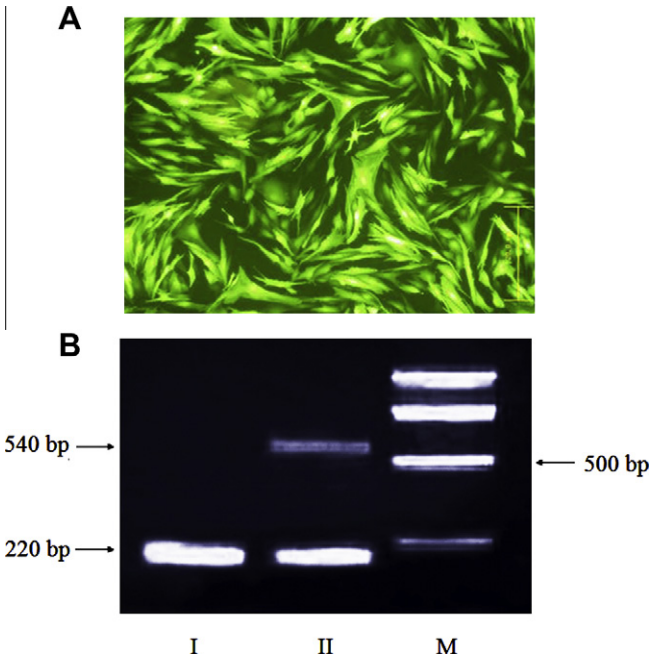

Fig. 1. Gene products of EGFP/hAm transduction in hBMSCs after the 72-h incubation. (A) FCM assay showing the expression of EGFP in the hBMSCs infected with LV-GFP. (B) hAm mRNA(540 bp) was expressed in the hBMSCs infected with LV-hAm, whereas LV-GFP has no effect on hAm mRNA expression. The RT-PCR assay results indicate that LV-hAm efficiently induced hAm mRNA expression in hBMSCs. I: hBMSCs infected with LV-GFP II: hBMSCs infected with LV-hAm M: Marker.

3.2. Genome-wide expression profile analysis of hBMSCs transduced with hAm

To gain insights into the mechanism by which hAm induces hBMSCs osteogenic differentiation, genome-wide expression profile analysis was carried out in LV-hAm infected hBMSCs and control cells using a cDNA microarray. As shown in Fig. 2, hAm transduction resulted in extensive differential gene expression in hBMSCs. Of ~37,805 genes analyzed, we identified a large number of genes with significantly different expression in LV-hAm infected cells compared to the control cells. Based upon potential involvement in osteogenic differentiation, four of the differentially expressed genes were selected for further analysis and characterization. The Diffscore data in Fig. 3A clearly indicates significant differences in gene expression profiles between the two groups of cells. A fold change in the transcript indicates that the selected gene has been markedly up-regulated in LV-hAm infected cells compared to control cells. Transcript levels of the selected genes were quantified by quantitative real-time PCR, normalized to the β-actin control using the $2^{-\Delta\Delta Ct}$ method and depicted as $\log_2$ of the fold-change value between LV-hAm infected BMSCS and control cells (Fig. 3B). Consistent with the results of microarray analysis, the quantitative real-time PCR data confirms the differential expression profile of the selected genes in the LV-hAm infected cells compared to control cells.