

Expression and subcellular localization of histone deacetylases in mesenchymal stem-like cells from exfoliated deciduous teeth

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The acetylation of histones and transcription factors plays a crucial role in cell functional activity. Balance between histone acetyltransferases (HATs) and histone deacetylases (HDACs) is closely related to diverse cellular processes, including differentiation, proliferation, and apoptosis. Expression of different classes of HDACs is strictly dependent on the cell type, and differentiated cells possess different members of the HDAC family. In our study, we employed mesenchymal stem-like cells (MSC) derived from human exfoliated deciduous teeth. Expression of HDACs at mRNA level was analysed using the RT-PCR method. Next, we have assessed the protein levels of different classes of HDACs and their localization within the cell. The cytoplasm and nuclear fractions of cell lysates were subjected to western blot analysis. Since the functional activity of class II HDACs is determined by their shuttling between the nucleus and the cytoplasm, we have analysed the subcellular localization of HDAC4 in response to the well-known activators of differentiation, i. e. bone morphogenetic protein 2 (BMP-2) and phorbol 12-myristate-13-acetate (PMA), using fluorescent confocal microscopy. Our results suggest that class I and II HDAC family members are expressed and produced in MSC-like cells. The predicted HDAC4 translocation from the cell nucleus was not induced by PMA or BMP-2 in our model. However, our findings open new insights into a possible targeting for HDACs in future transcription activations / derepression studies.

Key words: mesenchymal stem cell, MSC, HDAC

INTRODUCTION

Mesenchymal stem cells (MSC) are characterized by their ability to differentiate into lineages of mesenchymal tissues, including bone, cartilage, muscle and fat under appropriate stimuli [1, 2]. Initially, bone-marrow-derived MSCs were characterized. Since then, populations of the MSC-like cells with similar characteristics (morphology, differentiation potential and immunophenotype) have been isolated from various tissues (adipose, umbilical cord, dental pulp, synovium, etc.) [3–6]. Recent studies have demonstrated that MSC-like cells can be isolated from virtually all organs and tissues. Authors assume that MSC-like cells are distributed throughout the organism, possibly occupying the perivascular niche [7, 8]. In the present study, we have used MSC-like cells derived from human exfoliated deciduous teeth. Exfoliated deciduous teeth-derived dental pulp is a relatively new and unexplored source of MSC-like cells. Importantly, these

cells demonstrate a very high *in vitro* proliferation potential and are able to regenerate bone tissue *in vivo* [9, 10]. Thus, MSC-like cells derived from exfoliated deciduous teeth can be used as a promising experimental model for studying osteogenic differentiation and bone regeneration.

The process of cell differentiation is driven by specific genetic programs responsible for mature, specialized phenotype formation. Regulated changes in the chromatin structure play a central role in this process. Histone acetylation, catalyzed by histone acetyltransferases (HATs), promotes gene transcription by relaxing the chromatin structure. The stimulatory effect of HATs is counterbalanced by histone deacetylases (HDACs) which promote chromatin condensation and transcriptional repression [11–14]. Class I HDACs (1–3, 8, 11) are ubiquitously expressed. By contrast, the class II HDACs (4, 5, 7, 9) display tissue-restricted (muscle, brain, T and B lymphocytes, chondrocytes) expression patterns [15–17]. In addition, class II HDACs contain an N-terminal regulatory region which mediates interactions with specific transcription factors and also serves as a target for post-transcriptional modifica-

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tions connecting signals from the extracellular environment and chromatin remodelers [18]. Ca^{2+} / calmodulin-dependent kinases (CaMKs I, II, and IV) and protein kinase D (PKD) phosphorylate serines on N-terminal regulatory region of class II HDACs, creating the binding site for the ubiquitous 14–3–3 adaptor protein which further facilitates subsequent export of II class HDACs from the nucleus. This results in hyperacetylation of specific transcription factors, derepression of the target genes and initiation of specific differentiation programs [15, 17, 19].

Besides phosphorylation, class II HDACs may also be subjected to ubiquitination and sumoylation. Unlike ubiquitination, modification of N-terminal region by a small ubiquitin-related modifier (SUMO) does not lead to protein degradation, but is rather associated with protein–protein interactions, subcellular protein distribution and stability [20, 21].

Little is known about the role of HDACs in the differentiation of MSC. A recent study has demonstrated that HDAC inhibitors, valproic acid and trichostatin A, accelerate osteogenic differentiation of the MSCs. This effect was linked to an increased expression of osteogenic differentiation-related genes Runx2, osterix, osteopontin, and bone morphogenetic protein 2 (BMP-2) [22]. However, the HDAC inhibitors used in this study display a broad activity spectrum. Therefore, the precise role of distinct classes of HDACs during the process of MSC differentiation remains largely unknown. Interestingly, HDAC4 is expressed in pre-hypertrophic chondrocytes and regulates endochondral bone formation by interacting with the transcription factor Runx2 which is an important regulator of osteo- and chondro- genesis [12, 18].

Since MSCs participate in chondro-, osteo-, and adipogenesis, we speculate that class II HDACs might be involved in these processes. Class II HDACs demonstrate tissue-restricted expression patterns and are regulated by signaling kinases. However, its involvement in MSC differentiation remains to be elucidated. In the present study, we aim to investigate the expression and subcellular distribution of class II HDACs in proliferating MSC-like cells derived from exfoliated deciduous teeth.

MATERIALS AND METHODS

Isolation of MSC-like cells from human exfoliated deciduous teeth. MSC-like cells were isolated according to protocol approved by the Lithuanian Bioethics Committee. Briefly, the pulp from a normal exfoliated deciduous tooth of a 5-year-old child was separated and digested in collagenase solution (3 mg/ml) for 1 h at 37 °C. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Biochrome) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal bo-

vine serum (Biochrome) at 37 °C in a humidified atmosphere containing 5% of CO_2 .

Induction of osteogenic differentiation. MSC-like cells were seeded at a density $5 \times 10^3/cm^2$ in DMEM (low glucose) supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium pyrophosphate in 35-mm diameter plastic dishes and cultured until subconfluent. Differentiation was then induced by adding an osteogenic-induction medium prepared according to manufacturer's instructions (StemCell Technologies) with addition of dexamethasone (10^{-8} M) and ascorbic acid (50 µg/ml). When the cell multilayering became evident, β -glycerophosphate was added into the medium (10^{-6} M). Cell cultures were treated with osteogenic-induction medium for two weeks, the medium was changed twice a week. Osteogenic differentiation was assessed by hydroxy-apatite crystal staining with silver nitrate (von Kossa). The cells were first fixed for 10 min with 1% formalin in PBS and stained with 1% silver nitrate for 30 min, followed by 5% sodium thiosulfate for 5 min. Control cells were cultured without differentiation stimuli and were stained in the same manner.

Flow cytometry. MSC-like cells were tested for the presence of the set of MSC markers. Single cell suspension of MSC-like cells (approximately 5×10^5) in 200 µl of PBS buffer was incubated with 2 µl of phycoerythrin (PE)-conjugated antibodies against CD14, CD105, CD73, CD34, or fluorescein isothiocyanate (FITC)-conjugated antibodies against CD45, CD90 (Santa Cruz Biotechnology). After three washes, fluorescence was evaluated by flow cytometry in FACSaria (Becton Dickinson, BD).

Reverse-transcriptase (RT)-PCR analysis. Endogenous mRNA of MSC-like cells was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. We used the First strand cDNA synthesis kit with (dT)₁₈ primer (Fermentas) to generate the first-strand cDNA. The reaction product (cDNA) was subjected to PCR analysis with the primers shown in Table 1. PCR reactions (35 cycles) were performed in 25 µl of reaction mix (5 µl cDNA, 1 µl Thermo-Start Taq DNA polymerase (Abgene), 200 nM dNTP and 500 nM forward and reverse primers, 1× reaction buffer with 1.5 mM $MgCl_2$). An automated Mastercycler personal amplifier (Eppendorf) was used for PCR amplification reactions. The final products were separated by agarose gel electrophoresis, incubated in ethidium bromide, and photographed under UV illumination. For sizing the PCR products, GeneRuler™ 50 bp, GeneRuler™ 1 kb Plus and Lambda DNA / Hind III (Fermentas) DNA markers were used.

Western blot analysis. Cells were grown to 70–80% confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin

Table 1. Oligonucleotides used in expression analysis of HDACs mRNA

	Forward	Reverse	Amplicon
b-actin	5'-TGACGGGGTACCCACACTGTGCC-3'	5'-TAGAAGCATTTCGCGTGGACGATG-3'	600 bp
HDAC1	5'-CAAGTCCACATCAGTCCTTCC-3'	5'-TGCGGCAGCATTTAAGGTT-3'	100 bp
HDAC2	5'-AGTCAAGGAGGCGGCAAAA-3'	5'-TGCGGATTCTATGAGGCTTCA-3'	100 bp
HDAC3	5'-CCGAAATGTTGCCCGCTGTG-3'	5'-AGGTGCATGGTTCAGCATCTT-3'	220 bp
HDAC4	5'-AAGGAGTGGACATTGCT-3'	5'-GATTCAGCAGCTCCACT-3'	85 bp
HDAC5	5'-TCACTGTCACTCAACTCAC-3'	5'-CAGGAATAGAGGATGTGC-3'	134 bp
HDAC7	5'-CTTCTCCACAAGGACAAG-3'	5'-CTCCAGGTTCTGTAGG-3'	150 bp

and 10% fetal bovine serum (Biochrome) at 37 °C in a humidified atmosphere containing 5% of CO₂. Following stimulation with 100 nM phorbol 12-myristate-13-acetate (PMA) (Sigma) for 60 min, the cells were washed three times with ice-cold PBS and lysed in lysis buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Triton X-100, 20 mg/ml aprotinin, 1 mM PMSE, 2 mM NaVO₄). Lysates were clarified by centrifugation at 20 000 × g for 15 min. Isolation of cytosolic and nuclear protein fractions was performed in parallel using the Nuclei EZ kit (Sigma) according to the manufacturer's instructions. All samples were fractionated in 10% SDS-PAGE gel and transferred to the PVDF membrane (Bio-Rad). Approximate sizing of proteins and transfer efficiency were performed using Precision Plus Protein Dual Color Standards (Bio-Rad). Primary rabbit polyclonal anti-HDAC1 and anti-HDAC4 (Cell Signaling Technology) antibodies were used at a 1 : 500 dilution. Secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG (Santa Cruz) was used at 1 : 2000 dilution. Immunoreactivity was detected by reaction with BCIP / NBT substrate (Sigma).

Immunocytochemistry. Cells grown on coverslips were incubated with 100 nM PMA (Sigma) or 100 ng/ml bone morphogenetic protein 2 (BMP-2) (R & D Systems) for 60 min at 37 °C. Control cells were left in the cell culture medium alone. The

coverslips were rinsed three times in ice-cold PBS (pH 7.6) and fixed in 4% (w/v) paraformaldehyde for 15 min at room temperature. Then the cells were washed three times in PBS and permeabilized with 1% Triton X-100 for 15 min. Next, the cells were blocked in 5% goat serum (v/v) (DAKO) for 60 min at room temperature, washed with PBS again and incubated with the primary rabbit anti-HDAC4 antibody (Cell Signaling Technology) for 90 min at 37 °C (dilution 1 : 200). Finally, the cells were washed three times with PBS, incubated with the secondary Alexa 488 coupled goat anti-rabbit (Molecular Probes) antibody at a concentration of 15 µg/ml and mounted with Fluorescent mounting medium (DAKO). Coverslips were subsequently examined by confocal laser scanning microscopy using a Nikon Eclipse TE2000U microscope.

RESULTS AND DISCUSSION

In the present study, we have used MSC-like cells derived from human exfoliated deciduous teeth. Cells were isolated according to a protocol described elsewhere, with minor modifications [9]. The isolated cells displayed a typical fibroblastoid morphology and a high proliferation potential (Fig. 1). Our *in vitro* studies of MSC-like cells revealed their ability to differentiate into os-

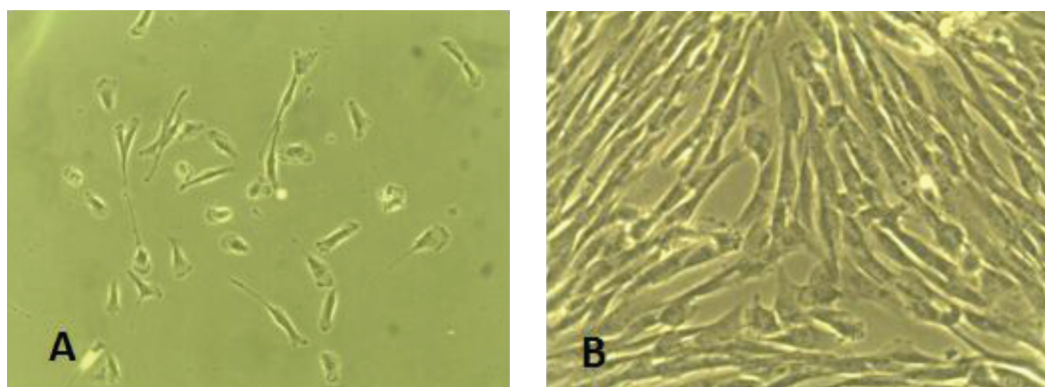


Fig. 1. Morphology of MSC-like cells isolated from human exfoliated deciduous teeth. Cells were cultured in DMEM medium containing 10% FBS for 3 days (A) or for 12 days (B). Phase-contrast images (magnification 100×) represent typical adherent fibroblastoid colony (A) and a subconfluent cell layer (B)

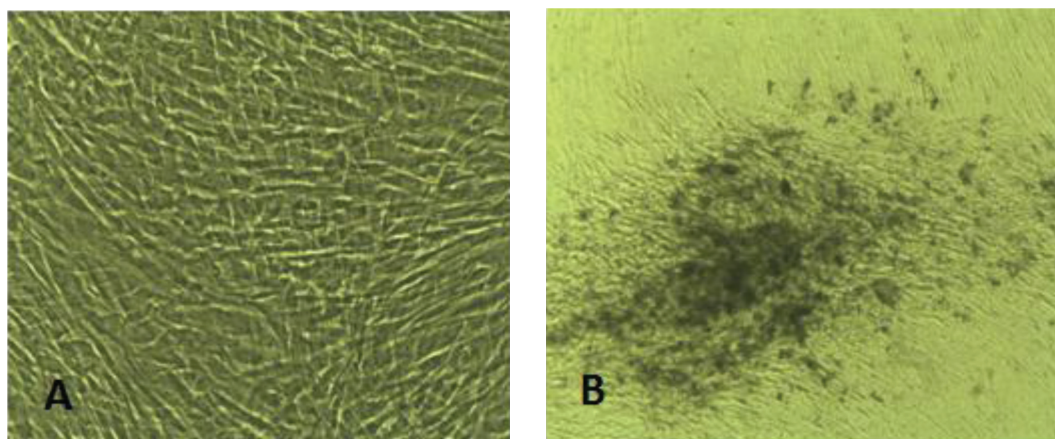


Fig. 2. Osteogenic differentiation properties of MSC-like cells. *In vitro* osteogenesis. Cells were grown in DMEM media containing 10% FBS (A) or exposed to osteoinducing medium for 14 days (B). Mineral depositions at day 14 were stained by von Kossa method (B)

teocytic cells. The cells cultured under osteogenic conditions formed multilayers within the first week. Staining with silver nitrate (von Kossa) revealed characteristic patterns of mineralized matrix two weeks later (Fig. 2). We then analysed the expression of markers recognized as typical of MSCs. According to the International Society for Cellular Therapy [1], MSC should express CD105, CD73 and CD90 and a lack of expression of CD45, CD34, CD14 surface molecules. For that reason, sets of specific positive and negative markers were analysed using FACS (Table 2). We found that our MSC-like cells resembled the recognized phenotype of MSC.

According to previous reports, expression of HDACs depends on cell type and regulates cell differentiation [13, 17, 22]. However, there is no direct evidence of expression of all classes of HDACs in MSC. We have analysed HDAC mRNA expression patterns by the RT-PCR method using specific primers to class I HDAC (HDAC1, 2, 3) and class II HDACs (HDAC4, 5, and 7) (Table 1). The housekeeping β -actin gene was used as a reference gene. Surprisingly, we have found mRNA expression of all analysed HDACs at a detectable level (Fig. 3). This is the first report to show that both class I and class II HDACs are expressed in MSC-like cells in our model.

Table 2. Expression of cell surface markers by MSC-like cell

Marker	Positive cells (% of total cell count)
CD105	52.4
CD90	96.2
CD73	98.5
CD45	1.4
CD34	5.9
CD14	8.1

We next examined the expression and subcellular localization of HDAC proteins in MSC-like cells derived from human exfoliated deciduous teeth. We chose HDAC1 and HDAC4 (representing class I and II HDACs, respectively) for these studies. Cell lysates were analysed by Western blot using specific antibodies against HDAC1 and HDAC4 (Fig. 4).

According to our results, both, HDAC1 and HDAC4 proteins are expressed in MSC-like cells. In agreement with other studies, we have found that HDAC1 localizes within the cell nucleus only, while HDAC4 is present in the cytoplasm and the nucleus. Unexpectedly, a major intranuclear immunoreactive band of the HDAC4 protein was detected at approx. 175 kDa, whereas other

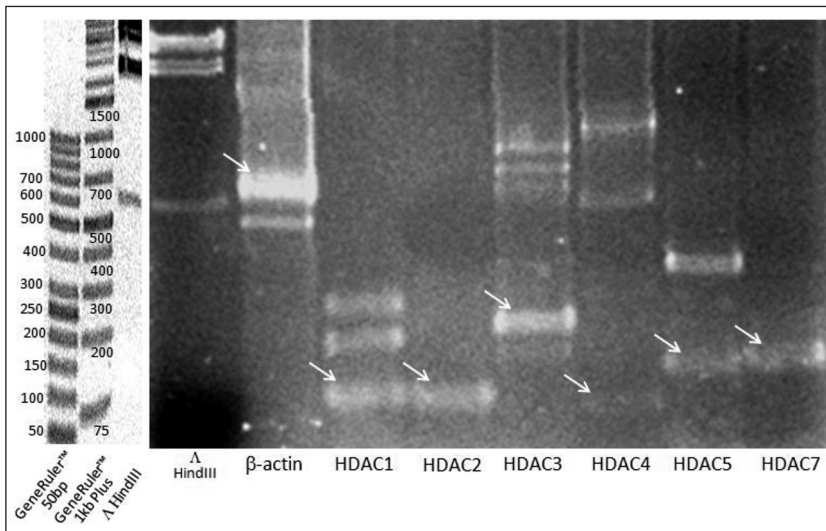


Fig. 3. Expression profile of different classes of HDACs mRNA. RT-PCR was performed on total mRNA isolated from MSC-like cells. Oligo(dT)₁₈ primer was used. The first-strand cDNA generated was used in PCR with primer-pair targeting indicated HDACs, or beta-actin for the control. The final products were separated by 1.5% agarose gel electrophoresis, incubated in ethidium bromide, and photographed under UV illumination. The numbers refer to the size of DNA fragments in bp. Arrows indicate specified PCR products at the molecular size of fragments indicated in Table 1

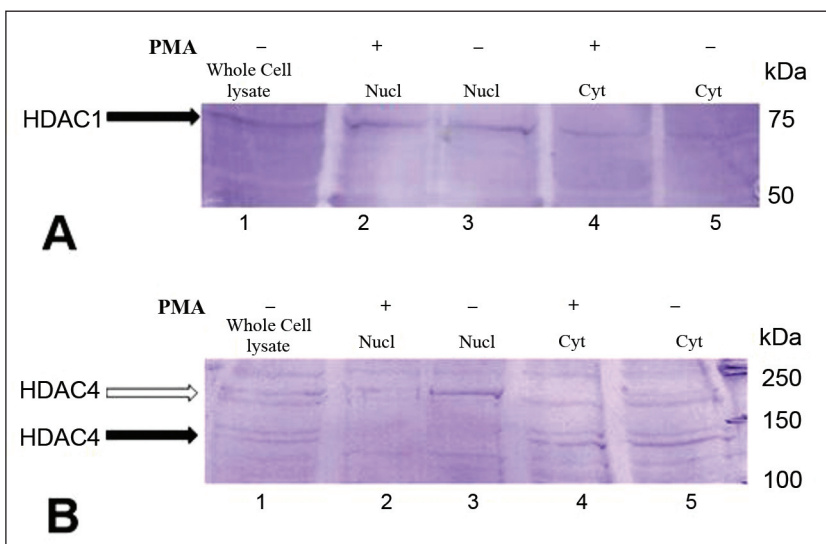


Fig. 4. Expression and localization of HDAC1 and HDAC4 proteins in subcellular fractions. Nuclear (lanes 2 and 3) and cytoplasm (lanes 4 and 5) fractions of PMA-treated (100 nM for 60 min) (+) or untreated (-) cells and also total cell lysates (lane 1) were subjected to western blot analysis using anti-HDAC1 (A) and anti-HDAC4 (B) antibodies. Protein bands of HDAC1 and HDAC4 are indicated by arrows. White arrow indicates possibly modified HDAC4

studies report 140 kDa as a typical molecular weight of native HDAC4. A possible explanation for this phenomenon could be HDAC4 modification by SUMO protein. According to Kirsh [21], sumoylated HDAC4 migrates as a 175 kDa protein band, and its specific function and subcellular distribution are different from those of the native form. Importantly, we found that in cytoplasmic HDAC4 the molecular weight was 140 kDa. We speculate that the native and the sumoylated isoforms of HDAC4 could be differently distributed in separate cellular compartments.

Unlike class I HDACs, the function of class II HDACs is primarily determined by their subcellular localization. The phosphorylation of class II HDACs by protein kinase D (PKD) triggers their export from the nucleus [23–26]. Therefore, we have investigated whether PMA, a well-known activator of PKD, may induce nuclear export of HDAC4 protein in MSC-like cells [27]. We found a insignificant decrease of the nuclear HDAC4 protein in PMA-treated cells, while the cytoplasmic HDAC4 (molecular weight 140 kDa) remained largely unchanged.

Next, subcellular localization of HDAC4 protein in MSC-like cells treated with several activating agents was analysed by confocal microscopy (Fig. 5). In addition to PMA, we employed BMP-2, an inducer of osteogenic differentiation in MSC cells

[18, 28]. Confocal microscopy experiments revealed the nuclear localization of HDAC4 protein after BMP-2 stimulation. Neither PMA nor BMP-2 was able to induce subcellular redistribution of HDAC4 protein in our model. These data are in agreement with our western blotting results where a strong immunoreactive HDAC4 band was detected at 175 kDa in the nuclear fraction. The presence of HDAC4 protein within the nucleus, detected microscopically, indirectly supports our western blotting results where a modified (possibly sumoylated) HDAC4 protein was detected also in the nuclear fraction. Our data demonstrate that neither PMA nor BMP-2 are able to induce subcellular redistribution of HDAC4 protein in MSC-like cells. Further analysis of HDAC inactivation signaling pathways will be in the focus of our future studies.

In sum, our study shows that the morphology, culture characteristics, and phenotypic marker expression profile of cells from human exfoliated deciduous teeth are similar to those of mesenchymal stem cells. We for the first time report expression of class I and class II HDACs in MSC-like cells at both mRNA and protein levels. Although the expression of class II HDACs has been reported to be tissue-restricted, members of this class (HDAC 4, 5, and 7) as well as class I HDACs (1,

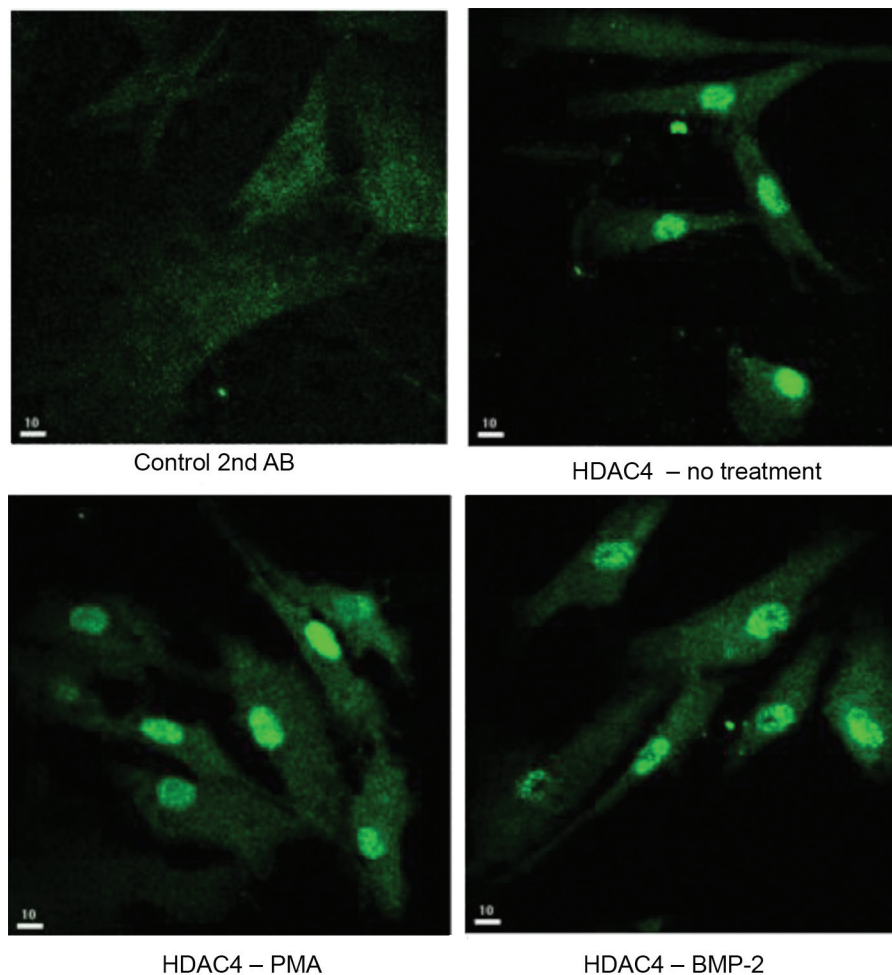


Fig. 5. Distribution of HDAC4 in MSC-like cells. Subconfluent cells were fixed and incubated with anti-HDAC4 polyclonal antibody, then visualized with Alexa 488-conjugated goat anti-rabbit secondary antibody. Confocal images demonstrate the nuclear localization of HDAC4 in untreated, PMA-treated (100 nM for 60 min), and BMP-2-treated (100 ng/ml for 60 min) MSC-like cells. Bar: 10 μ m

2, and 3) were found in our cells. Due to HDAC4 importance in osteogenic differentiation, we have assessed the expression and localizations of HDAC4 in cells treated with a strong PKD activator (PMA) and an inductor of osteogenic differentiation (BMP-2). According to our data, the possibly sumoylated HDAC4 protein accumulates in the nucleus of the MSC-like cells, and its localization is not changed by PMA or BMP-2 treatment. We speculate that more complex mechanisms are responsible for the HDAC4 activity regulation in cells. Our data elucidate presence of integrated regulatory mechanisms of transcription derepression / activation in MSC-like cells and indicate several candidate targets, i. e. HDACs, for the future studies.

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HISTONŲ DEACETILAZIŲ RAIŠKOS IR VIDULĄSTELINĖS LOKALIZACIJOS TYRIMAI IŠ PIENINIO DANTIES IŠSKIRTOSE MEZENCHIMINĖSE KAMIENINĖSE LĄSTELĖSE

Santrauka

Histonų ir transkripcijos veiksnių acetilinimas yra vienas iš esminių ląstelių veiklos reguliavimo būdų. Ląstelėje histonų acetiltransferazių (HAT) ir deacetilazių (HDAC) balansas yra susijęs su diferenciacija, proliferacija ar apoptoze. Diferencijuotos ląstelės pasižymi skirtinga įvairių tipų HDAC raiška, aktyvavimu, lokalizacija ir funkcija. Šiame darbe tyrėme iš pieninio danties pulpos išskirtas ląsteles, kurias priskyrėme mezenchiminių kamieninių ląstelių tipui (MKL tipui). Atvirkštinės transkripcijos PGR būdu (RT-PCR) nustatėme I ir II HDAC klasės atstovų (HDAC1, HDAC2, HDAC3 ir HDAC4, HDAC5, HDAC7) raišką mRNR lygyje. Tirdami HDAC1 ir HDAC4 baltymų raišką branduolinėje ir citoplazminėje ląstelių frakcijose patvirtinome branduolinę HDAC1 lokalizaciją bei aptikome HDAC4 pasiskirstymo ląstelių frakcijoje priklausomybę nuo jo modifikacijos. Kadangi HDAC4 funkcinis aktyvumas susijęs su baltymo lokalizacijos pokyčiais, fluorescencinės konfokalinės mikroskopijos metodu tyrėme HDAC4 lokalizacijos atsaką paveikus forbolo esteriu (PMA) ir kaulų morfogenetiniu baltymu 2 (BMP-2). Remdamiesi rezultatais galime teigti, kad tirtos I ir II klasės histonų acetilazės yra gaminamos MKL tipo ląstelėse. Darbo metu imunoblotais ir konfokaline mikroskopija nustatyta HDAC4 lokalizacija ląstelėse. Pastebėta, kad mūsų modelyje nei po PMA, nei po BMP-2 poveikio branduolinė HDAC4 lokalizacija pastebimai nepakito. Visi gauti duomenys rodo, kad tirtose MKL tipo ląstelėse egzistuoja kompleksiškas HDAC4 lokalizacijos ir funkcinio aktyvumo reguliavimo mechanizmas, kurio ištyrimas padės ateityje išaiškinti potencialias MKL diferenciacijos galimybes.