Biochemical and Biophysical Research Communications 428 (2012) 271–277

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Chemical and genetic blockade of HDACs enhances osteogenic differentiation of human adipose tissue-derived stem cells by oppositely affecting osteogenic and adipogenic transcription factors

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ARTICLE INFO

Article history:
Received 4 October 2012
Available online 16 October 2012

Keywords:
hASCs
HDACs
Runx2
PPARγ
Osteo-differentiation

ABSTRACT

The human adipose-tissue derived stem/stromal cells (hASCs) are an interesting source for bone-tissue engineering applications. Our aim was to clarify in hASCs the role of acetylation in the control of Runt-related transcription factor 2 (Runx2) and Peroxisome proliferator activated receptor (PPAR) γ. These key osteogenic and adipogenic transcription factors are oppositely involved in osteo-differentiation. The hASCs, committed or not towards bone lineage with osteoinductive medium, were exposed to HDACs chemical blockade with Trichostatin A (TSA) or were genetically silenced for HDACs. Alkaline phosphatase (ALP) and collagen/calcium deposition, considered as early and late osteogenic markers, were evaluated concomitantly as index of osteo-differentiation. TSA pretreatment, useful experimental protocol to analyse pan-HDAC-chemical inhibition, and switch to osteogenic medium induced early-osteoblast maturation gene Runx2, while transiently decreased PPARγ and scarcely affected late-differentiation markers. Time-dependent effects were observed after knocking-down of HDAC1 and 3: Runx2 and ALP underwent early activation, followed by late-osteogenic markers increase and by PPARγ/ALP activity diminutions mostly after HDAC3 silencing. HDAC1 and 3 genetic blockade increased and decreased Runx2 and PPARγ target genes, respectively. Noteworthy, HDACs knocking-down favoured the commitment effect of osteogenic medium. Our results reveal a role for HDACs in orchestrating osteo-differentiation of hASCs at transcriptional level, and might provide new insights into the modulation of hASCs-based regenerative therapy.

1. Introduction

The human adipose-tissue derived stem/stromal cells (hASCs) differentiate towards various lineages including bone, dependent on the stimuli (osteogenic supplements, collagen scaffolds) and growth factors [1,2]. Based on the regenerative properties and the scarcely invasive procedure for collection of adipose tissue, hASCs are important tools for therapy of bone diseases [3]. No significant differences between hASCs and bone-marrow derived mesenchymal stem cells from the same patient are observed with regard to the yield of adherent cells, their growth kinetic, cell senescence, differentiation capacity, and gene transduction efficiency [3]. Numerous preclinical applications for hASCs in musculoskeletal tissue engineering are reported [4–7].

Notwithstanding the growing interest in the differentiation plasticity and therapeutic potential of hASCs, molecular mechanisms favouring engagement towards osteogenic lineage have been scarcely investigated. There is few evidence on the epigenetic mechanisms involved in the control of transcription-factor network driving osteo-differentiation, through the expression of osteoblast-maturation genes and extracellular matrix mineralization.

Our funding hypothesis is that osteogenesis is a complex process associated with dramatic changes of gene expression, and the epigenetic control via histone acetylation might be a key molecular mechanism for osteo-differentiation of hASCs. Histone deacetylases (HDACs)/acetylases (HATs) spatio-temporal interplay is critical for gene expression changes [8]. HDACs influence histone acetylation and chromatin condensation, but they act also as co-repressors or co-activators of transcription factors [9–11]. In the present paper we add insights on the role of acetylation in hASCs differentiation towards bone lineage by regulating transcription factors.

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http://dx.doi.org/10.1016/j.bbrc.2012.10.044

HDAC1, 3, 4 and 6 are known to inhibit Runt-related transcription factor 2 (Runx2) activity in different pre-osteoblastic cell lines and bone-marrow stem cells [2,12,13]. Runx2, the master osteoblastic transcription factor, seems involved in osteogenic differentiation of hASCs [14,15]. During osteogenesis, total HDAC-enzymatic activity and HDAC1 expression decrease [12]. Noteworthy, HDAC3 and 7 play opposite roles in osteoclast differentiation, favouring and inhibiting osteoclastogenesis [16].

HDAC1 and 3 (Class I HDACs) are broadly expressed, and localize in cell nuclei like those of stem cells, contributing to transcription-factor regulation for example as Runx2 co-repressors [9,17]. In contrast, HDAC4 and 6 (Class II HDACs) are expressed in mature osteoblasts with a tissue-restricted expression pattern: cytosol/nuclear shuttle occurs in response to signalling-pathway stimulation [17,18]. The specific role of HDAC1 and 3 as activators or repressors of the transcriptional activity of Peroxisome proliferator activated receptor (PPAR) γ in hASCs osteo-differentiation is largely unknown.

Our study deals with the effect of HDAC blockade on the activities of Runx2 and PPARγ, key transcription factors for the divergent osteogenic and adipogenic differentiation pathways, respectively [19]. The aim was to clarify whether acetylation state influenced osteo-differentiation at transcriptional level, and whether a co-operation with the osteogenic commitment occurred. Adequate supplements in the culture medium (osteogenic medium) allowed to obtain osteogenic commitment [20,21]. We examined the effects of specific HDAC1 and 3 knocking-down versus pan-HDACs inhibition with Trichostatin A (TSA), on Runx2 and PPARγ transactivating activities as well as on early (alkaline phosphatase, ALP) and late (calcium and collagen deposition) osteogenic markers [22,23].

ALP activity plays a pivotal role in calcified tissue formation by regulating phosphate transport [24]. Calcium deposition and osteogenic-gene expression in pluripotent stromal cells derived from adipose tissue and bone marrow are enhanced by HDACs blockade with valproic acid [25], and matrix mineralization occurs early in HDAC3 suppressed pre-osteoblasts [13].

Our results suggest a new methodological approach to favour osteogenic differentiation by oppositely and specifically affecting the activities of Runx2 and PPARγ by genetic knock-down of HDACs.

2. Materials and methods

2.1. Reagents

DMEM-high glucose, foetal bovine serum (FBS), TSA and troglitazone from Sigma–Aldrich (St. Louis, MO). Anti-HDAC1 and anti-HDAC3 antibodies from Abcam (Cambridge, UK) and Cell Signaling (Beverly, MA). Lipofectamine 2000 and Optimem from Invitrogen (Carlsbad, CA). Antibodies against collagen, type I, α1 (COL1A1), osteopontin, adipocyte-fatty acid-binding protein (A-FABP) and lipoprotein lipase (LPL) from Santa Cruz-Biotechnology (Santa Cruz, CA).

2.2. Cell preparation, culture conditions and osteo-differentiation

The hASCs were isolated from the subcutaneous-adipose tissue of 3 healthy female donors (range 30–50 yrs, BMI < 30 without any pathological obesity), undergoing elective lipoaspiration, after written consent and Institutional-Review Board authorization. Primary cultures establishment: after digestion of raw lipoaspirates (50–100 ml) with 0.075% type I collagenase, hASCs were separated by centrifugation (1200×*g* for 10 min), filtered and plated (100,000 cells/cm^2) in basal medium (10% FBS). For the experiments, 10,000 cells/cm^2 were re-plated. To induce osteo-differentiation, hASCs were cultured in osteogenic medium consisting of basal medium supplemented with 10 mM glycerol-2-phosphate, 10 nM dexamethasone, 150 μM L-ascorbic acid-2-phosphate, and 10 nM cholecalciferol: exposure to osteoinductive medium lasted for a maximum of 14 days. hASCs cultured in basal medium were the controls [20,21].

2.3. Plasmids and siRNAs

To evaluate Runx2 and PPARγ activities, we used the gene reporters driven by 6-Runx2 binding sites (p6OSE2-Luc) and 3-PPRE binding sites (PPREx3-tk-Luc) generously given by J.B. Kim (Seoul National University, South Korea) and R.M. Evans (Howard Hughes Medical Institute, La Jolla, CA). siRNA sequences (Eurofin MWG Operon, Ebersberg, Germany): 5′-CGUACGCGGAAUACUUCGATT-3′ (siRNALuc, used as control); 5′-CAGCGACUGUUUGAGAACCTT-3′ (HDAC1.1) and 5′-CUAAUGAGCUUCCAUACAATT-3′ (HDAC1.3) for siRNAs HDAC1; 5′-GAUGCUGAACCAUGCACCUTT-3′ for siRNA HDAC3.

2.4. Cell transfection and evaluation of luciferase activity

Gene reporters (200 ng/0.5 ml) and siRNAs (150 nM) were transfected alone or in combination, using Lipofectamine 2000 dissolved in Optimem, when the cells reached 50–70% of confluence (efficiency of transfection more than 70%) [26]. For all the observation periods, i.e. 2, 7 or 14 days of culture in basal or osteogenic medium: (A) The gene reporters were transfected during the last 24 h. For PPARγ-activity assay, 20 μM troglitazone was added to the culture medium twice during the 24 h PPRELuc-transfection. The absolute values for Runx2 and PPARγ luciferase activities, shown as ratios calculated by the software, were obtained using as internal control pRL-TK *Renilla*-luciferase [26]. Two different protocols were used for TSA: (i) in cells transfected or not, TSA (1 or 2.5 μM) was added for 1 or 2 days at the end of the experimental period; (ii) 2 days pretreatment with 1 μM TSA was performed, and then hASC were switched to osteogenic medium for 2, 7 and 14 days, and the transfection performed during the last 24 h. (B) HDAC siRNAs and siRNALuc were transfected twice, with a time-period interval of 24 h, before the switch to the osteogenic medium. The silencing effect of HDAC siRNAs lasts for 72 h [27].

2.5. Western Blot assay

Samples of total extracts from cells transfected with siRNAs of HDACs were analysed at 14 days in basal medium [26].

2.6. Evaluation of osteogenic differentiation

ALP activity was measured in cell lysates (0.1% Triton X-100), using as substrate 1 mM p-nitrophenylphosphate in alkaline buffer (100 mM diethanolamine and 0.5 mM $MgCl_2$, pH 10.5), and evaluating the absorbance at 405 nm. Calcium deposition was measured staining with 40 mM Alizarin Red-S (ARS, pH 4.1) and, then, destaining with 10% cetylpyridinium chloride in 0.1 M phosphate buffer (pH 7.0); total collagen production was evaluated using 0.1% (w/v) Sirius Red F3BA in saturated picric acid for 1 h at room temperature, solubilizing with 0.1 M NaOH: the absorbances were assayed at 550 nm. Calf-skin type I collagen was used as standard.

2.7. Statistical analysis

The data were analysed using ANOVA, with $P < 0.05$ considered significant. Differences from controls were evaluated on original experimental data.

3. Results

3.1. Effect of TSA pretreatment on Runx2 and PPARγ activities as well as on osteo-differentiation markers under basal and osteogenic conditions

As shown in Fig. 1, we tested 1 μM TSA that activated ALP (Supplementary Figure), without giving signs of cell sufferance also for time periods longer than 14 days (data not shown). In particular, we performed 1 μM TSA pretreatment for 2 days, before switching to the osteogenic medium, because of the remarkable efficacy of this protocol on osteo-differentiation of hASCs [23] and the absence of hASCs apoptosis (data not shown). As index of osteo-differentiation, we examined the transactivating activity of Runx2 as well as early and late markers; as adipogenic marker we evaluated PPARγ-transactivating activity.

Basal activity values for Runx2, PPARγ and ALP are reported (Fig. 1A). To give more relevance to the TSA effect, in Fig. 1B the luciferase activities were shown as fold-variations of the ratios between TSA pretreated and untreated hASCs. Taking into consideration 2, 7 and 14 days after TSA pretreatment, Runx2Luc increased in basal medium at 7 days, and in osteogenic medium between 2 and 14 days. In contrast, diminution (−62%) of PPARγ activity occurred at 7 days in osteogenic medium, in respect to basal medium. Of note, osteogenic medium *per se* caused a fall down (−80%) of PPARγ activity only at 14 days (data not shown).

A possible differentiation of hASCs towards an immature-Runx2 positive osteoblast population led to study ALP activity, calcium and collagen deposition (Fig. 1C and D). TSA pretreatment in basal medium activated ALP after 7 days. Notwithstanding osteogenic medium *per se* enhanced ALP activity, TSA pretreatment incremented the stimulation (+64%) at 7 days (Fig. 1C). Exposure to osteogenic medium increased calcium and collagen deposition in hASCs, in comparison to basal medium, while TSA pretreatment seemed inhibitory for calcium deposition at 14 days, without affecting collagen deposition (Fig. 1D).

All the experiments were repeated with different hASCs–primary cultures established from the three donors with similar results.

In conclusion, the rapid increase of Runx2 activity by TSA under osteogenic medium, might be really related to an early differentiating osteoblast population showing activation also of ALP, an osteoblast-maturation gene. Noticeably, calcified matrix production and collagen deposition increased at late times of osteogenic commitment, without favouring effects of TSA pretreatment. It is known that TSA broadly compromises the activity of HDACs, having different biological significance, albeit with varying efficiencies [28]. For example cell proliferation is regulated more by Class I (1, 2, 3 and 8) rather than by Class II (4–7, 9 and 10) HDACs [29].

3.2. Genetic suppression of HDAC1 and 3 stimulated hASCs differentiation towards osteoblasts through transcriptional molecular mechanisms

Since the pan-HDAC inhibitor TSA seemed only to affect the early phases of osteoblast maturation, we examined whether specific HDAC1 and 3 knocking-down with siRNAs might favour osteoblast-terminal differentiation (Figs. 2 and 3).

The transfection of siRNAs for HDAC1 and 3 reduced the respective HDAC protein levels (Fig. 2A). To check the reproducibility of the knocking-down, we used two different oligonucleotides for HDAC1, i.e. 1.1 and 1.3, similarly effective in reducing HDAC1-protein level [26]. ALP was activated by siRNA HDACs at 7 days of culture in both the utilized media (Fig. 2B). siRNALuc did not modify the stimulatory effect exerted by osteogenic medium *per se*. Calcium and collagen depositions were enhanced at 14 days by HDAC3 silencing in osteogenic medium (Fig. 2C). siRNA HDAC1 and 3 combination was tested, but resulting toxic for hASCs, the data are not shown.

Under HDAC-genetic silencing, Runx2 and PPARγ activities were also evaluated (Fig. 3A). siRNALuc has been used as control. siRNA HDAC1 transfection slightly increased Runx2 activity under basal medium. Silencing of both HDACs activated Runx2 activity under osteogenic medium: the stimulatory effect was higher for HDAC1 siRNA (+260%) than for HDAC3 siRNA (+60%). The combination of siRNAs was less effective due to the toxicity (data not shown). PPARγ showed a persistent diminution (until 14 days) after siRNA HDACs transfection. Even if we did not observe substantial differences for PPARγ activity depending on culture medium, it is noteworthy that in osteogenic medium siRNA HDAC3 progressively inhibited PPARγ activity, diminishing (−50%) at 14 days.

All the experiments were repeated with different hASCs–primary cultures established from the three donors with similar results.

As shown in Fig. 3B, we evaluated the expression of a panel of genes, indicators of osteo-differentiation and adipo-differentiation. The steady-state protein levels of COL1A1 and osteopontin, target genes of Runx2, increased in siRNA HDAC1-silenced hASCs, while the expression of A-FABP and lipoprotein lipase, target genes of PPARγ, diminished in siRNA HDAC3-silenced hASCs.

Fig. 3C summarizes the principal results obtained with siRNA HDACs. The data are shown as fold-variations relative to siRNALuc control value, taken as 1 both under basal and osteogenic medium. Some differences were observed for the molecular effects of HDAC1 and 3 silencing. siRNA-HDAC1 transfection of osteo-induced hASCs, increased concomitantly the early-osteoblast maturation genes ALP and Runx2 at 7 days. Interestingly, HDAC1 knock-down under basal medium was sufficient to enhance early marker ALP and to cause the precocious deposition of calcium. The HDAC3 silencing at 14 days enhanced the expression of late markers of osteogenesis, i.e. calcium and collagen deposition, favouring the effect of osteogenic medium. At transcriptional level, the differentiation towards osteoblasts/osteocytes seemed to involve the reduction of PPARγ activity in both culture medium conditions.

4. Discussion

Here we show that osteo-differentiation of hASCs was under the control of HDAC1 and HDAC3: osteogenic and adipogenic markers Runx2 and PPARγ were oppositely affected by HDACs blockade. Chemical or genetic inhibition of HDACs seemed important for the control of transcriptional mechanisms favouring the engagement of hASCs towards bone lineage. Two main aspects deserve further discussion: the role of specific silencing of HDAC1 and 3 in osteogenic-terminal differentiation; the patterns of early and late differentiation markers under our experimental conditions, index of the formation of an osteoblast-like population from hASCs and the terminal differentiation.

First, Runx2 and PPARγ activities were studied with gene reporters, that permitted to evaluate the transactivating functions mirroring gene expression downstream. In particular, we examined transcription factors implicated in divergent differentiation pathways, i.e. the osteoblast-specific Runx2 [2], and PPARγ that is critical for adipogenesis [19]. The target genes of Runx2 are collagen1α1, osteocalcin and osteopontin, important for bone calcification; ALP is, instead, regulated indirectly by Runx2. At this purpose, it has been suggested that Wnt5a is implicated in the control of ALP and Runx2 activities [15]. PPARγ activation precludes osteogenesis, and PPARγ gene could become a target of drugs aiming to enhance bone mass by suppressing its expression.

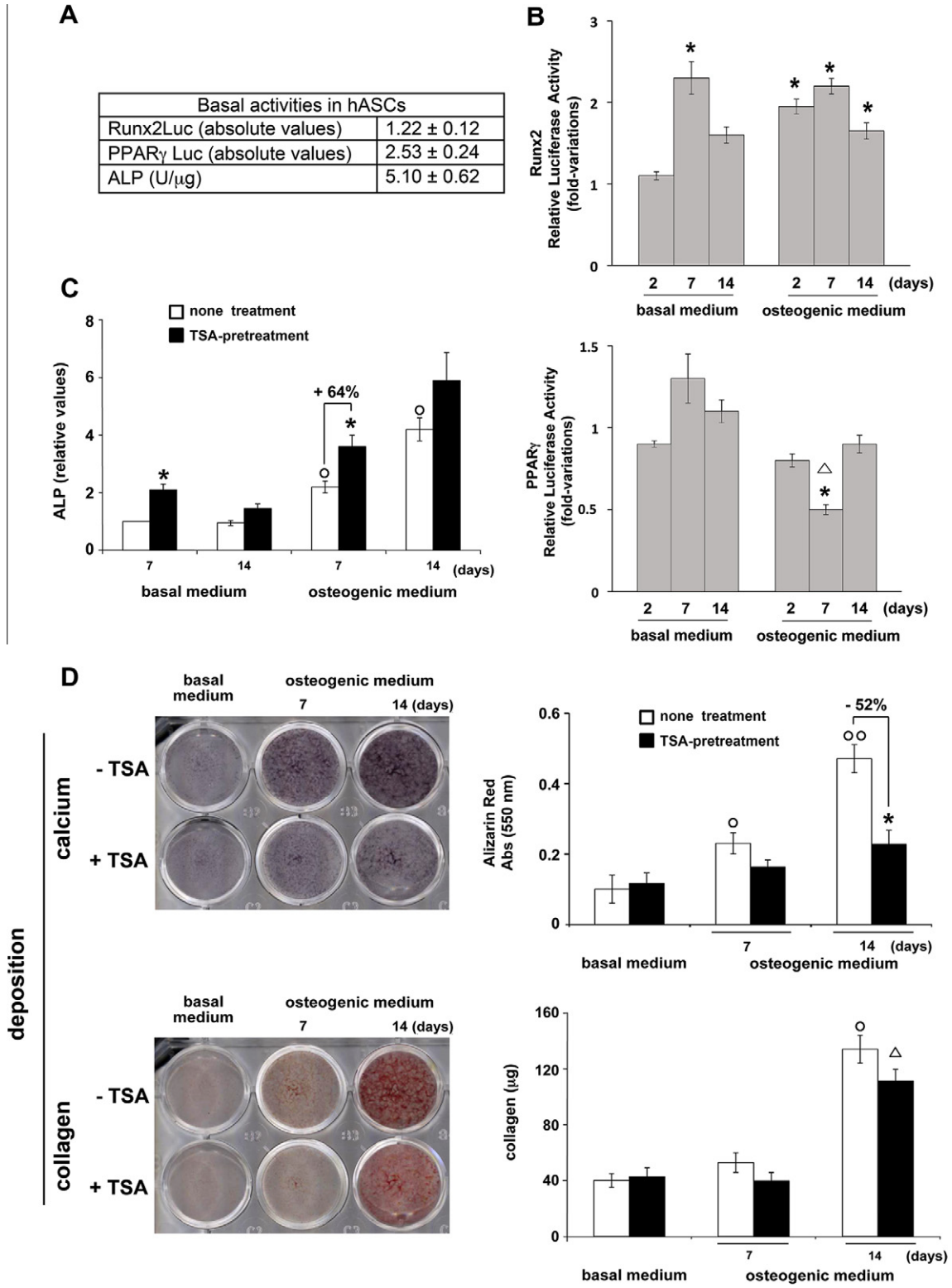

Fig. 1. Effects of TSA-pretreatment on Runx2 and PPARγ luciferase activities, and osteogenic differentiation markers. (A) Basal absolute values of the studied activities are shown. The experiments were performed three times, and the data are shown as means ± SE. (B) Relative-luciferase activity indicated the fold-variations of the ratios between the values of TSA pretreated and untreated cells, transfected with the reporter genes. The data are the means ± SE of three independent experiments performed in triplicate. $^{*}P < 0.05$ vs. the value at 2 days in basal medium; $^{\Delta}P < 0.05$ vs. the value at 7 days in basal medium. (C, D) Relative ALP activity was evaluated considering the value of untreated cells in basal medium as 1. The data are the means ± SE of experiments performed in triplicate. Calcium deposition was measured with Alizarin Red staining, and collagen deposition with Sirius Red assay. Representative images of treated hASCs in plates are shown after staining. Histograms show the means ± SE of three independent experiments. For basal-medium experiments (D), the data obtained at 7 and 14 days were similar. $^{\circ}P < 0.05$; $^{\circ\circ}P < 0.005$ vs. the value of untreated cells in basal medium: (first white bar). $^{*}P < 0.05$ vs. the corresponding value of TSA-untreated cells in osteogenic medium; $^{\Delta}P < 0.05$ vs. the value of TSA-pretreated cells committed to osteogenesis for 7 days.

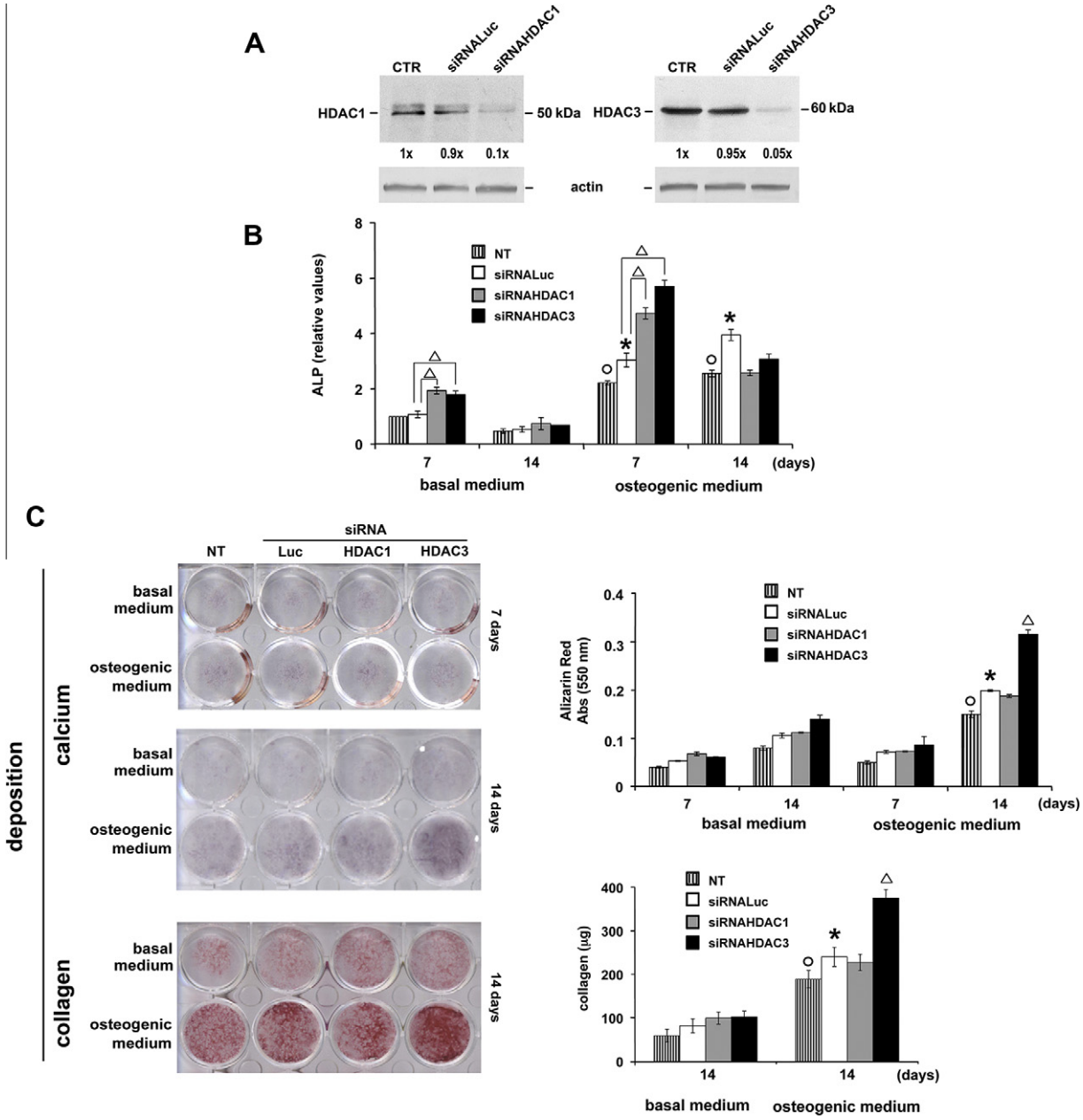

Fig. 2. Osteo-differentiation markers under HDAC1 and 3 knocking-down. (A) Western Blots of total-protein extracts from hASCs transfected or not with the indicated siRNAs; anti-HDAC1 and anti-HDAC3 antibodies were used for immunoblotting. Normalization was performed with anti-actin antibody. The numbers at the bottom indicate the fold-variations relative to the first lane, considered as 1. The experiments were repeated three times with similar results. (B, C) hASCs were transfected with the indicated siRNAs. Relative ALP activity was evaluated considering the value of untreated cells in basal medium as 1 (NT). The data are the means ± SE of experiments repeated three times. Calcium deposition was measured with Alizarin Red staining, and collagen deposition with Sirius-Red assay. Representative images of treated hASCs in plates are shown after staining. Histograms show the means ± SE of three independent experiments. $^{\circ}P < 0.05$ vs. the value of the first bar (NT in basal medium); $^{*}P < 0.05$ gives the significance of siRNALuc in osteogenic medium vs. basal medium at 7 or 14 days; $^{\Delta}P < 0.05$ vs. the respective siRNALuc value.

The knocking-down of HDAC1 and 3 oppositely affected Runx2 and PPARγ, resulting enhanced and inhibited both under basal and osteogenic media. A remarkable effect of HDAC1 silencing on Runx2 activity was observed, also when hASCs were already committed to osteogenesis by osteoinductive medium. In differentiating hASCs, HDACs seemed to function as transcription factor regulators: HDAC1 and 3 were PPARγ activators and Runx2 repressors. Thus, our data extended to hASCs the inhibitory role of HDAC1 and 3 on Runx2 activity, as reported for other cell types [2,12,13]. It is known that HDACs can repress Runx2 activity by either protein–protein interaction [13,30] or by promoting Runx2 degradation through deacetylation [31]. Mounting evidence shows that Runx2-protein levels and activity are regulated independently of mRNA expression: post-translational modifications, including phosphorylation, ubiquitination and acetylation are indeed involved [32]. HDAC3, one of the best studied HDACs, appears to be a very important factor in multiple co-repressor complexes controlling gene expression [10,11]. A generic effect of HDACs silencing on chromatin condensation might be excluded because we used gene-reporter constructs, that were reconstituted systems.

Second, HDACs genetic inhibition seemed more efficient than chemical blockade not only in inducing immature osteoblast population, but especially in conferring an osteogenic phenotype, as

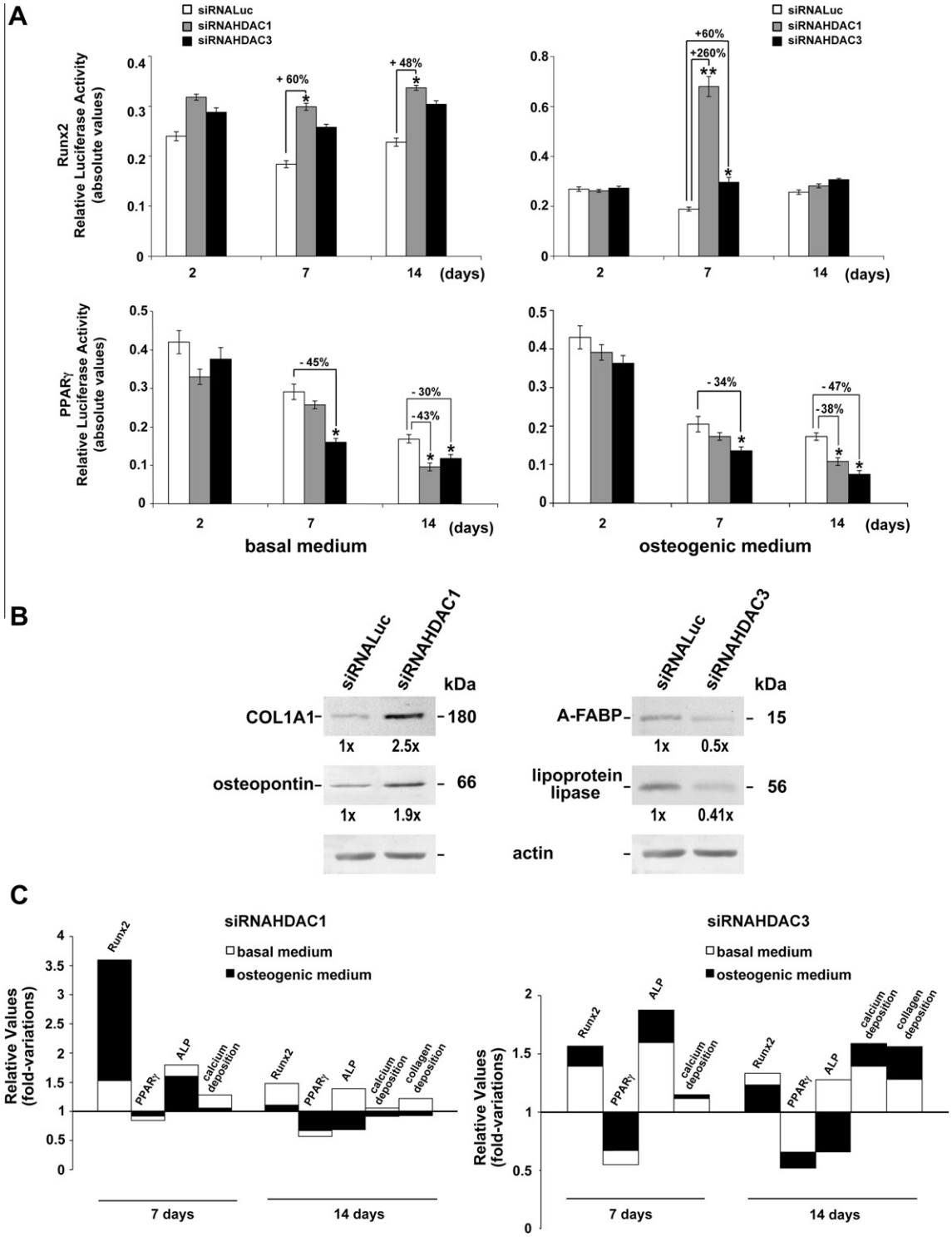

Fig. 3. Transcription factor activities under HDAC1 and 3 knocking-down. (A) hASCs were first transfected with the indicated siRNAs, and then with Runx2 or PPARγ expression vector at the end of the observation period for 24 h. The data are the means ± SE of three independent experiments performed in triplicate. $^{*}P < 0.05$; $^{**}P < 0.005$ vs. the value of the respective siRNALuc. (B) Western Blots of total-protein extracts from hASCs transfected with the indicated siRNAs. Actin was used for normalization. The numbers at the bottom indicate the fold-variations relative to siRNALuc, considered as 1. The experiments were repeated three times with similar results. (C) Schematic representation of the relative changes of the molecular parameters analysed in hASCs transfected with siRNAs for HDAC1 and 3. The data are shown as fold-variations in respect to corresponding siRNALuc value, taken as 1 both under basal and osteogenic medium. The absolute values are shown in the figures of the present paper.

indicated by the late markers calcium and collagen deposition. In fact, siRNA HDAC3 showed an additive effect with osteogenic medium in the triggering of differentiation towards bone lineage, also because of the strong inhibitory effect on PPARγ activity. Of note, one of the principal effects of HDAC3 silencing was the progressive enhancement of calcium deposition, starting already from the differentiation stage characterized by ALP activation, an enzyme important for calcified tissue formation. Our data also indicate that ALP was regulated by acetylation, being known until now its control by methylation [33].

Notwithstanding osteogenic medium *per se* activated ALP, HDACs silencing as well as TSA exposure further stimulated ALP activity concomitantly with Runx2-transactivating function. hASCs seemed, therefore, to differentiate towards an immature-osteoblast population through the control at gene level of matrix mineralization and expression of osteoblast genes. However, only hASCs silenced for HDACs in osteoinductive medium, assumed a phenotype producing late osteogenic markers. In contrast, the HDACs silenced cells in basal medium seemed to maintain an osteoblastic phenotype, expressing the osteoblast maturation genes ALP and Runx2.

Our study reveals molecular aspects possibly useful for bone differentiation from stem cells endowed of PPARγ and Runx2 activities, but practically devoid of Osterix activity (data not shown) consistent with Wu et al. [34]. In Osterix-expressing osteoblastic lineage, however, HDAC3 conditional knock-out is detrimental to skeletal health [35].

In conclusion, the wide effect of HDAC-genetic blockade, oppositely influencing Runx2 and PPARγ activities and their target genes, might better engage hASCs to undertake the osteogenic lineage towards terminal differentiation. These data led to suppose for HDACs a role as targets for therapy in regenerative medicine of skeletal pathologies with bone loss.

Acknowledgments

Authors thank Dr. Alessandro Degrate for his contribution to the statistical analysis. Grants: CARIPLO Foundation, 2010-0737; Ministero della Salute, Ricerca Corrente 4029 and 4064.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.044.

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