

Ratjadone C-Mediated Nuclear Accumulation of HDAC4: Implications on Runx2-Induced Osteoblast Differentiation of C3H10T1/2 Mesenchymal Stem Cells

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Z. Naturforsch. **69c**, 471–478 (2014) / DOI: 10.5560/ZNC.2014-0065

Received March 26 / November 7, 2014 / published online December 22, 2014

Histone deacetylases (HDACs) are a group of enzymes that deacetylate ϵ -N-acetyl lysine residues of histone and non-histone proteins and play an important role in gene regulation. HDAC4, a class-IIa HDAC, has been reported to shuttle between nucleus and cytoplasm in response to various cellular stimuli. The nucleo-cytoplasmic shuttling of HDAC4 is critical, and an anomalous nuclear localization might affect the cellular differentiation program. While the subcellular localization of HDAC4 has been reported to be vital for myoblast differentiation and chondrocyte hypertrophy, nuclear accumulation of HDAC4 during Runx2-induced osteoblast differentiation of stem cells has not been characterized. Ratjadone C is a natural compound that inhibits the nuclear export of proteins. Here, we show that Runx2 is a more potent transcription factor than Osterix in inducing osteoblast differentiation. Under the influence of ratjadone C, HDAC4 is retained in the nucleus and co-localizes with Runx2. However, forced nuclear accumulation of HDAC4 by ratjadone C or overexpression of the nuclear resident form of HDAC4 does not inhibit osteoblast differentiation, suggesting that the Runx2-induced osteogenic program of C3H10T1/2 cells is not affected by HDAC4. Even though phosphorylation of HDAC4 affects its compartmentalization and the stemness of progenitor cells, we found that total HDAC4 and phosphorylated HDAC4 remain cytoplasmic under both osteogenic and non-osteogenic conditions. Collectively, this work demonstrates that, regardless of the nucleo-cytoplasmic presence of HDAC4, the Runx2-induced osteoblast differentiation program of C3H10T1/2 cells remains unaffected. Additionally, the ratjadone C-mediated nuclear retention assay can potentially be used as a screening tool to identify novel regulatory mechanisms of HDAC4 and its functional partners in various pathophysiological conditions.

Key words: HDAC4, Runx2, Stem Cells, Ratjadone C

Introduction

There is a continuous and balanced epigenetic modification of histones and non-histone proteins in eukaryotic cells. This widespread posttranscriptional modification event comprising acetylation and deacetylation of proteins is regulated by two counteracting en-

zymes, *i.e.* the histone acetyl transferases (HATs) and the histone deacetylases (HDACs) (Peserico and Simone, 2011). Based on the sequence homology to yeast HDACs, these proteins can be grouped into four different classes, *i.e.* classes I, II, III, and IV. Class II HDACs can be further subdivided into classes IIa and IIb. The members of subclass IIa, consisting of

HDAC4, HDAC5, HDAC7, and HDAC9, respond to various environmental cues and co-interact with other members of the HDAC family to mediate epigenetic transitions (Delcuve *et al.*, 2012). Class-IIa HDACs exhibit tissue-specific expression patterns and frequently undergo nucleo-cytoplasmic shuttling to regulate their target genes and act as transcriptional repressors (Clocchiatti *et al.*, 2011).

HDAC4 has been reported to play significant roles in musculo-skeletogenesis and is expressed in cells of the mesenchymal lineage, such as osteoblasts, chondrocytes, and myocytes (Miska *et al.*, 2001; Vega *et al.*, 2004). *In vivo*, HDAC4 is expressed in prehypertrophic chondrocytes and inhibits Runx2-mediated chondrocyte hypertrophy. Mice lacking HDAC4 display ectopic and early onset of chondrocyte hypertrophy and premature ossification, similar to the phenotype observed in chondrocytes constitutively expressing Runx2 (Vega *et al.*, 2004). On the other hand, overexpression of HDAC4 prevents hypertrophy and ossification as seen in Runx2 knockout mice (Vega *et al.*, 2004). *In vitro*, HDAC4 has been shown to play a critical role in muscle formation and myoblast fusion during which it translocates from the cytoplasm to the nucleus to repress MEF2-dependent transcription and differentiation (McKinsey *et al.*, 2000; Miska *et al.*, 2001). Thus, differential compartmentalization seems to be an effective mechanism of HDAC4 mediating epigenetic changes or acting as transcriptional repressor.

Although nucleo-cytoplasmic shuttling of HDAC4 has been well documented for chondrocytes and myoblasts, its aberrant presence in the nucleus during Runx2-induced osteogenic differentiation has not been studied hitherto. Hence, we used ratjadone C to induce nuclear retention of HDAC4 and to examine whether thereby Runx2-mediated osteogenic differentiation of C3H10T1/2 stem cells can be inhibited.

Materials and Methods

Materials

The exportin-1 inhibitor, ratjadone C (cat. no. ALX-270-369), in native form was purchased from Enzo Life Sciences (Lausen, Switzerland). Anti-flag mouse monoclonal antibodies (cat. no. F-1804) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-HDAC4 (cat. no. sc-11418) and anti-phosphorylated HDAC4 (anti-p-HDAC4; cat. no. sc-101691) rabbit polyclonal antibodies were purchased from Santa Cruz

Biotechnology (Santa Cruz, CA, USA). Fast Blue BB salt (cat. no. F3378) and naphthol AS-MX phosphate (cat. no. N4875) were purchased from Sigma-Aldrich.

Generation of cell lines, cell culture, and osteogenic differentiation

The generation of C3H10T1/2 cells stably expressing flag-tagged Runx2 and their control cells have been reported elsewhere (Deepak *et al.*, 2011). C3H10T1/2 cells stably expressing flag-tagged-Osterix or co-expressing flag-tagged Runx2 and HDAC4 were generated using the same procedure as described earlier (Deepak *et al.*, 2011). The plasmid directing the expression of the GFP-HDAC4-3SA mutant protein was obtained from Addgene (Cambridge, MA, USA; cat. no. 45637) by courtesy of Dr. Tso-Pang Yao of Duke University, Durham, NC, USA (Cohen *et al.*, 2007). The GFP-HDAC4-3SA plasmid (4 μ g) was transfected into Runx2 overexpressing C3H10T1/2 cells using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). As a control, the corresponding amount of the pcDNA 3.1(+) plasmid was transfected into the Runx2 overexpressing C3H10T1/2 cells. All cell lines used in this study were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), 100 IU ml⁻¹ penicillin, and 100 μ g ml⁻¹ streptomycin, and incubated at 37 °C in a humidified atmosphere with 5% CO₂. For osteogenic differentiation, 10 mM β -glycerophosphate and 50 μ g ml⁻¹ ascorbic acid were added to the growth medium which was changed every alternate day.

Western blot and immunofluorescence

Cells were homogenized in RIPA lysis buffer (BIOSS, Beijing, China) supplemented with a protease inhibitor cocktail, followed by Western blotting as described previously (Shi *et al.*, 2013). Blots were developed using the ECL Plus Western blotting detection system (GE Healthcare, Piscataway, NJ, USA). Immunofluorescence microscopy was performed as described earlier (Zhang *et al.*, 2012).

Alkaline phosphatase activity staining and von Kossa staining

For alkaline phosphatase (ALP) staining, cells were fixed in 10% formaldehyde followed by incubation in

Tris buffer (0.2 M, pH 8.3) with AS-MX phosphate (Sigma-Aldrich) as substrate and Fast Blue BB salt (Sigma-Aldrich) as a stain. ALP-positive cells were stained blue/purple. Von Kossa staining for mineralization was performed as described earlier (Yoshida *et al.*, 2012).

Results and Discussion

Runx2 is more potent than Osterix in inducing osteoblast differentiation

Osteoblasts are specialized cells of skeletal lineage originating from mesenchymal progenitors that secrete and deposit the bone matrix (Raggatt and Partridge, 2010). Runx2 and Osterix are both essential for osteoblast differentiation of various cell lineages including mesenchymal stem cells *in vitro* (Komori, 2011). Runx2 is considered the master regulator of osteoblast differentiation, whereas Osterix has been shown to be indispensable during the late stage of osteoblast differentiation and acts downstream of Runx2 (Komori *et al.*, 1997; Nakashima *et al.*, 2002; Sun *et al.*, 2006). We and others have established that overexpression of Runx2 in mesenchymal stem cells commits the cells

towards osteogenic lineage and induces the expression of osteoblast marker genes *in vitro* (Deepak *et al.*, 2011; Fujita *et al.*, 2004; Nakashima *et al.*, 2002). To find a potent osteogenic factor for studying the role of HDAC4 in osteogenesis, we first compared the potential of Runx2 and Osterix in inducing the osteogenic differentiation of C3H10T1/2 mesenchymal stem cells. Two separate C3H10T1/2 cell lines retrovirally overexpressing Runx2 and Osterix were generated. As can be seen in Fig. 1A, equal protein levels of Runx2 and Osterix were expressed in C3H10T1/2 cells. Intriguingly, we found that Runx2, but not Osterix, strongly induced osteoblast differentiation as evidenced by robust ALP activity and mineralization (Figs. 1B, C). This observation suggests that Runx2 rather than Osterix would be a more efficient and important therapeutic tool in metabolic bone diseases or fracture healing.

HDAC4 expression is maintained from mesenchymal stem cells to osteoblast-related cells

In our previous *in vivo* study we hypothesized that the Runx2 level (protein and presumably activity) must be downregulated to permit osteoblast maturation (Liu

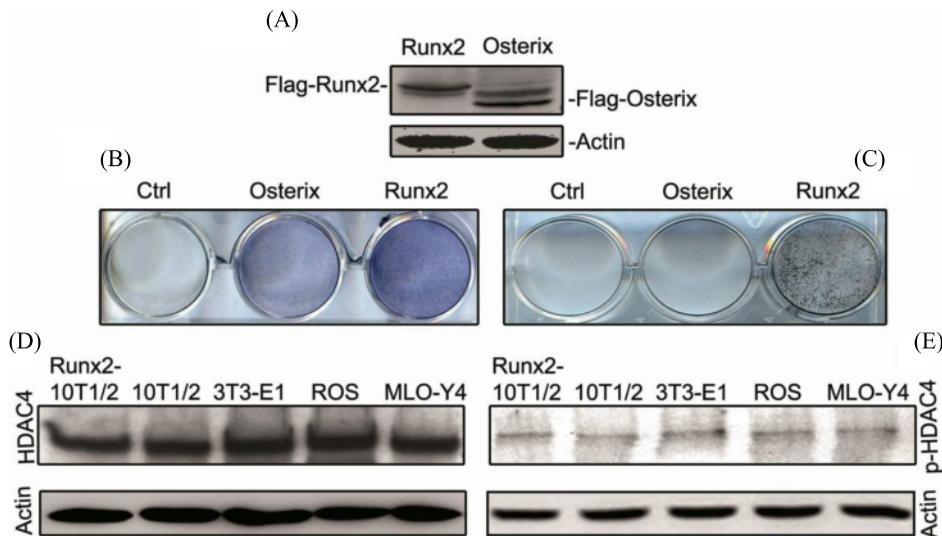


Fig. 1. Induction of *in vitro* osteoblast differentiation and expression of HDAC4 and p-HDAC4 in various cell lines directed by Runx2 and Osterix. (A) Flag-tagged Runx2 and Osterix were overexpressed in C3H10T1/2 mesenchymal stem cells. The two proteins were visualized in a Western blot using anti-flag antibodies. (B, C) Osteoblast differentiation directed by Runx2 and Osterix. C3H10T1/2 mesenchymal stem cells overexpressing Runx2 or Osterix were stained for ALP activity or were allowed to differentiate in osteogenic medium containing 10 mM β -glycerophosphate and 50 μ g ml⁻¹ ascorbic acid and then stained for mineralization through von Kossa staining. (D, E) Western blots of HDAC4 and p-HDAC4 in the following cell lines: C3H10T1/2 (10T1/2), MC3T3-E1 (3T3-E1), ROS17/2.8 (ROS), and MLO-Y4.

et al., 2001). We have now extended our study by searching for negative epigenetic regulators that can affect the Runx2 activity. HDAC4 is a class-IIa histone deacetylase, and its expression and role in myoblast differentiation and chondrocyte hypertrophy have been widely studied. HDAC4 has also been shown to negatively regulate Runx2 (Vega *et al.*, 2004).

We used Western blotting to analyse the levels of HDAC4 in osteoblast-related cell lines: C3H10T1/2, a mesenchymal stem cell line; MC3T3-E1, a committed osteoblast precursor cell line; ROS17/2.8, an osteogenic sarcoma cell line; MLOY4, an osteocytic cell line. Interestingly, we found that HDAC4 expression was maintained at very similar levels in all cell lines (Fig. 1D). This suggests that HDAC4 expression is highly conserved among the osteoblast-related cell lines ranging from mesenchymal stem cells to osteocytes.

In vitro studies reported elsewhere suggest that the functions of class-IIa HDACs are regulated by intracellular trafficking and phosphorylation (Grozinger and Schreiber, 2000; Li *et al.*, 2012; Wang and Yang, 2001). To find out whether the level of phosphorylated HDAC4 (p-HDAC4) is maintained in a way similar to that observed for total HDAC4 protein, we performed a Western blot analysis of the osteoblast-related cell lines. To our surprise, the levels of p-HDAC4, phosphorylated at S632, were similar in all cell lines (Fig. 1E). In addition, we noticed that Runx2 overexpression had no effect on the levels of HDAC4 and p-HDAC4 in either wild-type (WT) or Runx2 overexpressing C3H10T1/2 cells (Figs. 1D, E).

Therefore, HDAC4 seems to be an interesting candidate for studying the Runx2 function, as it is expressed in mesenchymal stem cells as well as in all cell lines of the osteogenic lineage including the one at the terminal stage of differentiation.

Ratjadone C triggers nuclear accumulation of HDAC4 and p-HDAC4

Nucleo-cytoplasmic shuttling of HDAC4 plays an essential role in myoblast differentiation and chondrocyte hypertrophy (McKinsey *et al.*, 2000). It remains cytoplasmic during myoblast differentiation and relocates to the nucleus after myoblast fusion, and the inappropriate presence of HDAC4, as a result of its overexpression, can suppress myogenesis (Miska *et al.*, 2001). McKinsey *et al.* (2000) demonstrated that HDAC4 and HDAC5 are phosphorylated through CaMK signaling, whereby their nu-

clear export is initiated leading to myoblast differentiation. *In vivo*, nuclear localization of HDAC4 in consequence of its overexpression has been reported to inhibit chondrocyte hypertrophy and perturb the DNA binding activity of Runx2 (Vega *et al.*, 2004). These findings suggest that nucleo-cytoplasmic trafficking of HDAC4 is critical for cell differentiation.

Ratjadone C, a natural compound isolated from a strain of the myxobacterium *Sorangium cellulosum*, blocks the export of nuclear proteins through a CRM1-dependent mechanism (Koster *et al.*, 2003). In this study, we established that Runx2 directs osteoblast differentiation more potently than Osterix. Moreover, we also found that HDAC4 and its phosphorylated form are widely expressed in mesenchymal to osteocyte cell lines. Hence, we sought to find out if ratjadone C can cause an aberrant nuclear accumulation of HDAC4. We first elucidated the lowest dose of ratjadone C (1 ng ml^{-1}) that can efficiently inhibit the nuclear export of HDAC4 in C3H10T1/2 mesenchymal stem cells without cytotoxicity (data not shown). Within 1 h of ratjadone C treatment, 100% of the C3H10T1/2 nuclei stained positively for HDAC4. Five h after removal of ratjadone C, the nuclei of 80% of the cells were still positive for HDAC4, while after 11 h most cells displayed HDAC4 presence in the cytoplasm (Fig. 2A).

Conserved serine residues in HDAC4 undergo phosphorylation thereby creating docking sites for 14-3-3 proteins (Grozinger and Schreiber, 2000). The N-terminal domain of HDAC4 contains canonical 14-3-3 binding sites, namely S246, S467, and S632 (Healy *et al.*, 2011). Binding of 14-3-3 to HDAC4 results in dissociation of the deacetylase from protein complexes in the nucleus and exposure of the nuclear export sequence to CRM1, leading to the nuclear export of HDAC4 (Healy *et al.*, 2011). The status of HDAC4 phosphorylation in Runx2 overexpressing C3H10T1/2 cells was examined by an immunofluorescence assay. The p-HDAC4 protein phosphorylated at S632 was found to be localized in the cytoplasm, whereas in ratjadone C-treated cells its localization was exclusively nuclear (Fig. 2B).

These findings indicated that ratjadone C caused the nuclear accumulation of both HDAC4 and p-HDAC4 (S632), the latter actually being expected to be present in the cytoplasm of C3H10T1/2 cells. Once imported into the nucleus, HDAC4 was finely distributed in this organelle and exactly co-localized with Runx2 (Fig. 2C).

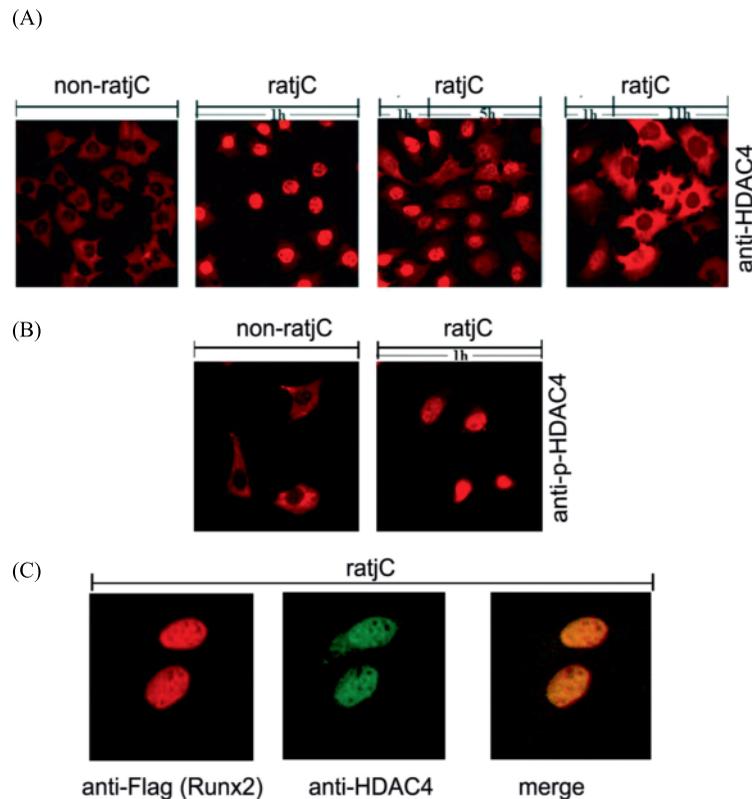


Fig. 2. Localization of HDAC4 and p-HDAC4 in Runx2 overexpressing cells. (A) Effect of ratjadone C (ratjC) on localization of HDAC4. Runx2 overexpressing C3H10T1/2 mesenchymal stem cells were treated with 1 ng ml^{-1} ratjadone C for 1 h and then incubated in the absence of ratjadone C for up to 11 h. Non-ratjC, control treatment. (B) Effect of ratjadone C on localization of p-HDAC4. See legend to (A) for details. (C) Localization of HDAC4 and Runx2 in cells exposed to ratjadone C. See legend to (A) for details.

HDAC4 remains phosphorylated and cytoplasmic during osteogenic differentiation

HDAC4 predominantly localizes to the cytoplasm during myoblast differentiation, as a result of its phosphorylation at conserved serine residues, and relocates to the nucleus once fusion has occurred (Miska *et al.*, 2001). Since we found that HDAC4 was phosphorylated and present in the cytoplasm of undifferentiated C3H10T1/2 stem cells, we wondered if this epigenetic modulator can undergo nucleo-cytoplasmic shuttling during osteogenic differentiation.

Runx2 overexpressing C3H10T1/2 mesenchymal stem cells cultured in differentiation medium were allowed to differentiate for 3 days and then designated differentiating osteoblasts. In these differentiating osteoblasts, both total HDAC4 and p-HDAC4 (S632) were still cytoplasmic, but exposure to ratjadone C for 1 h caused nuclear accumulation (Fig. 3). After re-

moval of ratjadone C, both forms of HDAC4 were still in the nucleus after 5 h, but were found localized to the cytoplasm after 12 h (Fig. 3). These results suggest that functions of HDAC4 in differentiating osteoblasts arising from mesenchymal stem cells might be different from those reported for differentiating myoblasts originating from committed precursors such as C2C12 cells (McKinsey *et al.*, 2000; Miska *et al.*, 2001).

In summary, the above results in the context of osteoblast differentiation of C3H10T1/2 mesenchymal stem cells clearly demonstrated that Runx2 (the master regulator of osteoblast differentiation) had no effect on the HDAC4/p-HDAC4 protein levels. Runx2 or the osteogenic medium-induced osteoblast differentiation did not affect the HDAC4/p-HDAC4 cytoplasmic localization. This strongly suggests that HDAC4 is not directly involved in Runx2-mediated osteoblast differentiation.

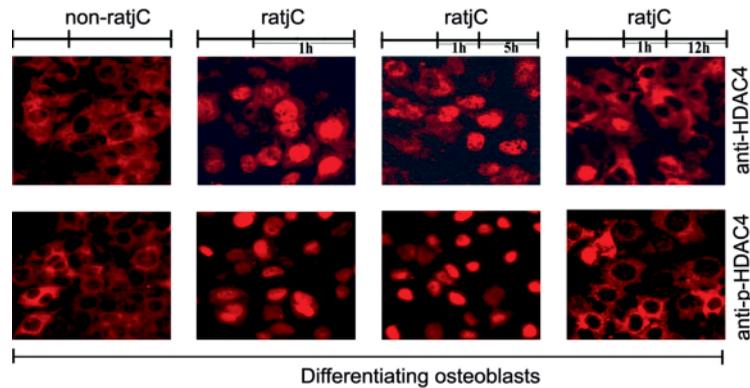


Fig. 3. Phosphorylation status and localization of HDAC4 in differentiating osteoblasts. Runx2 overexpressing C3H10T1/2 mesenchymal stem cells were cultured in osteoblast differentiation medium as described in Materials and Methods. On the third day of differentiation, cells were treated with 1 ng ml^{-1} ratjadone C (ratjC) for 1 h as described in Fig. 2A and probed for HDAC4 and p-HDAC4 localization. Non-ratjC, control treatment.

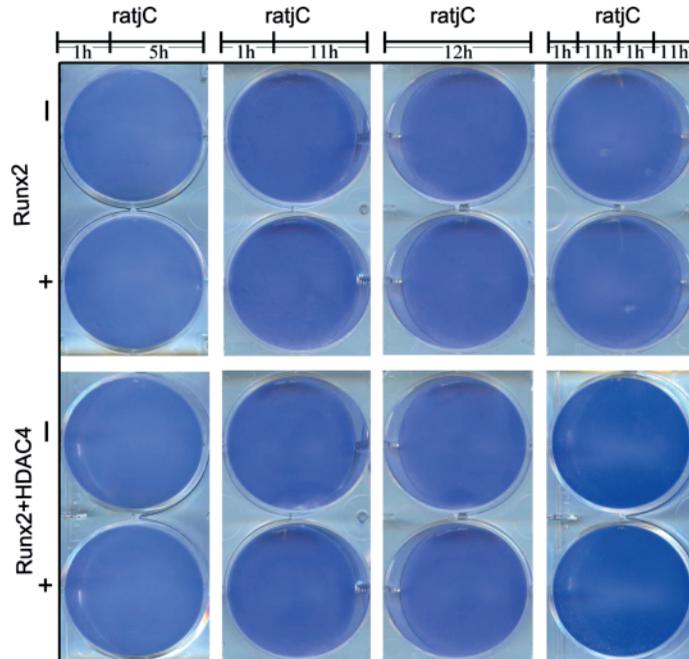


Fig. 4. Effect of ratjadone C (ratjC) on Runx2-induced osteoblast differentiation. C3H10T1/2 mesenchymal stem cells overexpressing Runx2 and co-expressing flag-tagged Runx2 and HDAC4 were exposed to ratjadone C at the indicated time points, and osteoblast differentiation was assessed through ALP staining.

Forced nuclear localization of HDAC4 is insufficient to abrogate Runx2-mediated osteoblast differentiation

Alkaline phosphatase (ALP) is a key marker of differentiating osteoblasts (Robins, 1998). Having established that both the phosphorylated and total HDAC4 proteins remained cytoplasmic in osteogenic and non-osteogenic conditions, and furthermore, that ratjadone

C could efficiently induce the nuclear accumulation of HDAC4, we addressed the question whether ratjadone C has an effect on osteoblast differentiation. C3H10T1/2 cells overexpressing Runx2, or both Runx2 and HDAC4, were exposed to ratjadone C for 1 h and stained for ALP after removal of the inhibitor. Long-term exposure of C3H10T1/2 cells to ratjadone C resulted in inhibition of proliferation, but treatment

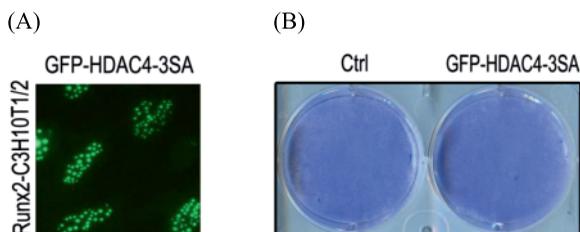


Fig. 5. Effect of the expression of the nuclear resident form of HDAC4 on Runx2-induced osteoblast differentiation. The GFP-HDAC4-3SA plasmid directing the expression of the nuclear resident form of HDAC4 was overexpressed along with Runx2 in C3H10T1/2 mesenchymal stem cells. (A) Nuclear localization of GFP-HDAC4-3SA. (B) Control (Ctrl) and GFP-HDAC4-3SA overexpressing cells were assessed for osteoblast differentiation by ALP staining.

for 1 h followed by 11 h without ratjadone C did not affect cell viability (data not shown). Even though the nuclear import of HDAC4 and its co-localization with Runx2 suggested a functional interaction between HDAC4 and Runx2, we failed to observe any visible effects on Runx2-mediated osteoblast differentiation of C3H10T1/2 cells. As can be seen in Fig. 4, the intensity of the stain reflecting ALP activity induced by Runx2 expression was not altered by the retention of HDAC4 in the nucleus in response to ratjadone C treatment. Further, Runx2 protein levels remained unchanged even after HDAC4 nuclear accumulation (data not shown).

Ratjadone C is a general nuclear export inhibitor and might affect other factors involved in osteoblast differentiation. To resolve this ambiguity, we transfected C3H10T1/2 cells with a plasmid driving the expression of the nuclear resident form of HDAC4 (GFP-HDAC4-3SA) which contains serine to alanine mutations at S246/S467/S632 (binding sites for 14-3-3 proteins). As can be seen in Fig. 5A, the mutated form of HDAC4 was retained specifically in the nucleus of Runx2 overexpressing C3H10T1/2

cells. The 3SA mutant form of HDAC4 did not affect the Runx2-mediated osteoblast differentiation as evidenced by ALP activity staining (Fig. 5B). These results corroborate our finding that ratjadone C-induced nuclear accumulation of HDAC4 does not affect the Runx2-mediated osteoblast differentiation in C3H10T1/2 mesenchymal stem cells. Moreover, this also excludes the possibility that unaffected osteoblastogenesis might be due to the non-selective accumulation of other proteins in the nucleus.

Conclusion

The transcription factor Runx2 has a higher potency than Osterix to induce osteoblast differentiation. The mode of action of HDAC4 in the Runx2-mediated osteogenic differentiation of mesenchymal stem cells is possibly different from its function in chondrocyte or myoblast differentiation. The differential role of HDAC4 in mesenchymal stem cells and in osteoblast differentiation might be due to the possibility that crucial signals essential for the HDAC4 function are not influenced by ratjadone C. Elucidation of the distinct functions of nuclear HDAC4 in the differentiation of mesenchymal stem cells as compared to chondrocyte and myoblast differentiation requires further studies. We propose that the *in vitro* assay of the ratjadone C-mediated nuclear retention of HDAC4 designed in this study can be used for further analysis of the HDAC4 function and identification of novel HDAC4 partners.

Acknowledgement

We thank the following agencies for their support: The National Natural Science Foundation of China (30270660), the Jilin Province Science and Technology Development Project (20040114), the Fok Ying Tung Education Foundation (91025), and the Training Fund of NENU'S Scientific Innovation (NENUSTC08015).

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