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Histone Deacetylase-1 Expression is Upregulated in Tbx3 Overexpressing Cells

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***Abstract*— Tbox3, a T-box containing transcription factor is associated with ulnar mammary syndrome. Recent studies suggest that Tbx3 is related with bone formation and is a negative regulator of osteoblastogenesis. The purpose of this study was to find the targets that may be involved in Tbx3 mediated downregulation of osteogenesis. Reverse transcription PCR, Immunoblotting and ALP staining was used to assess the expression and the affects of the target gene. HDAC1 mRNA levels in C3h10t1/2 cells overexpressing Tbx3 were increased by 2> fold during proliferation whereas differentiating cells showed 3> fold increase. Protein levels of HDAC1 were also remarkably higher during both stages. Cells showed ALP positive staining in the presence of HDAC inhibitor TSA. Taken together these results suggest that HDAC1 can be used as a target for overcoming the repression mediated by Tbx3 on osteogenesis.**

Keywords- C3h10t1/2; HDAC1; Tbx3;osteoblast;TSA

I. INTRODUCTION

Bone formation is a complex developmental process involving the differentiation of mesenchymal stem cells to osteoblasts[1], an imbalance in bone formation can lead to osteoporosis. Osteoporosis is a major communal health threat, an estimated 75 million people in Europe, USA and Japan are affected by the disease [2]. By 2050, the worldwide incidence of hip fracture in men is projected to increase by 310% and 240% in women [3]. A number of transcription factors and mutations have been shown to be related with osteogenesis but there's still a need for finding new molecular targets for the treatment and prevention of bone related diseases.

Tbx3 belongs to the T-box family of genes that arose through gene tandem duplication and cluster dispersion from a single ancestral gene [4]. Mutations in Tbx3 gene leads to Ulnar mammary syndrome associated with hypoplasia of the terminal phalanx of the fifth digit, to the complete absence of forearm and hand [5]. Tbx3 is highly upregulated in bone, in a growth hormone induced condition and negatively regulates osteoblast differentiation [6]. Histone deacetylases (HDAC) function by catalyzing the removal of acetylated lysine residues from histone and non-histone proteins, thereby silencing the gene expression. Various HDACs have been shown to play crucial roles in bone formation [7], interact with master gene Runx2 and repress osteogenic promoters like osterix and osteocalcin [8]. In this study we investigated whether HDAC1 plays a role in Tbx3 mediated negative regulation of osteogenesis, since we have already established that the repression of the osteogenic genes by Tbx3 is not direct and a second factor might be involved [9]. The results attained in this study show that levels of HDAC1 are increased in the presence of Tbx3 and inhibition of HDAC1 may be useful for overcoming Tbx3 mediated repression.

II. MATERIAL AND METHODS

A. Cell Culture

Mouse mesenchymal C3h10t1/2 stem cells were maintained in Dulbecco's Modified Eagle's Medium (Gibco) containing 10% fetal bovine serum (Gibco), 50 units/ml penicillin-G, and 50 µg/ml streptomycin and incubated at 37°C in humidified atmosphere with 5% CO_2 .

B. Plasmid Transfection, Retroviral Production and Differentiation

R2 (cells overexpressing Runx2) and RT cells (cells overexpressing Runx2 along with Tbx3) transfected with pCDNA+TBX3-HA construct and pMX-Runx2-puro plasmids have been described previously [9]. For differentiation 10mM β-glycerophosphate and 50µg/ml ascorbic acid was added to the growth medium and was changed every other day.

C. Trichostatin A (TSA)

TSA, 5mM ready to use solution was purchased from Sigma Aldrich and was mixed at a concentration of 100nM in the culture medium.

D. Western Blot

Western blot was performed as described earlier [10], except for the use of anti-HDAC-1 and anti-Actin antibodies.

E. Semi-Quantitative RT-PCR

Trizol was used to isolate total cellular RNA. One microgram of the RNA was used for cDNA synthesis using Reverse Transcriptase (Promega) and Oligo$(dT)_{20}$ primers,

according to manufacturer instructions. PCR was performed with Premix 2.0 (Takara Biotech). mRNA levels of HDAC1 were quantified and 18s RNA served as internal control. The mRNA analysis was done on day 0 (PM) and day 7 (DM). Quantitative graphs were generated by plotting the results obtained from ImageJ software.

F. ALP Staining

Cells were fixed in 10% formaldehyde for 20 minutes followed by 30 minute incubation in Tris Buffer (0.2 M, pH 8.3) with AS-MX phosphate (Sigma, St. Louis, USA) as a substrate and Fast Blue (Sigma, St. Louis, USA) as a stain. The ALP positive cells stained blue/purple.

G. Statistics

Statistical analyses were performed using t-test. $p<0.05$ was considered to be significant.

III. Results & Discussion

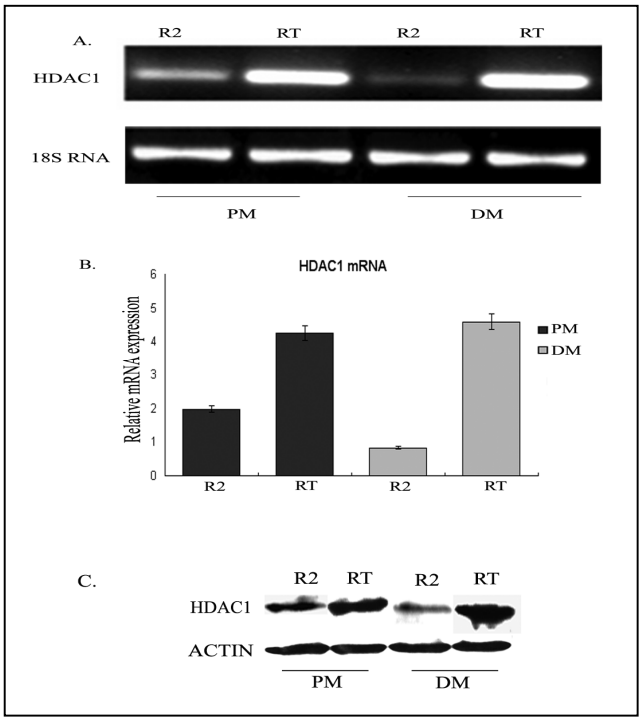

Figure 1. Increased levels of HDAC1 mRNA and protein.

A, HDAC1 mRNA expression levels in C3h10t1/2 cells overexpressing Tbx3 along with Runx2 (RT) and Runx2 alone (R2). B, HDAC1 mRNA bands were quantified using ImageJ analysis software and graphs were generated, P<0.05. C, HDAC1 protein expression levels in R2 and RT cells. (PM- cells grown in proliferation medium till confluency, DM- cells grown in differentiation medium for 7 days).

A. Levels of HDAC1 mRNA and protein are increased in Tbx3 overexpressing cells

Overexpression of Runx2 in C3h10t1/2 cells results in differentiation towards osteoblastic lineage [11]. We generated two C3h10t1/2 cell lines one overexpressing Runx2 alone (R2), used as osteoblast differentiating model and another expressing Runx2 along with Tbx3 (RT), to study the effects mediated by Tbx3 on osteogenesis. RT cells grown in proliferation medium showed 2>fold increase in the HDAC1 mRNA levels as compared to R2 cells, whereas RT cells grown in differentiation medium showed even higher 3>fold increase in the HDAC1 mRNA levels. Protein levels of HDAC1 followed the same pattern and were remarkably higher in RT cells as compared to R2 cells. To our knowledge this is the first evidence that HDAC1 mRNA and protein levels are upregulated in Tbx3 dependent manner.

A number of explanations can be given on higher HDAC1 levels. Tbx3 is a repressor and can interact with HDAC1, both Tbx3 and HDAC1 are involved in cell proliferation and have been reported to negatively regulate osteoblast differentiation, HDAC1 has been shown to repress osterix and osteocalcin promoter [8, 13]. Additional studies can be helpful in knowing the mechanism through which Tbx3 is able to upregulate HDAC1 levels.

B. Inhibition of HDAC1 activity by TSA accelerates osteoblast differentiation

To assess whether the increase in HDAC1 levels is indeed related to inhibition of osteoblast differentiation we used a potent HDAC inhibitor Trichostatin A (TSA) [12]. 100nM of TSA was added in the differentiating medium and cells were grown 4 days for ALP staining. TSA treated Tbx3 overexpressing cells showed ALP positive staining whereas untreated cells remained unstained. This indicates that HDAC1 is indeed a target of Tbx3 and is recruited by Tbx3 to repress osteoblastogenesis.

Taken together this study provides evidence that 1) HDAC1 mRNA and protein levels are increased in Tbx3 dependent manner and 2) Inhibition of HDAC1 results in osteoblast differentiation in Tbx3 overexpressing cells. HDAC1 can be targeted for treating bone related diseases and for overcoming the repression mediated by Tbx3 on osteogenesis.

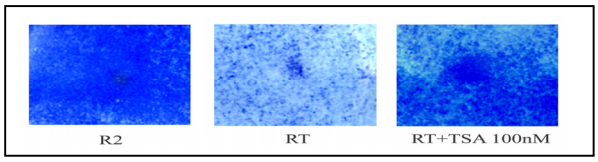

Figure 2. TSA treated Tbx3 overexpressing C3h10t1/2 cells show Alkaline Phosphatase positive staining.

RT and R2 cells were differentiated in osteogenic medium and RT+TSA cells were differentiated by adding 100nM TSA in the differentiating medium. R2 cells (control) show high differentiation and ALP staining while RT cells show poor differentiation. TSA treated RT+TSA cells show increased differentiation.

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