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# RE1-Silencing Transcription Factor (*Rest*) is a Novel Regulator of Osteoblast Differentiation

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## ABSTRACT

RE1-silencing transcription factor (*Rest*) has been identified as a master negative regulator of neuronal differentiation. Nothing is known about *Rest* function in bone cells. In this study, we examined the *Rest* expression levels and role during osteoblast differentiation. We found that *Rest* is abundantly expressed in bone marrow stromal cells, calvarial osteoblasts, and MC3T3-E1 osteoblasts. Treatment of primary osteoblasts with ascorbic acid (AA) down regulated *Rest* mRNA expression at an early stage, but not in later stages of differentiation. Consistent with treatment of primary cultures, AA treatment of MC3T3-E1 cells significantly reduced *Rest* protein expression at day 3 and at day 8 after initiation of osteoblast differentiation. Treatment of bone marrow stromal cells with BMP-2 and dexamethasone, but not IGF-I for 3 days greatly decreased *Rest* mRNA expression. To test the function of *Rest* during osteoblast differentiation, *Rest* expression was knocked down in MC3T3-E1 cell subclones segregated on the basis of ALP activity (differentiation status) using lentivirus expressing shRNA against *Rest*. An 80% knockdown of *Rest* expression decreased *Osterix* (*Osx*) expression by 52–57% and as a result, increased both basal and AA induced ALP expression and activity in the subclone that expresses low basal level of ALP (undifferentiated). By contrast, a 98% knockdown of *Rest* expression in cells that express high basal levels of ALP (differentiated cells) caused a significant reduction in *Osx* expression, basal and AA induced ALP expression and activity. These data suggest that *Rest* regulates early osteoblast differentiation via modulating *Rest* expression that is independent of *Osx* expression. J. Cell. Biochem. 116: 1932–1938, 2015. 

**KEY WORDS:** *REST*; OSTEOBLAST; SHRNA KNOCKDOWN; OSTERIX; ALP

Osteoporosisis a common disease characterized by compromised bone strength that contributes to frequent fragility fractures of the hip, spine, forearm, and other skeletal sites. It is estimated that one in two women and one in five men aged 50 years and older are expected to have an osteoporosis-related bone fracture in their remaining life time [van Staa et al., 2001; Bone Health and Osteoporosis, 2004; Strom et al., 2011]. Bone strength is determined by two major factors, bone density, and bone quality, and is maintained by a dynamic bone remodeling process throughout life time. Bone remodeling comprises two opposite processes: bone formation by osteoblasts and resorption by osteoclasts. Osteoporosis develops when the rate of bone resorption exceeds bone formation and causes a loss of bone mass [Glaser and Kaplan, 1997; Raisz, 2005].

*Osterix* (*Osx*) is one of two master transcription factors that control osteoblast differentiation and bone formation [Nakashima et al., 2002; Hojo et al., 2010; Wuelling and Vortkamp, 2010]. Mice with total disruption of *Osx* showed no bone formation and died at birth [Nakashima et al., 2002]. Mice with osteoblast-specific disruption of *Osx* developed osteopenia during adulthood [Baek et al., 2009]. Conditional knockout of *Osx* in chondrocytes resulted in impaired endochondral bone formation [Oh et al., 2012; Cheng et al., 2013b]. In our previous studies, we found that 7–8 week old mice with a mutation in the *GULO* gene (responsible for ascorbic acid [AA] biosynthesis) exhibited severe spontaneous osteoporotic fractures because of the AA deficiency, reduced *Osx* expression, and impaired osteoblast differentiation [Mohan et al., 2005]. We have also found that AA regulates osteoblast differentiation and *Osx* expression by modulating *Nrf1* nuclear translocation and *Phd2* activity [Xing

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et al., 2007; Xing et al., 2011]. However, mice with a disruption of *Phd2* or *Nrf1* showed only mild bone phenotypes [Kim et al., 2010; Cheng et al., 2014]. Because the skeletal phenotypes in either *Osx* knockout mice or *GULO* mutant mice are much more severe than *Phd2* or *Nrf1* knockout mice, it is likely that other factors besides *Nrf1* and *Phd2* play critical roles in regulating *Osx* or other regulators of osteoblast differentiation and bone formation.

*Rest* (RE1-silencing transcription factor), also known as the neuron Restrictive silencing factor or NRSF, is a Kruppel type zinc finger transcription factor with eight zinc fingers and two repressor domains located at the N- and C-terminals, respectively. It functions as a master transcription repressor that maintains transcriptional silence of a range of neuronal genes in undifferentiated neuronal and non-neuronal cells [Chen et al., 1998; Coulson, 2005; Lunyak and Rosenfeld, 2005]. *Rest* is down regulated to activate *Rest* target genes that are critical for the differentiation of neural stem cells [Su et al., 2004; Lunyak and Rosenfeld, 2005]. *Rest* usually binds to the 21 bp repressor element 1 (RE1) in the promoters of target genes, and then assembles inhibitory transcriptional regulators by recruiting corepressors such as CoRest and mSin3A through the two repressor domains. The repressor complex then modifies histones and other chromatin remodeling proteins to block transcription enhancer's access to their DNA binding sites [Coulson, 2005; Lunyak and Rosenfeld, 2005]. Thus, *Rest* plays a prominent role in transcriptional repression. In addition to neurons, *Rest* also acts as a transcriptional repressor in pancreas and heart tissues [Kemp et al., 2003; Kuwahara et al., 2003]. However, the functions of *Rest* in bone cells are unknown. In the present study, we examined *Rest* expression and its function during osteoblast differentiation. We found that *Rest* regulates early osteoblast differentiation via modulating *Rest* expression that is independent of *Osx* expression.

## MATERIALS AND METHODS

### ANTIBODIES AND BIOLOGICAL REAGENTS

Antibodies against REST (07-579, EMD Millipore, Billerica, MA) were used at a dilution of 1:1,000. Antibodies against β-actin (A5441, Sigma–Aldrich, St. Louis, MO) were used at a dilution of 1:10,000. Recombinant BMP-2 was purchased from R&D Systems (Minneapolis, MN). Recombinant IGF-1 was extracted from yeast as reported previously [Qin et al., 1998]. MISSION® shRNA Lentiviral Transduction Particles against *Rest* (CCGGGTGTAATCTACAATAC-CATTTCTCGAGAAATGGTATTGTAGATTACACTTTTTG) and control non-target shRNA were purchased from Sigma–Aldrich.

### CELL CULTURES

Primary calvarial osteoblasts (COBs) were isolated by a series of digestions according to a previously published procedure [Cheng et al., 2014]. Briefly, calvaria were dissected from 3–5 day old wild type animals and digested with 2 mg/ml collagenase A (Sigma–Aldrich) and 1 mg/ml hyaluronidase (Sigma–Aldrich) for 20 min. The first round of digestion was discarded and the second and third rounds of digestion (30 min each) was harvested and plated in α-minimal essential medium (α-MEM) containing 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). Bone marrow stromal (BMS) cells were derived from femurs and tibias of 4-week-old wild type mice as described before [Cheng et al., 2014]. Primary osteoblasts and MC3T3-E1 cells were plated at $1.5 \times 10^4/cm^2$ ($1.5 \times 10^5$/well) in 35 mm 6-well culture plates or 100-mm dishes in α-MEM medium containing 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). The cells were cultured up to 80–90% confluence prior to treatment to induce differentiation.

### OSTEOBLAST DIFFERENTIATION

Primary osteoblasts or MC3T3-E1 cells were grown to 80% confluence and changed to serum free α-MEM medium containing 0.1% BSA, penicillin (100 units/ml), and streptomycin (100 μg/ml) for 24 h. The cells were then treated with control medium containing 10 mM β-glycerophosphate (BG) alone or differentiation medium containing 10 mM BG and 50 μg/ml AA. Cell differentiation was also induced by treating osteoblast cells with 30 ng/ml BMP-2, $10^{-8}$ dexamethasone (DEX), or 30 ng/ml IGF-1 for 24-72 h.

### QUANTITATIVE REAL TIME RT-PCR

RNA samples were extracted from primary osteoblasts or MC3T3-E1 cells with Trizol reagent (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. An aliquot of RNA (300 ng) was reverse-transcribed using SuperScript® II reverse transcriptase (Invitrogen, Grand Island, NY). Quantitative real time RT-PCR was performed as previously described [Cheng et al., 2012]. The ΔΔCT method was used to calculate relative gene expression with the housekeeping gene PPIA serving as an internal control as previously described [Cheng et al., 2012]. The primer sequences used for real time RT-PCR are listed in Table 1.

### WESTERN BLOTTING

MC3T3-E1 Cells were lysed with a lysis buffer containing 50 mM HEPES [pH 7.5], 100 mM NaCl, 10 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1x protease inhibitor cocktail (Sigma). Thirty microgram of total protein from each sample was loaded

TABLE 1. Primer Sequences Used for Real Time RT-PCR

| Gene | Primer | Sequences |
|---|---|---|
| Rest | Forward | 5′-GCGAACTCACACAGGAGAACG-3′ |
| | Reverse | 5′-GGTCACTTCATGCTGATTAGAGG-3′ |
| Osx | Forward | 5′-AGAGGTTCACTCGCTCTGACGA-3′ |
| | Reverse | 5′-TTGCTCAAGTGGTCGCTTCTG-3′ |
| ALP | Forward | 5′-ATGGTAACGGGCCTGGCTACA-3′ |
| | Reverse | 5′-AGTTCTGCTCATGGACGCCGT-3′ |
| PPIA | Forward | 5′-CCATGGCAAATGCTGGACCA-3′ |
| | Reverse | 5′-TCCTGGACCCAAAACGCTCC-3′ |

and separated by SDS-PAGE under reducing conditions. The blots were incubated with anti-*Rest* antibody overnight at 4°C or anti-β-actin antibody for 1 h at room temperature to detect protein expression as previously described [Cheng et al., 2013a]. β-actin was used as a loading control. Image J [Schneider et al., 2012] was used to quantify the intensity of the bands detected by *Rest* antibody. Data were normalized to β-actin expression.

### VIRAL TRANSDUCTION

MC3T3-E1 cells were grown to 25–30% confluence and transduced with scramble control or *Rest* shRNA lentivirus at multiplicity of infection (MOI) of five by adding a pre-made viral particle stock ($1.5 \times 10^7$) into the 6-well culture plates in the presence of 8 μg/ml of polybrene for 2 days, followed by puromycin selection (5 μg/ml) for 3–5 days as previously described [Xing et al., 2011]. The puromycin selected cells are propagated and used for gene expression experiments and ALP activity assays.

### ALP ACTIVITY MEASUREMENT

Transduced MC3T3-E1 cells were treated with serum-free α-MEM containing 10 mM BG and 50 μg/ml AA, and incubated for 72 h before measuring the ALP activity as previously reported [Linares et al., 2011].

### STATISTICS

For pairwise comparisons, Student's *t* test was used for statistical analysis. For multiple comparisons, one way ANOVA was used for statistical analysis using the STATISTICA software (Statsoft, Tulsa, USA).

## RESULTS

### REST EXPRESSION IS DECREASED DURING EARLY OSTEOBLAST DIFFERENTIATION

In our previous studies, we found that AA is a critical regulator of osteoblast differentiation and bone formation [Mohan et al., 2005]. To investigate if *Rest* expression is altered during osteoblast differentiation, we treated primary COB cells with AA for 3, 6, 13, 19, and 24 days. Control cells were treated with growth medium without AA. We found that *Rest* mRNA expression was decreased at day 3 and 6 after AA treatment by 68 and 69%, respectively, as compared to AA untreated control cells ($P < 0.05$, Fig. 1A). However, *Rest* expression was comparable to the expression levels in control cells at day 13, 19, and 24 (Fig. 1A). We also examined *Rest* expression during MC3T3-E1 cell differentiation. Consistent with the primary calvarial osteoblasts, 3 days of AA treatment resulted in a 40% decrease in *Rest* protein expression as compared to the vehicle treated cells ($P < 0.05$, Fig. 1B and C), and 8 days after AA treatment resulted in a 58% reduction of *Rest* protein expression ($P < 0.05$, Fig. 1B and C).

To further examine if the observed down regulation of *Rest* expression during AA induced early osteoblast differentiation was also true for other osteogenic regulators, we treated BMS cells with BMP-2, DEX, and IGF-1 for 3 days, and measured *Rest* mRNA expression. In agreement with the AA-induced differentiation treatment, treatment of BMP-2 and Dex also inhibited *Rest* mRNA expression by 38% and 41%, respectively ($P < 0.05$, Fig. 2). However,

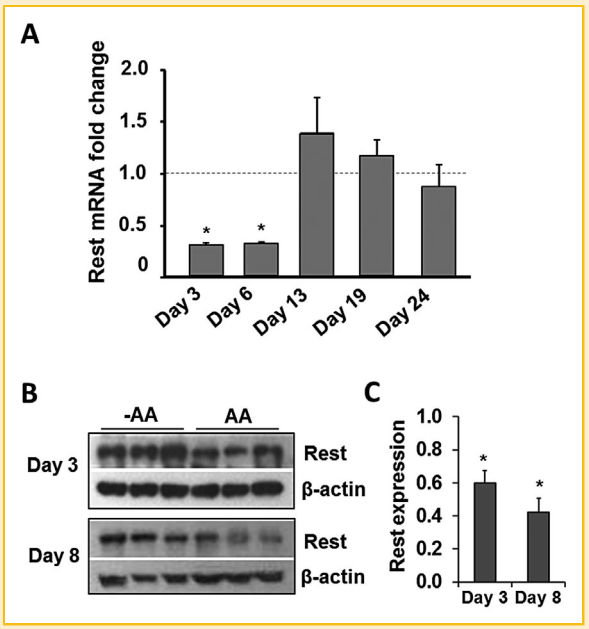


**Figure 1.** *Rest* is down regulated during early osteoblast differentiation induced by AA. (A) *Rest* mRNA expression in primary COB cells. Primary COB cells derived from 2 to 4 days old mouse calvaria were treated with a differentiation medium containing 10 mM β-glycerophosphate and 50 ug/ml AA for 3, 6, 13, 19, and 24 days and mRNA samples were extracted for real time RT-PCR. Data were normalized to cells without AA treatment. (B) *Rest* protein expression in MC3T3-E1 cells treated with or without AA. Cells were collected after 3–8 days of treatment and analyzed by western blotting using anti-REST antibody. β-actin was used as a loading control. (C) Quantification of *Rest* protein levels in Fig. B. Data was normalized to AA untreated cells. AA, ascorbic acid. n = 3/group. *, $P < 0.05$ vs control. Data are presented as mean ± SEM.

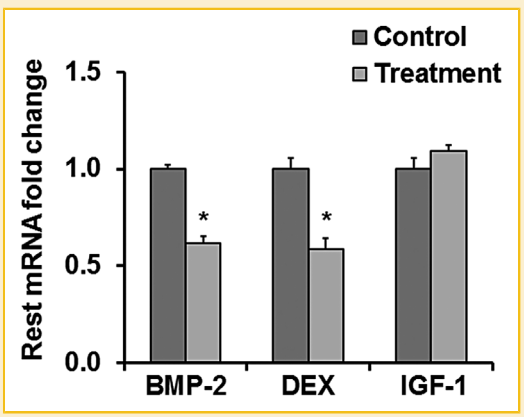


**Figure 2.** Effects of osteogenic regulator BMP-2, Dex, and IGF-1 on *Rest* expression during early osteoblast differentiation. BMS cells were treated with control medium or medium containing BMP-2, DEX, or IGF-1 for 72 h. BMP-2 and Dex treatments reduced *Rest* mRNA expression by 38% and 41% respectively, while IGF-1 treatment did not alter *Rest* expression. Data were normalized to control. n = 3/group. *, $P < 0.05$ vs control. Data are presented as mean ± SEM.

there was no significant change in *Rest* expression between the vehicle treated cells and IGF-1 treated osteoblasts (Fig. 2).

### REST REGULATION OF OSTEOBLAST DIFFERENTIATION IS STAGE-DEPENDENT

To determine the function of *Rest* in early osteoblast differentiation, we transduced MC3T3-E1 cells with lentiviral particles expressing shRNA directed at *Rest*. The cells infected with Lentivirus expressing scramble shRNA were used as a control. We first knocked down *Rest* expression in a subcone of MC3T3-E1 cells that have low basal ALP activity. We found that endogenous *Rest* expression was efficiently knocked down because there was an 88% ($P < 0.05$) reduction in *Rest* mRNA expression in *Rest* shRNA transduced cells as compared to scramble shRNA transduced cells (Fig. 3A). In agreement with the observation in Fig. 1, 24 h of AA treatment reduced *Rest* expression by 47% in scramble shRNA expressing cells ($P < 0.05$). Next we examined the expression of transcription factor *Osx*. AA treatment increased *Osx* expression by 80% ($P < 0.05$) in the scramble shRNA expressing cells (Fig. 3B). In AA untreated cells, *Rest* shRNA transduction reduced *Osx* expression by 57% ($P < 0.05$) compared to scramble shRNA transduction. Interestingly, AA treatment in the *Rest* knockdown cells also increased *Osx* expression by 65% ($P < 0.05$) compared to AA untreated cells, suggesting AA induction of *Osx* expression is independent of *Rest* expression (Fig. 3B). We next examined the expression of ALP mRNA in these cells. Ascorbic acid treatment with the scramble shRNA transduced cells increased the ALP mRNA level by 60% ($P < 0.05$) compared to AA untreated cells, suggesting a stimulation of osteoblast differentiation (Fig. 3C). ALP mRNA level was increased by 110% ($P < 0.05$) after *Rest* shRNA knockdown in AA untreated cells, and 330% ($P < 0.05$) in AA treated cells (Fig. 3C). The AA stimulation of ALP expression was not effected by *Rest* knockdown. We also examined ALP activity in the control and *Rest* knockdown cells. ALP activity was increased by 51% after AA treatment in the scramble shRNA transduced cells ($P < 0.05$), and further increased by 110% ($P < 0.05$) after *Rest* shRNA knockdown (Fig. 3D).

To determine if the effect of *Rest* on osteoblast differentiation was stage-dependent, we knocked down *Rest* expression in a MC3T3-E1

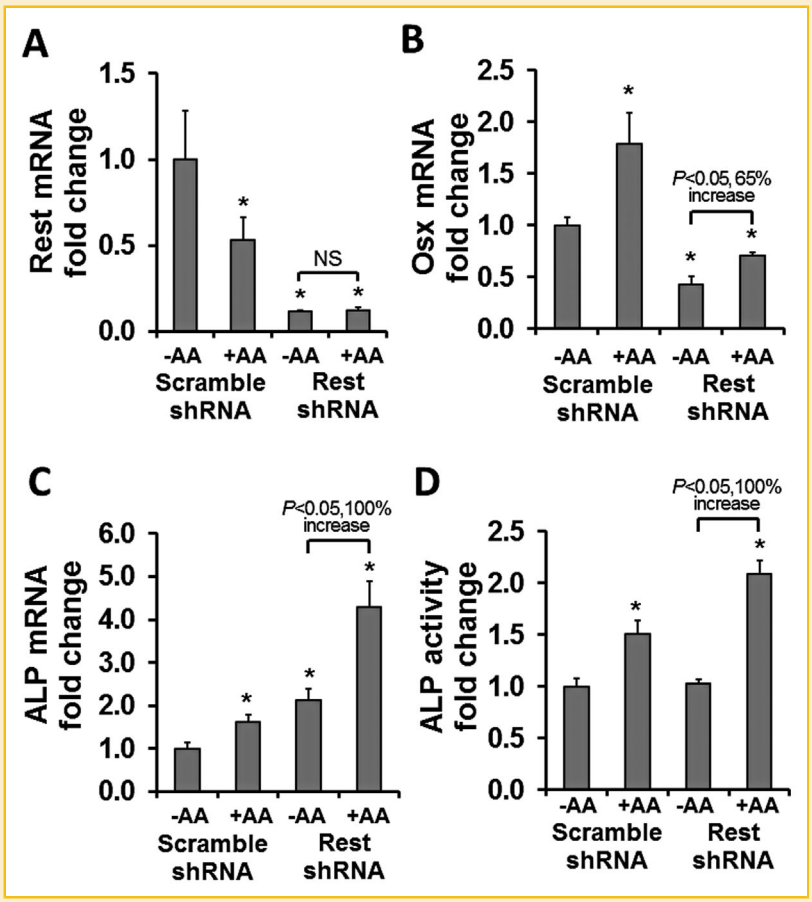


**Figure 3.** Effect of *Rest* shRNA knockdown on osteoblast differentiation in MC3TC-E1 cells expressing low basal ALP activity. A MC3T3-E1 subclone expressing low basal ALP activity was transduced with scramble control or *Rest* shRNA, and treated with or without AA for 24 h followed by quantitative real time PCR. For ALP activity, cells were treated for 72 h. All data were normalized to scramble shRNA transduced cells without AA treatment. (A) In scramble shRNA treated cells, AA treatment decreased *Rest* expression by 47%. *Rest* shRNA transduction reduced *Rest* expression by 88%. (B) *Osx* expression was increased by 80% after AA treatment in scramble shRNA tranduced cells. *Rest* shRNA knockdown reduced the *Osx* mRNA level by 57% in AA untreated cells, and 30% in AA treated cells. (C) AA treatment increased the ALP mRNA level in scramble shRNA transduced cells. *Rest* shRNA knockdwon further increased the ALP mRNA level. (D) ALP activity showed a similar pattern of regulation as ALP mRNA in Figure C. n = 3/group. *, $P < 0.05$ vs scramble shRNA cells without AA treatment. Data are presented as mean ± SEM.

subclone that has a high basal level of ALP activity, representing osteoblasts at an advanced differentiation stage. *Rest* shRNA transduction knocked down 98% ($P < 0.05$) of *Rest* expression (Fig. 4A). Consistent with previous observations, *Rest* expression was decreased by 40% ($P < 0.05$) after AA treatment in the scramble shRNA transduced cells (Fig. 4A). The effect of *Rest* knockdown on *Osx* expression was very similar between the two subclones of MC3T3-E1 osteoblasts. Ascorbic acid treatment increased *Osx* expression by 100% ($P < 0.05$) in scramble transduced cells. *Rest* shRNA knockdown reduced *Osx* expression by 55% ($P < 0.05$) in AA untreated cells and 22% ($P < 0.05$) in AA treated cells (Fig. 4B). In general, *Rest* knockdown reduced *Osx* expression, but did not affect the AA stimulation of *Osx* expression. However, *Rest* knockdown showed an opposite effect on ALP expression in these two subclones of MC3T3-E1 osteoblasts. In the subclone with high ALP activity, *Rest* knockdown reduced ALP mRNA expression by 78% ($P < 0.05$) in the AA untreated cells and 58% in AA treated cells (Fig. 4C). The effect of *Rest* knockdown on ALP activity was similar to ALP mRNA expression in Fig. 4C. ALP activity was increased by 91% after AA treatment in the scramble shRNA transduced cells ($P < 0.05$), but decreased by 27% ($P < 0.05$) after *Rest* shRNA knockdown (Fig. 4D).

## DISCUSSION

*Rest* has been extensively studied in neuronal tissues as a master negative regulator [Chen et al., 1998; Kemp et al., 2003; Kuwahara et al., 2003; Coulson, 2005; Aoki et al., 2012]. In stem cells, *Rest* is highly expressed and suppresses the expression of genes responsible for cell differentiation. Although *Rest* is highly expressed in bone, its function in osteoblasts is not understood. In this study, we investigated the expression profile of *Rest* during osteoblast differentiation, as well as the effect of *Rest* knockdown on *Osx* expression, and osteoblast differentiation. We found *Rest* is abundantly expressed in osteoblastic lineage cells including primary COBs, BMS cells, and pre-osteoblast MC3T3-E1 cell lines. Interestingly, *Rest* expression was significantly down-regulated during early osteoblast differentiation in response to treatments of cells with osteogenic factors such as AA, BMP-2, and Dex. We did not find a significant change in *Rest* expression in mature osteoblasts. Knockdown of *Rest* expression significantly decreased *Osx* expression and increased ALP expression and activity in early differentiated cells. Our data suggest that osteogenesis regulators such as AA, BMP-2 and Dex stimulate osteoblast differentiation in

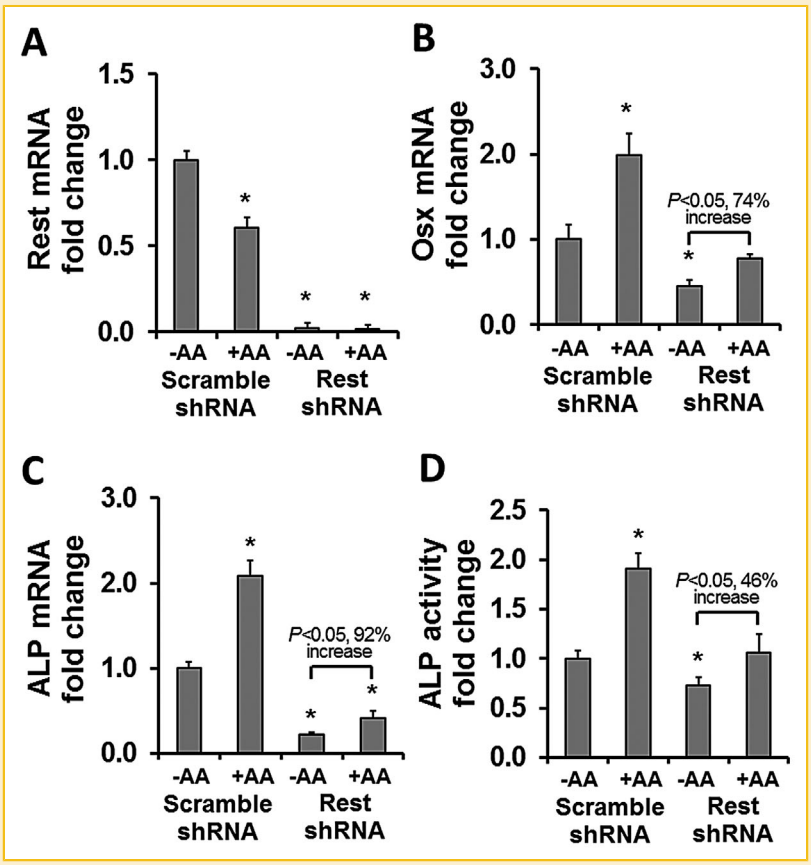


**Figure 4.** Effect of *Rest* shRNA knockdown on osteoblast differentiation in MC3TC-E1 cells expressing a high basal level of ALP activity. MC3T3-E1 subclone cells expressing high basal ALP activity were treated and analyzed as described in Figure 3. (A) In scramble shRNA transduced cells, AA treatment decreased *Rest* expression by 40%. *Rest* shRNA transduction reduced *Rest* expression by 98%. (B) Expression of *Osx* was increased by 100% after AA treatment in scramble shRNA transduced cells. *Rest* shRNA knockdown reduced *Osx* expression by 55% in AA untreated cells, and 22% in AA treated cells. (C) AA treatment increased ALP mRNA level in scramble shRNA transduced cells. *Rest* shRNA knockdwon reduced ALP mRNA expression by 78% in AA untreated cells, and 58% in AA treated cells. (D) ALP activity showed a similar pattern of regulation as ALP mRNA in Fig. C. n = 3/group. *, $P < 0.05$ vs control shRNA cells grown in control medium. Data are presented as mean ± SEM.

part by an *Osx*-independent mechanism through regulating *Rest* expression and relieving transcriptional repression of *Rest*-target genes that are responsible for determining cell fate in the transition from osteoprogenitor to pre-osteoblast, but not by directing osteoblast maturation.

While *Rest* is predicted to act as a repressor of osteoblast differentiation based on what is known regarding the role of *Rest* in neurons and other cell types, the role of *Rest* in regulating osteoblast differentiation appears to be complex. We knocked down the *Rest* expression in two different subclones of MC3T3-E1 cells, one with low basal ALP activity (pre-osteoblast), and one with high basal ALP activity (mature osteoblast). *Osx* expression is down regulated in *Rest* knockdown cells regardless of differentiation status. Thus *Rest* positively regulates *Osx* expression. However, the effect of *Rest* on the differentiation marker, ALP, a marker of mature osteoblasts, was regulated in a differentiation stage-dependent manor. *Rest* down regulated ALP mRNA and activity in the MC3T3-E1 cells at an early differentiation stage, while the reverse was true in the MC3T3-E1 cells at an advanced differentiation stage. Therefore these data strongly support an inhibitory role for *Rest* during early osteoblast differentiation. Such an inhibitory role appears to diminish during later stages of osteoblast differentiation.

Since *Osx* is a key transcription factor regulating maturation of osteoblasts, it is likely *Rest* influences osteoblast differentiation by interacting with signaling pathways other than via *Osx*. Impairment of *Rest* function in *Xenopus* embryos disturbed neural tube formation through BMP signaling [Olguin et al., 2006]. In PC12 cells, high expression of *Rest* correlates with reduced TSC2 levels and accumulation of β-catenin [Tomasoni et al., 2011]. BMP and β-catenin signaling play important roles in proliferation and differentiation of osteoprogenitors, and are potential interactors for *Rest*. Other transcription factors such as Runx2 and Dlx3/5/6, play critical roles in the fate switching from osteoprogenitors to osteoblasts. It is interesting to further investigate the interaction between *Rest* and these signaling pathways and transcription factors.

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