Sumoylation of the Tumor Suppressor Promyelocytic Leukemia Protein Regulates Arsenic Trioxide-Induced Collagen Synthesis in Osteoblasts

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Key Words
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Abstract

\textbf{Background/Aims:} Promyelocytic leukemia (PML) protein is a tumor suppressor that fuses with retinoic acid receptor-α (PML-RARα) to contribute to the initiation of acute promyelocytic leukemia (APL). Arsenic trioxide (ATO) upregulates expression of TGF-β1, promoting collagen synthesis in osteoblasts, and ATO binds directly to PML to induce oligomerization, sumoylation, and ubiquitination. However, how ATO upregulates TGF-β1 expression is uncertain. Thus, we suggested that PML sumoylation is responsible for regulation of TGF-β1 protein expression.

\textbf{Methods:} Kunming mice were treated with ATO, and osteoblasts were counted under scanning electron microscopy. Masson’s staining was used to quantify collagen content. hFOB1.19 cells were transfected with siRNA against UBC9 or RNF4, and then treated with ATO or FBS. TGF-β1, PML expression, and sumoylation were quantified with Western blot, and collagen quantified via immunocytochemistry.

\textbf{Results:} ATO enhanced osteoblast accumulation, collagen synthesis, and PML-NB formation \textit{in vivo}. Knocking down UBC9 in hFOB1.19 cells inhibited ATO- and FBS-induced PML sumoylation, TGF-β1 expression, and collagen synthesis. Conversely, knocking down RNF4 enhanced ATO- and FBS-induced PML sumoylation, TGF-β1 expression, and collagen synthesis.

\textbf{Conclusion:} These data suggest that PML sumoylation is required for ATO-induced collagen synthesis in osteoblasts.
is caused by the chromosomal aberration t(18, 20), which plays a role in initiating acute promyelocytic leukemia (APL) [1] by blocking hematopoietic progenitor cell differentiation [2]. As such, many treatments for APL are targeted at disrupting this fusion protein or its downstream signaling [3, 4]. In normal cells, PML forms discrete structures known as PML nuclear bodies (PML-NBs) together with a variety of other proteins such as p53, SUMO-1, and Daxx [5]. PML-NBs play critical roles in a variety of cellular processes. Furthermore, recent experiments using MEFs from PML−/− knockout mice revealed that PML regulates cell migration, adhesion, proliferation, and morphology [6].

The transforming growth factor (TGF)-β signaling pathway plays a critical role in maintaining cellular homeostasis by regulating processes such as apoptosis, senescence, differentiation, and growth inhibition. Altered TGF-β signaling has been implicated in the development or progression of a variety of cancers, such as colon, breast, pancreatic, prostate, and leukemia, including APL [7]. Interestingly, several studies have reported links between TGF-β and PML. For example, cytoplasmic PML regulates TGF-β signaling, and cells lacking PML are insensitive to TGF-β-induced growth arrest [8].

Arsenic trioxide (As₂O₃; ATO) is an antitumor agent that is used to treat a variety of tumors, including APL, myelodysplastic syndrome, multiple myeloma, as well as solid tumors [9]. It is particularly effective against cells that harbor the PML-RARα fusion protein [10]. Previous studies revealed that ATO could induce the differentiation of an APL cell line that harbors the t(15, 17) chromosomal aberration by inducing the sumoylation of PML-RARα [11, 12]. Sumoylation is a process that conjugates ubiquitin-like polypeptides named SUMO (small ubiquitin-related modifier) onto target proteins, which regulates a variety of physiological process including transcription, DNA repair and replication, the cell cycle, apoptosis, nucleocytoplasmic transport, and ribosome synthesis [13, 14]. Sumoylated PML then recruits the SUMO-dependent ubiquitin ligase, which leads to the ubiquitination and proteasomal degradation of PML-RARα [12].

Bone is a complex tissue that is composed of multiple cell types, including osteoclasts, osteoblasts, and osteocytes, and is also remodeled continuously for renewal and repair [15-17]. During this process osteoblasts generate collagen, which contributes to the formation of new bone. Previous studies revealed that ATO induces DNA tailing and causes oxidative stress in primary osteoblasts, and subsequently modulates the function and differentiation of osteoblasts [18, 19]. Conversely, another study demonstrated that ATO induces endoplasmic reticulum stress and subsequent apoptosis in osteoblasts; therefore, the authors concluded that it ATO might cause conditions such as osteoporosis by altering bone formation. As such, the role of ATO in bone is controversial [20].

We demonstrated recently that ATO upregulated the expression of TGF-β1 protein and promoted collagen synthesis in osteoblasts in vitro [21]. Furthermore, ATO increased TGF-β1 synthesis and secretion in guinea pigs in vivo [22], and upregulated TGF-β1 expression to induce apoptosis in cardiac fibroblasts [23]. However, the mechanism by which ATO upregulates TGF-β1 expression remains unknown. Furthermore, ATO binds directly to PML to induce its oligomerization to enhance the interaction between PML and the SUMO-conjugating enzyme UBC9, which increases the sumoylation and subsequent degradation of PML [24]. Because PML induced the sumoylation and formation of PML-nuclear bodies, we hypothesized that PML sumoylation regulates TGF-β1 protein expression.

Materials and Methods

Reagents and siRNA
ATO was purchased from Harbin YI-DA (Harbin, China). Fetal bovine serum (FBS) was obtained from Gibco (Beijing, China). siRNAs against UBC9 and RNF4 were purchased from GenePharma (Suzhou, China), and sequences are presented in Table 1. All culture media were obtained from Hyclone (Logan, UT).
Cell culture
The human osteoblast hFOB1.19 cell line was purchased from the Chinese Academy of Sciences (Shanghai, China), and cells were cultured in complete culture medium containing 0.3 mg/ml G418 in F12 medium with 10% FBS at 34°C under 5% CO₂. Any non-adherent cells were removed with media changes and adherent cells were retained in the culture dish for further growth. Media were changed every 3 days, as described previously [21].

Western blotting
hFOB1.19 cells were lysed in SDS buffer containing protease inhibitors (Huatesheng Biotechnology, Fushun, China) and isolated proteins were separated by SDS-PAGE and electro-transferred to nitrocellulose membranes which were incubated overnight at 4°C with the following primary antibody dilutions: PML (Santa Cruz, CA), SUMO1 (Santa Cruz), SUMO2/3 (Invitrogen, Carlsbad, CA), TGF-β1 (Abcam, Shanghai, China), UBC9 (Cell Signaling Technologies, Danvers, MA), RNF4 (Cell Signaling), β-actin (Research Diagnostics Inc., Flanders, NJ), and Gap (Research Diagnostics). After washing, membranes were incubated with the appropriate secondary antibodies (goat anti-rabbit and -mouse IgG; Abcam). Protein expression was quantified using Odyssey V3.0 software (LI-COR, Lincoln, NE).

Immunofluorescent staining
hFOB1.19 cells were cultured in six-well plates on glass cover slips and transfected with expression plasmids (GenePharma, Suzhou, China) using Lipofectamine 2000 (Invitrogen, Beijing, China) according to the manufacturer’s instructions. Six hours after transfection the culture media were switched to media containing 10% FBS but lacking penicillin-streptomycin, and cells were cultured for an additional 12 h. Cells were fixed 15 min after transfection using 4% paraformaldehyde in PBS for 20 min, and permeabilized using 0.5% Triton X-100 in PBS for 10 min. They were then blocked using 4% BSA in PBS for 20 min prior to incubation with mouse anti-PML antibodies (Santa Cruz) and Alexa 488-conjugated secondary antibodies (Invitrogen). Stained cells were mounted onto glass slides using Shandon Immu-Mount (Thermo Fisher Scientific, location) containing DAPI to visualize cell nuclei. Confocal laser microscopy was performed using an Olympus microscope (Tokyo, Japan) and the presence of PML-NBs was determined using Fluoview software v.5.0 (Olympus).

Collagen measurement
hFOB1.19 cells were cultured in six-well plates and transfected as described above. Total collagen content was determined by using a Sircol™ Collagen Assay kit (Biocolor Ltd., Northern Ireland) as described previously [21, 22]. Briefly, 0.05 M Tris buffer (pH 7.5) was used to lyse cells, and 1 mL of Sircol Dye reagent was used to stain the lysate (100 mL). Contents were mixed for 30 min and centrifuged for 10 min (at >10,000 × g) to pellet the bound dye. Each tube received 1 mL of the alkali reagent to dissolve the bound dye, and the absorbance value was read at 540 nm. Collagen content (mg) was converted to protein units using the linear calibration curve generated from standards (Vitrogen 100; Angiotech Biomaterials, Palo Alto, CA, USA) and was normalized to the total protein (mg) of each sample.

Animals
Mice were obtained from the Experimental Animal Center of Harbin Medical University (Grade II). Food and water were available freely throughout all experiments. Male Kunming mice weighting 20-25 g were divided randomly into three groups (n = 6/group): controls, experimental animals treated with 1 mg/
kg ATO (IV via tail vein every other day for 1 week), or experimental animals 1 mg/kg ATO (IV via tail vein every other day for 2 weeks). At the end of the treatment period mice were anaesthetized with sodium pentobarbitone (40 mg/kg, i.p.) and xylazine (12.5 mg/kg, i.p.), and anesthetic efficacy was confirmed by the absence of a tail pinch withdrawal.

**Histological analyses and electron microscopy**

Femurs were harvested from all mice at the end of the experiment (at 1 and 2 weeks) and bone cross-sections were fixed in 4% formalin and decalcified by incubation in EDTA-2Na for 2 weeks. Hematoxylin and eosin (H&E) staining was applied to visualize osteoblasts as reported previously [25], and Masson’s staining with transmission electron microscopy (TEM) was used to quantify collagen using routine methods [26]. Finally, scanning electron microscopy (SEM) was used to assess osteoblast differentiation.

**Statistical analysis**

Data are presented as means ± standard deviations (SDs). Differences between groups were evaluated using Student’s t-tests, and P < 0.05 was used to define statistically significant differences. Statistical analyses were performed using GraphPad Prism 5 software (La Jolla, CA).

**Results**

**ATO promotes collagen synthesis and stimulates PML-NB formation in bone tissue**

To assess the effects of ATO on bone histology, collagen synthesis, and PML sumoylation in vivo, Kunming mice femurs were harvested and analyzed using Masson’s staining and EM. Figure 1A shows that control femurs had little collagen accumulation, and that most osteoblasts were pre-osteoblasts (left panels). However, ATO increased collagen accumulation significantly in a time-dependent manner, as indicated by increased staining sections in mice treated for 1 and 2 weeks (Fig. 1A upper panels and 1B; P < 0.05). Furthermore, ATO increased osteoblast formation in a time-dependent manner. After 1-week treatment, occasional bone cells were observed and the endoplasmic reticulum was developed. These effects were greater in mice that received ATO for 2 weeks, in which osteocytes covered most of the surface of the trabecular bone (Fig. 1A; lower panel).

Because studies suggest that PML protein and nuclear bodies (PML-NBs) formation play instrumental roles in tumor suppression [27, 28] and that ATO treatment leads to PML degradation and enhances PML-NBs formation [29, 30], we performed immunohistochemical staining for PML in control and ATO-treated mice (Fig. 1C). Data show that ATO increased PML-NBs formation slightly after both 1 and 2 weeks.

**ATO and FBS induce PML sumoylation and PML-NB formation**

Because previous data revealed that ATO stimulated PML sumoylation and subsequent degradation [29, 31], we measured PML sumoylation in hFOB1.19 human osteoblasts. Data revealed that expression of high molecular weight forms of PML (>110 kDa) increased in parallel with SUMO-1 and SUMO-2/3 as cells were incubated with ATO and FBS over time. Thus, ATO increased PML sumoylation, consistent with previous reports [32, 33].

Data to assess the effects of ATO on PML-NBs formation in hFOB1.19 cells (Fig. 2C), show that both FBS and ATO increased PML-NBs formation compared with control cells; however, ATO had a greater effect.

**Knocking down UBC9 prevents ATO- and FBS-induced PML sumoylation**

Because UBC9 is a SUMO-conjugating enzyme [34], we investigated its role in ATO- and FBS-induced PML sumoylation. Western blot confirmed that UBC9 expression was reduced significantly (~60%) compared with untransfected (ctl) and NC-transfected cells (Fig. 3A; P < 0.05). Transfecting hFOB1.19 cells with si-UBC9 alone had no effect on PML expression or sumoylation (Fig. 3B). As expected, treatment with ATO for 4 h increased PML expression and sumoylation in control and NC-transfected cells (Fig. 3B). However, these
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**Fig. 1.** ATO promotes collagen synthesis and stimulates PML-NBs formation in bone tissue. Kunming mice were treated as described in Materials and Methods. Femur tissue was visualized with Masson’s staining and EM (A and B). Arrows in the upper and lower panels of A indicate collagen and osteoblasts, respectively. Immunohistochemical staining for PML (green) in the bones of control and ATO-treated mice; sections were counterstained with DAPI (blue) to visualize the cell nuclei (C). * $P < 0.05$ vs. ctl.

**Fig. 2.** ATO and FBS induce PML sumoylation and PML-NBs formation. hFOB1.19 human osteoblasts were treated as described in Materials and Methods (0, 1, 3, or 4 h (A) or 10% FBS for 12 h (B)), and analyzed with antibodies against PML, SUMO-1, and SUMO-2/3; GAPDH was used as a loading control. (C) Cells were treated as depicted in Materials and Methods and PML-NBs formation was measured immunofluorescently (green); sections were counterstained with DAPI (blue) to visualize the cell nuclei, $n = 3$. 
Knocking down UBC9 reduced ATO- and FBS-induced TGF-β1 expression and collagen synthesis

ATO is reported to upregulate TGF-β1 expression [21, 22] and UBC9 is thought to regulate TGF-β expression and signaling [35]. So we used transfection studies to assess scrambled control (NC)- or UBC9-siRNA treated with ATO (Fig. 4A) or FBS (Fig. 4B). TGF-β1 was measured and we concluded that transfection with si-UBC9 had no effect on TGF-β1 expression. Also, ATO and FBS both increased TGF-β1 expression significantly compared with control cells (P < 0.05); however, both ATO- and FBS-induced TGF-β1 expression was inhibited be pre-transfection with si-UBC9 (Fig. 4A, B).

To learn whether UBC9 was important for ATO- and FBS-induced collagen synthesis in cultured osteoblasts, hFOB1.19 cells were treated with either FBS or ATO and relative collagen content increased significantly compared with control cells (both P < 0.05). These effects were inhibited significantly by pre-transfection with si-UBC9.
Knocking down RNF4 promotes ATO- and FBS-induced PML sumoylation

RNF4 is an E3 ubiquitin ligase that ubiquitinates sumoylated PML in PML-NBs [12]. We investigated its role in ATO-induced PML sumoylation. After knockdown of RNF4 with specific siRNA (si-RNF4), hFOB1.19 cells were transfected with scrambled control (NC)- or RNF4-siRNA, and Western blot confirmed that RNF4 expression was reduced significantly (~30%) compared with untransfected (ctl) and NC-transfected cells (Fig. 5A; $P < 0.05$).

Also, transfecting hFOB1.19 cells with si-RNF4 alone had no effect on PML expression or sumoylation (Fig. 5B and 3D) and treatment with ATO increased expression and sumoylation of PML compared with control and NC-transfected cells (Fig. 5B). However, pre-transfection with si-RNF4 increased ATO-induced PML expression and sumoylation. Similar effects were observed in FBS-treated hFOB1.19 cells (Fig. 5D). Briefly, FBS treatment increased PML expression and sumoylation, which was enhanced by pre-transfection with si-RNF4.

Studies to understand whether RNF4 is important in ATO- and FBS-induced PML-NBs formation revealed that transfection with si-RNF4 alone had no effect on PML-NBs formation, (Fig. 5C and 3E). However, transfection with si-RNF4 but not NC enhanced both ATO- (Fig. 5C) and FBS-induced (Fig. 5E) PML-NBs formation. Thus, RNF4 inhibits ATO- and FBS-induced PML sumoylation and PML-NBs formation.

Knocking down RNF4 enhances ATO- and FBS-induced TGF-β1 expression and collagen synthesis

Finally, we investigated whether RNF4 plays a role in ATO-induced upregulation of TGF-β1. hFOB1.19 cells transfected with scrambled control (NC)- or RNF4-siRNA and then treated with ATO (Fig. 6A) or FBS (Fig. 6B) were assayed for TGF-β1. As expected, transfection with si-RNF4 alone did not change TGF-β1 expression and ATO and FBS both increased TGF-β1 expression significantly compared with control cells ($P < 0.05$). However, both ATO- and FBS-induced TGF-β1 expression was inhibited by pre-transfection with si-RNF4 (Fig. 6A and 6B).

Finally, (Fig. 6C) we treated hFOB1.19 cells with either FBS or ATO and noted increased relative collagen content of cells compared with control cells (both $P < 0.05$). Interestingly, si-RNF4 transfection enhanced ATO-induced TGF-β synthesis after 4 h, but not 24 h and si-RNF4 increased FBS-induced TGF-β1 synthesis.
Discussion

Our work confirms that ATO enhanced osteoblast accumulation, and increased collagen synthesis and PML-NBs formation in vivo and in vitro. Knocking down SUMO-conjugating enzyme UBC9 with specific siRNA inhibited ATO- and FBS-induced PML sumoylation and TGF-β1 expression. Furthermore, knocking down RNF4, the poly-SUMO-specific E3 ubiquitin ligase required for arsenic-induced PML degradation using siRNA enhanced ATO- and FBS-induced PML sumoylation and TGF-β1 expression. In addition, increased PML-NBs formation for serum response was verified [36]. Therefore, we assessed whether osteoblasts can affect the formation of PML-NBs. In hFOB1.19 cells, serum can increase the formation of PML-NBs after sumoylation.

The PML-RARα fusion protein is found in approximately 98% of patients with APL [37], so many APL treatments are designed to disrupt or inhibit PML-RARα downstream signaling [1]. A major treatment modality is all-trans retinoic acid (ATRA) therapy, which is a natural ligand for RARs. ATRA therapy functions by destroying PML-RARα, which eradicates or induces the terminal differentiation of leukemia-initiating cells (LICs). This induces the complete remission in almost all PML-RARα-positive APL patients [36, 38]. However, for patients the ATRA binding site in PML-RARα becomes mutated, reducing the sensitivity of LICs to ATRA-induced terminal differentiation and causes relapse. Thus, additional treatments are needed [39].
ATO is a highly effective treatment for APL, often used to treat relapsing patients after first-line ATRA therapy [10]. For ATO-treated APL, patients undergoing induction therapy receive daily infusions of ATO (0.15 mg/kg) until achieving bone marrow remission, or for a maximum of 28 days. Pharmacokinetic data indicate that mouse plasma concentration increased after administration of ATO (1 mg/kg, a commonly used dose), peaking at 1.5–3.4 mol/L [21, 22, 40]. Recent studies also suggest complete remission for APL patients with combination treatment with ATO and ATRA, compared with 95% of patients who received ATRA-chemotherapy [40]. Furthermore, 2-year survival rates exceeded those of patients who received ATO-ATRA. Therefore, ATO may have promise but more studies are needed.

A goal of APL front-line treatment is bone marrow remission [41], so the effects of therapies have on bone is important. Bone is a complex tissue comprised of multiple cell types, including osteoclasts, osteoblasts, and osteocytes, and undergoes continuous bone remodeling during renewal and repair [15]. During this process, osteoblasts generate collagen, which contributes to the formation of new bone. Therefore, that ATO can stimulate collagen formation and differentiation in osteoblasts is beneficial, indicating that ATO may offer benefits that ATRA does not [41].

We previously reported that ATO upregulated the expression of TGF-β1 protein and promoted collagen synthesis in osteoblasts in vitro, and hypothesized that PML might regulate ATO-induced activation of the TGF-β pathway [21]. This work confirmed the mechanism of action of ATO in osteoblasts, and increases our understanding of arsenic as a chemotherapeutic agent to treat bone-related diseases, as well as its safety and toxicity.

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**Disclosure Statement**

The authors have declared that no competing interests exist.
References


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