Oncogene (2016), 1–10

www.nature.com/onc

ORIGINAL ARTICLE

The E3 ubiquitin protein ligase MDM2 dictates all-*trans* retinoic acid-induced osteoblastic differentiation of osteosarcoma cells by modulating the degradation of RARα

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Retinoic acid receptor alpha (RARα) has a critical role in the differentiation process of osteosarcoma cells induced by all-*trans* retinoic acid (ATRA). However, degradation of RARα through ubiquitin proteasome pathway weakens the differentiation efficiency of osteosarcoma cells. In this study, we discover that murine double minute-2 (MDM2) acts as an E3 ubiquitin ligase to target RARα for degradation. We observe that MDM2 is required for RARα polyubiquitination and proteasomal degradation because downregulation of MDM2 by short hairpin RNA results in the accumulation of RARα, and MDM2 overexpression promotes the degradation of RARα. We also demonstrate that the N-terminal domain of MDM2 (amino acids 1–109) is the major RARα-binding site. Importantly, endogenous MDM2 levels are not only upregulated in human primary osteosarcoma blasts but are also inversely correlated with the level of osteopontin, which is a marker of bone differentiation. Moreover, MDM2 impairs the ATRA-induced osteoblastic differentiation of osteosarcoma cells, whereas an inhibitor of the MDM2 ubiquitin ligase synergizes with ATRA to enhance the differentiation of osteosarcoma cells and primary osteosarcoma blasts. Therefore, our study indicates that MDM2 serves as an E3 ubiquitin ligase to regulate the degradation of RARα and suggests that MDM2 is a novel therapeutic target for ATRA-based differentiation therapeutic approaches in osteosarcoma.

Oncogene advance online publication, 18 January 2016; doi:10.1038/onc.2015.503

INTRODUCTION

Retinoic acid is a vitamin A derivative that has major effects on biological processes, such as cell differentiation and embryo pattern formation.[1] Numerous experiments have shown that all-*trans* retinoic acid (ATRA) and its derivatives are potent agents that induce the osteoblastic differentiation of mouse and rat mesenchymal stem cells and preosteoblasts.[2,3] Our group and others have found that ATRA induces the osteoblastic differentiation of osteosarcoma cells *in vivo* and *in vitro*.[4–6] All of these studies suggest that ATRA can restore normal osteogenesis, and ATRA-based differentiation therapeutics might be feasible for osteosarcoma. However, no clinical applications for ATRA for patients with osteosarcoma have been reported. Hence, research strategies that seek to further sensitize osteosarcoma cells to retinoids and increase the effectiveness of differentiation therapy for osteosarcoma patients are key avenues of investigation.

The biological effects of ATRA are mediated by two families of nuclear receptors, retinoic acid receptor (RAR) and retinoid X receptor (RXR), which work as RAR/RXR heterodimers and bind to retinoic acid response elements in the promoter regions of retinoid-responsive genes.[7] Recently, our group and others have reported that RARα exert an important role in the ATRA-induced differentiation process of osteosarcoma cells.[4,8,9] However, previous studies have demonstrated that RARs are degraded by the ubiquitin-proteasome pathway (UPP),[9–11] which might limit ATRA-mediated differentiation effects on osteosarcoma. Thus these observations led us to study the mechanism of the degradation of RARα in osteosarcoma cells.

The human homolog of murine double minute-2 (MDM2) is an oncogene overexpressed in different types of malignancies,[12,13] and it has an important role in the development and progression of cancers. MDM2 mainly functions, if not exclusively, as an E3 ligase.[14,15] This protein targets various substrates for mono- and/or poly-ubiquitylation, thereby regulating their activities, for instance, by controlling their localization and/or levels by proteasome-dependent degradation.[16–20] In addition, the MDM2 gene was found to be amplified in 30–40% of human sarcomas, including osteosarcoma.[21,22] Therefore, we were encouraged to investigate whether MDM2 acts as an E3 ligase to regulate the stabilization of RARα and further illustrate the function of MDM2 in the ATRA-induced differentiation process.

Here we report that MDM2 has a critical role in modulating the stability of the RARα protein. Moreover, MDM2 impairs ATRA-induced osteoblastic differentiation in osteosarcoma cells and primary osteosarcoma blasts. Thus our findings reveal a critical and novel mechanism involved in the ATRA-induced differentiation of osteosarcoma where MDM2 impairs osteosarcoma differentiation by modulating the stability of RARα. Additionally, our results identify MDM2 as a potent therapeutic target of differentiation therapy in osteosarcoma.

RESULTS

RARα degradation is accompanied by MDM2 upregulation and cytoplasmic translocation during ATRA-induced differentiation

Constitutive degradation of RARα has been reported to weaken the ATRA differentiation efficiency,[23] therefore, we first validated RARα degradation in ATRA-treated osteosarcoma cells. As expected, ATRA treatment induced rapid degradation of RARα in U2OS cells (Figure 1a). Interestingly, the MDM2 protein expression

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Received 21 December 2014; revised 30 November 2015; accepted 4 December 2015

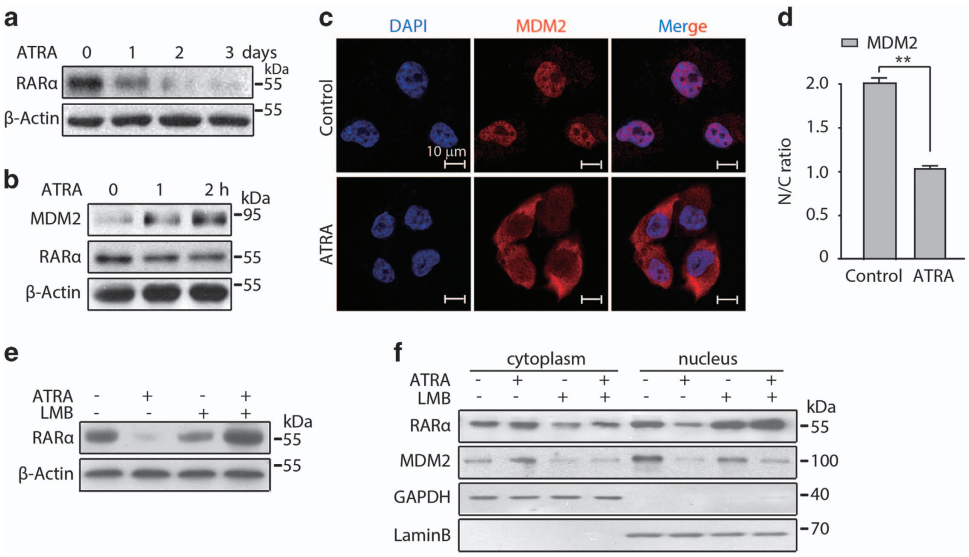

Figure 1. ATRA-triggered RARα degradation is accompanied by MDM2 upregulation and cytoplasmic translocation. (**a**) ATRA triggers degradation of RARα. U2OS cells were treated with 1 μM ATRA for 0–3 days, and RARα expression was determined by western blotting. (**b**) ATRA upregulates MDM2. U2OS cells were treated with 1 μM ATRA for 0–2 h, and the expression levels of MDM2 and RARα were determined by western blotting. (**c**) ATRA promotes the cytoplasmic translocation of MDM2. U2OS cells were treated with ATRA, and the subcellular localization of MDM2 was assessed by immunofluorescence. (**d**) MDM2 nuclear: cytoplasmic (*N/C*) ratios for immunofluorescently stained U2OS cells. The data are presented as the mean ± s.d. of three independent experiments. $^{**}P < 0.01$. (**e**) LMB inhibits ATRA-induced RARα degradation. U2OS cells were pretreated with LMB for 2 h followed by ATRA treatment for 6 h. RARα expression was determined by western blotting. (**f**) LMB inhibits ATRA-induced MDM2 translocation and RARα degradation. U2OS cells were pretreated with LMB for 2 h followed by ATRA treatment for 6 h. Nuclear and cytoplasmic fractions were prepared and analyzed by immunoblotting with the indicated antibodies.

level in U2OS cells was significantly upregulated after ATRA treatment accompanied by downregulation of the RARα protein (Figure 1b). Hence, we were prompted to investigate whether the upregulation of MDM2 was related to the degradation of RARα. It has been reported that MDM2 is an ubiquitin ligase that, when localized to the cytoplasm, ligates ubiquitin to substrates, such as p53 and itself, thus targeting both proteins for proteasomal degradation.[14,24] Therefore, we determined that MDM2 shuttles between the nucleus and cytoplasm in immunofluorescence assays. As illustrated in Figure 1c and Supplementary Figure S1, MDM2 mainly existed in the nucleus in U2OS cells, while ATRA treatment caused MDM2 to shuttle from the nucleus to the cytoplasm. The cytoplasm translocation of MDM2 was also quantified by calculating nuclear-to-cytoplasmic pixel intensity ratios from immunofluorescently stained cells (Figure 1d). Furthermore, when U2OS cells were pretreated with leptomycin B (LMB), a specific nuclear export inhibitor followed by ATRA treatment, the ATRA-induced degradation of RARα was significantly blocked by LMB (Figure 1e), which strongly indicated the involvement of MDM2 cytoplasmic translocation in the degradation of RARα upon ATRA treatment. Nuclear and cytoplasmic fractions were prepared from U2OS cells and the distribution of RARα and MDM2 proteins were further analyzed. The results indicate that RARα and MDM2 proteins are present in both the nucleus and cytoplasm; ATRA treatment caused RARα and MDM2 to shuttle from the nucleus to the cytoplasm, whereas LMB obviously inhibited this translocation (Figure 1f). Altogether, our results suggest that ATRA-induced RARα degradation may be regulated by MDM2.

MDM2 promotes the degradation of RARα

To further validate the MDM2-mediated regulation of RARα degradation, we manipulated the MDM2 levels in U2OS cells and then determined the stability of RARα. As shown in Figure 2a, both ATRA treatment and transfection with a myc-MDM2 plasmid led to significant reduction in the RARα protein level compared with control cells, and MDM2 transfection further enhanced the ATRA-induced reduction of RARα. However, MDM2 C464A, a RING finger mutant with cysteine changed to alanine leading to loss of its E3 ligase activity, lost the ability to downregulate the RARα protein level (Figure 2b), indicating that the E3 ligase activity of MDM2 is involved in the downregulation of RARα. Consistent with these results, knockdown of MDM2 expression by a specific short hairpin RNA (shRNA) remarkably upregulated the RARα protein levels (Figure 2c). Moreover, the depletion of MDM2 by transfection with shRNA plasmids also prolonged the half-life of endogenous RARα protein in U2OS cells (Figure 2d). Thus these results suggest that MDM2 promotes the degradation of RARα, and the E3 ubiquitin ligase activity of MDM2 might be required for the degradation of RARα.

MDM2 directly interacts with endogenous and exogenous RARα

To gain insight into the mechanisms underlying the degradation of RARα mediated by MDM2, we first examined whether MDM2 regulates RARα via direct interaction. Immunofluorescence results demonstrate that endogenous MDM2 partially colocalizes with endogenous RARα in the nucleus in control U2OS cells. Significantly, endogenous MDM2 localized in cytoplasm were co-stained for endogenous RARα after ATRA treatment, suggesting that interaction between these two proteins may exist (Figure 3a). Consistently, exogenous MDM2 also colocalized with exogenous RARα in COS7 cells transfected with myc-MDM2 and Flag-RARα (Supplementary Figure S2). Furthermore, this interaction was further validated by immunoprecipitation (IP). U2OS cell extracts were subjected to IP with anti-RARα or anti-IgG (anti-immunoglobulin G) antibody as control. As shown in Figure 3c, MDM2 was precipitated by anti-RARα but not by the control, indicating that endogenous MDM2 may interact with endogenous RARα. Similarly, ectopically expressed MDM2 and RARα were coprecipitated in

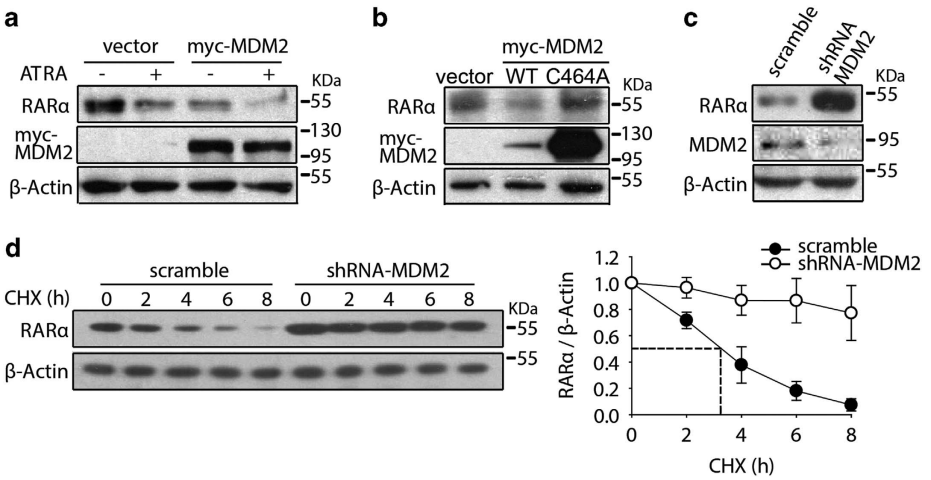

Figure 2. MDM2 reduces RARα protein levels. (**a**) Overexpression of MDM2 decreases the RARα protein levels. U2OS cells were transfected with vector or myc-MDM2 plasmids followed by ATRA treatment for 12 h. RARα and MDM2 expression were determined by western blotting. (**b**) Mutated MDM2 (C464A) loses the ability to decrease RARα. U2OS cells were transfected with vector, MDM2 or MDM2-C464A constructs for 48 h, and then the expression levels of RARα and MDM2 were determined. (**c**) Depletion of MDM2 elevates the protein levels of RARα. U2OS cells were transfected with scramble or shRNA MDM2, and the protein levels of RARα and MDM2 were detected. (**d**) Knock down of MDM2 decreases the degradation of RARα. U2OS-shRNA scramble or U2OS-shRNA MDM2 cells were treated with 10 μg/ml cycloheximide (CHX) for the indicated times, and the protein level of RARα were determined. The relative RARα levels normalized to β-Actin were indicated as mean ± s.d.

COS7 cells overexpressing both proteins, but little or no coprecipitation occurred in cell lysates immunoprecipitated with anti-IgG antibody (Figure 3d). In total, these data support our hypothesis that MDM2 and RARα specifically interact.

To define the MDM2 region required for RARα binding, COS7 cells were transfected with RARα together with four MDM2 deletion constructs fused to a myc-tag. Cell extracts were then immunoprecipitated with an anti-myc antibody, and the results demonstrated that deletion of MDM2-Δ1 (1–109) abolished its interaction with RARα, while wild-type and other MDM2 deletions could interact with RARα (Figure 3e). Consistent with this result, no decrease in RARα protein levels was observed with MDM2-Δ1 overexpression, while wild-type MDM2 significantly downregulated RARα expression (Figure 3f). Previous studies reported that the 1–109-residue amino-terminal domain of MDM2 is required for binding to p53,[25] which overlaps with the region of MDM2 that interacts with RARα. In addition, nutlin-3, a reported MDM2 antagonist, binds to MDM2 in its p53-binding pocket.[26] Thus we investigated whether the interaction between MDM2 and RARα could be disrupted by nutlin-3. As presented in Figure 3g, nutlin-3 could significantly decrease the interaction between MDM2 and RARα. Furthermore, while the MDM2 protein levels were significantly increased by nutlin-3, which was in agreement with other reports,[27] the RARα protein levels decreased by ATRA were reversed by nutlin-3 treatment (Figure 3h). Both of these results indicate that the 1–109-residue amino-terminal domain of MDM2 is critical for RARα binding. In summary, our data clearly demonstrate that RARα may be directly conjugated to MDM2.

MDM2 promotes RARα polyubiquitination *in vivo* and *in vitro*

To confirm the role of MDM2 as an E3 for RARα polyubiquitination, we established RARα *in vivo* and *in vitro* ubiquitination assays. U2OS cells were overexpressed with MDM2 protein. And 24 h later, the cells were treated with ATRA for 12 h, followed by MG132 treatment for an additional 8 h. As demonstrated in Figure 4a, polyubiquitinated RARα accumulated in ATRA plus MG132-treated cells, while MDM2 overexpression further enhanced the accumulation of polyubiquitinated RARα (lane 6 vs lane 10). Consistent with these results, knockdown of MDM2 expression by a specific shRNA significantly reduced RARα ubiquitination (Figure 4b, lane 6 vs lane 10). Therefore, these results demonstrate that MDM2 mediates the polyubiquitination of RARα *in vivo*. To further assess whether the E3 ligase activity of MDM2 is required for RARα polyubiquitination, we overexpressed a mutant MDM2 (C464A) construct in U2OS cells. Our results demonstrated that RARα ubiquitination was significantly decreased in U2OS cells transfected with mutant MDM2 (C464A) when compared with cells transfected with wild-type MDM2 (Figure 4c, lane 6 vs lane 10). Furthermore, *in vitro* ubiquitination assays were also performed to confirm the role of MDM2 as an E3 for the polyubiquitination of RARα. In this assay, polyubiquitination of RARα was observed only in the presence of E1, E2, MDM2 and ubiquitin (Figure 4d). This result corroborates the *in vivo* ubiquitination results and indicates that MDM2 functions as an ubiquitin E3 ligase for RARα.

MDM2 impairs the ATRA-induced osteogenic differentiation of osteosarcoma cells

As the degradation of RARα has been reported to limit ATRA-mediated differentiation,[4,10,11] we next asked whether MDM2 has a critical role in ATRA-induced cell differentiation. We first determined the expression levels of MDM2 in primary osteosarcoma tissues using immunohistochemistry assay. As shown in Figures 5a and b, 19 of the 23 osteosarcoma cases (82.6%) were determined to demonstrate positive expression for MDM2. Moreover, the correlation between the degree of differentiation and MDM2 expression was detected by analyzing the expression level of osteopontin (OPN), a marker of bone differentiation. As illustrated in Figures 5c and d, all four cases with negative MDM2 expression were found positive for OPN expression. Among the remaining 19 cases with moderate-to-intense MDM2 expression, only 5 cases showed positive OPN expression. Four representative cases (OS5–OS8) are displayed in Figure 5c. An inverse correlation between these two proteins is also found to be statistically significant ($R = -0.712$, $P < 0.001$). These results suggest a connection between high MDM2 expression levels and poor differentiation degree in primary osteosarcoma tissues.

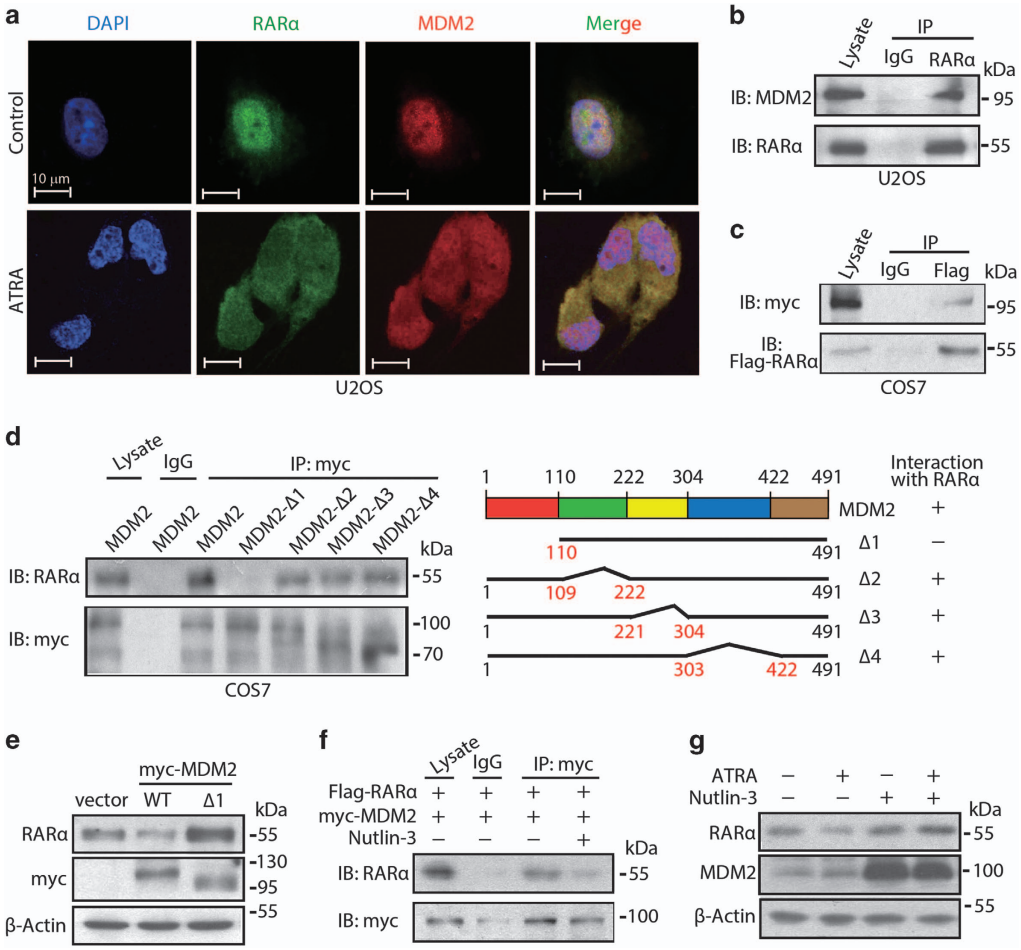

Figure 3. MDM2 forms a complex with RARα *in vivo* and *in vitro*. (**a**) Colocalization of endogenous MDM2 and RARα. U2OS cells were treated with ATRA for 6h and then subjected to immunofluorescence staining with anti-RARα and anti-MDM2 antibodies. (**b**) Interaction between endogenous MDM2 and RARα. U2OS cell lysates were immunoprecipitated with anti-RARα antibody followed by immunoblotting with anti-MDM2 antibody. (**c**) Interaction between ectopic MDM2 and RARα. COS7 cells were transfected with Flag-RARα and myc-MDM2 plasmids for 48 h, and cell lysates were then immunoprecipitated with anti-Flag antibody followed by immunoblotting with an anti-myc antibody. (**d**) The N-terminus (1–109) of MDM2 is required for RARα binding. COS7 cells were co-transfected with RARα and different MDM2 mutants. Cell lysates were immunoprecipitated with anti-myc antibody followed by immunoblotting with anti-RARα antibody. Diagram of the different MDM2 deletion mutant constructs are illustrated. (**e**) Mutated MDM2 (Δ1–109) loses the ability to decrease RARα. U2OS cells were transfected with the MDM2 or MDM2 (Δ1–109) constructs for 48h. Cell lysates were immunoblotted with anti-RARα and anti-myc antibodies. (**f**) Nutlin-3 blocks the interaction between MDM2 and RARα. U2OS cells were transfected with Flag-RARα and myc-MDM2 plasmids followed by nutlin-3 treatment, and cell lysates were then immunoprecipitated with anti-myc antibody followed by immunoblotting with an anti-RARα antibody. (**g**) Nutlin-3 reverses the degradation of RARα induced by ATRA. U2OS cells were treated with 1 μM ATRA in the presence of 10 μM nutlin-3 for 3 days, and cell lysates were then immunoblotted with anti-RARα or anti-MDM2 antibody.

Using overexpressing or silencing MDM2, the proliferation-inhibition and differentiation-induction effects of ATRA were evaluated. As illustrated in Figure 6a, compared with vector controls, MDM2 overexpression inhibited the sensitivity of U2OS cells to ATRA-inhibited proliferation, while MDM2 deletion further slowed the proliferation rate. As osteoblasts have the properties of OPN synthesis, runt-related transcription factor 2 (RUNX2) expression and high alkaline phosphatase (ALP) activity stimulation, western blotting analysis was performed to evaluate the production of OPN, RUNX2 and ALP, and BCIP/NBT staining was further used to detect ALP activity. Our results showed ATRA induced an apparent upregulation of OPN, RUNX2 and ALP expression as we previously reported,[4] whereas there were no active osteoblasts present in MDM2-overexpressing U2OS cells even after ATRA treatment (Figure 6b, left panel). Moreover, BCIP/NBT staining further verified that ALP-positive cells were increased after ATRA treatment, while cells with MDM2 overexpression demonstrated few ALP-positive cells (Figure 6c, left panel). Consistently, the silencing of endogenous MDM2 expression remarkably potentiated the cytodifferentiation activity of ATRA of U2OS cells as indicated by the protein levels of OPN, RUNX2 and ALP and the ALP activity (Figures 6b and c). Consequently, these results reveal that MDM2 impairs the ATRA-induced osteogenic differentiation of osteosarcoma cells.

MDM2 inhibitor synergizes with ATRA to promote the osteogenic differentiation of osteosarcoma cells

Finally, to test whether MDM2 may be used as a differentiation therapeutic target, we used HLI373, an inhibitor of MDM2 ubiquitin E3 ligase activity,[28] to investigate whether MDM2 inhibition could promote the osteogenic differentiation of osteosarcoma cells. First, the effects of HLI373 on the MDM2 E3 ligase activity were examined. U2OS cells were treated with serial concentrations of HLI373, and the RARα protein levels were

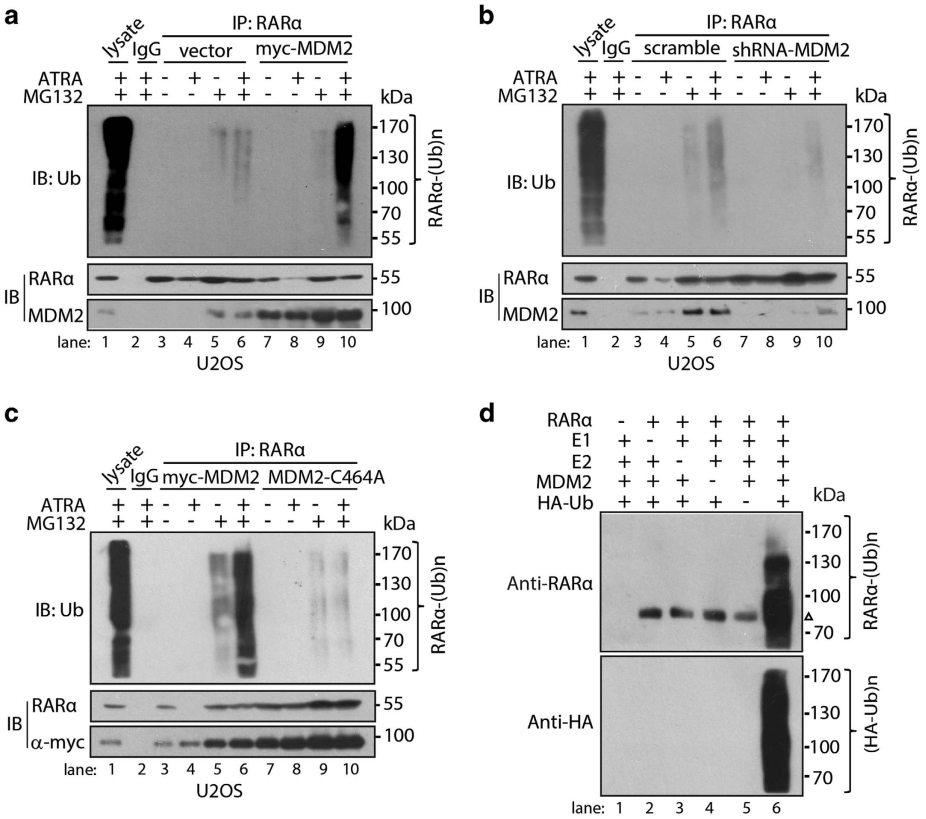

Figure 4. MDM2 is an E3 ligase for RARα and promotes RARα ubiquitylation and degradation. (**a**) Overexpression of MDM2 promotes ubiquitination of RARα. U2OS cells were transfected with empty vector or MDM2 followed by ATRA and MG132 treatment, and cell lysates were then immunoprecipitated with an anti-RARα antibody followed by immunoblotting with an anti-ubiquitin antibody. (**b**) Depletion of MDM2 inhibits the ubiquitination of RARα. U2OS cells were transfected with scramble or shRNA MDM2 followed by ATRA and MG132 treatment, and cell lysates were then immunoprecipitated with anti-RARα antibody followed by immunoblotting with an anti-ubiquitin antibody. (**c**) Mutated MDM2 (C464A) loses the ability to trigger the ubiquitination of RARα. U2OS cells were transfected with vector, MDM2 or MDM2-C464A plasmids followed by ATRA treatment, and cell lysates were then immunoprecipitated with anti-RARα antibody followed by immunoblotting with an anti-ubiquitin antibody. (**d**) MDM2 promotes the ubiquitination of RARα *in vitro*. *In vitro* ubiquitination assays were conducted as described in the Materials and Methods section. Ubiquitinated RARα was detected by IP.

increased accompanied by an accumulation of p53 and MDM2, two other substrates (Figure 7a). Similarly, HLI373 also could hinder the ubiquitination of RARα induced by ATRA treatment (Figure 7b, lane 4 vs lane 6), indicating that the E3 ligase activity of MDM2 was indeed inhibited by HLI373. To further clarify the role of MDM2 in ATRA-induced osteogenic differentiation of osteosarcoma cells, we treated U2OS with ATRA in the absence or presence of HLI373, and differentiation was then determined. As shown in Figure 7c, both in U2OS cells and primary osteosarcoma blasts (MDOS-4), ATRA treatment upregulated the protein levels of OPN and RUNX2, and the combined treatment of HLI373 and ATRA further enhanced the increase in OPN and RUNX2 expression. Moreover, HLI373 treatment also synergized with ATRA to promote ALP activity in U2OS cells when compared with cells treated with ATRA (Figure 7d). Taken together, these results not only support the involvement of MDM2 in ATRA-induced differentiation but also further suggest that targeting MDM2 may be a novel differentiation strategy for osteosarcoma.

DISCUSSION

The induction of terminal differentiation may be a promising alternative to conventional chemotherapy for osteosarcoma because ATRA can induce the osteoblastic differentiation of osteosarcoma cells and restore normal osteogenesis.[4–6,23] However, the degradation of RARα as induced by ATRA tremendously limits ATRA-induced cellular differentiation,[10,11] which reinforces the urgent need for exploring the regulatory mechanisms involved in RARα degradation. In this report, we demonstrate that interaction with MDM2 leads to strong stimulation of RARα polyubiquitination and degradation by proteasomes. MDM2 appears to function as an ubiquitin E3 ligase in this process, as the MDM2 RING domain mutant inhibits the ubiquitination of RARα. Furthermore, MDM2 is capable of stimulating RARα polyubiquitination under cell-free conditions. Moreover, we also provided evidence that silencing or inhibiting MDM2 promotes the differentiation of U2OS cells as induced by ATRA. Therefore, our findings reveal that the RARα expression level is directly regulated by MDM2-mediated ubiquitination. These results also suggest that MDM2 may serve as a potential therapeutic target for differentiation therapy in osteosarcoma.

Although proteasome-mediated RARα degradation has been previously reported[10,11] and several proteins, such as pin1, p38, sumo-1 and E2F1, have been reported to regulate the stability of RARα,[9,29–31] it has yet to be determined how RARα is degraded by proteasome and which E3 ligase targets RARα for degradation. MDM2, which is known as an E3 ubiquitin-protein ligase, is reported to mediate the ubiquitination of multiple proteins, including p53/TP53,[14] RB1,[20] Akt,[16] MDMX[17] and itself,[24] leading to their degradation by the proteasome. In our study, we found that MDM2 directly interacts with RARα and promotes the ubiquitination-proteasome-mediated degradation of RARα, which suggests that MDM2 may function as an ubiquitin E3 ligase for RARα. Thus we are the first to report that this E3 ligase targets

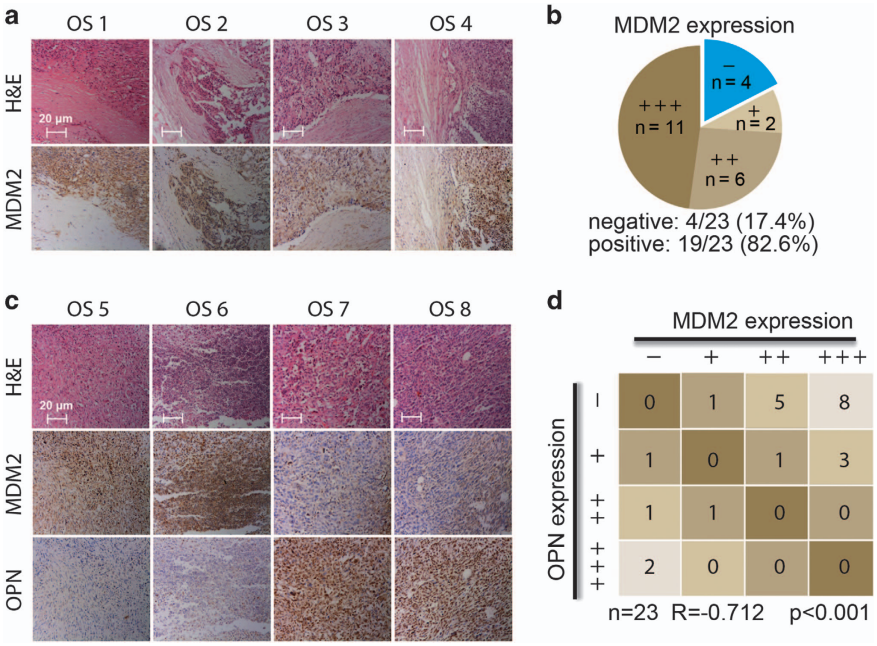

Figure 5. MDM2 expression is inversely correlated with the level of OPN in osteosarcoma tissues. (**a**) Four representative cases of immunohistochemical analysis of MDM2 expression in human primary osteosarcoma tissues. (**b**) The expression levels of MDM2 in 23 detected osteosarcoma tissues were graded and summarized in pie chart. (**c**) Correlation analysis of MDM2 and OPN in osteosarcoma tissues. Four representative cases are presented. (**d**) Statistical analysis of immunohistochemical results of MDM2 and OPN expression in 23 human osteosarcoma specimens. *P*-value was calculated by the Pearson correlation test. '–', negative expression; '+', low expression; '++', medium expression; '+++', high positive expression.

RARα for degradation. During the ubiquitination-proteasome-mediated degradation process for RARα, the N-terminus (1–109-residue amino) of MDM2 is critical for RARα binding. Moreover, we found that the interaction between MDM2 and RARα could be disrupted by nutlin-3, a reported molecular antagonist that binds to MDM2 in the p53-binding pocket. Because of nuclear magnetic resonance studies of unliganded (16–125) MDM2, which showed that the N-terminal residues 16–24 (TSQIPASEQ) form a partial helical 'lid' occluding the p53-binding site on apo-MDM2,[32] we predicted that the binding domain within MDM2 for RARα and p53 may overlap with one another. However, the role of p53 in the MDM2-mediated degradation of RARα should be further investigated.

RARα has an important role in ATRA-induced osteosarcoma differentiation, and insufficient RARα is not beneficial for differentiation progression. Thus strategies focusing on the stability of RARα may provide new approaches for differentiation therapy. In our study, we provide for the first time evidence for the involvement of MDM2 in controlling osteogenic differentiation by mediating the stability of RARα and suggest that MDM2 may serve as a potential therapeutic target for differentiation therapy in osteosarcoma. Similar to our findings in Figure 5, MDM2 has been reported to be amplified and overexpressed in a variety of human cancers with approximately one-third of osteosarcomas demonstrating amplification and overexpression of MDM2.[13,21,33] Moreover, the MDM2 protein has been found to induce cell proliferation, interfere with apoptosis in sarcoma cells, and induce spontaneous tumorigenesis in transgenic mice.[34] Anti-MDM2 antisense oligonucleotides have *in vitro* and *in vivo* antitumor activity and chemosensitizing and radiosensitizing effects in several human cancer models.[35] Therefore, targeting MDM2 may be a promising approach for cancer therapy. Thus, by liberating RARα from MDM2, one might stabilize RAR and activate the retinoid pathway, leading to growth arrest and differentiation. We have tested this hypothesis using RNA interfering (Figure 6) and macromolecular tools (Figure 7). Collectively, the data from our studies suggest that, once freed from MDM2, RARα rapidly accumulates in osteosarcoma cells, activates RARα target genes and results in proliferation arrest and differentiation. Different approaches may be exploited to release RARα from MDM2 control, including the inhibition of MDM2 expression, MDM2 ubiquitin ligase activity and MDM2–RARα binding. Further studies should be performed to confirm the *in vivo* effects of pharmacological inhibition of MDM2 in osteosarcoma cells.

MDM2 expression is found to be regulated via gene amplification, transcription, mRNA stability, protein translation and protein stability. Our results indicate that MDM2 protein expression is significantly induced by ATRA treatment in osteosarcoma cells (Figure 1). The importance of MDM2 as a negative regulator of RARα leads us to further elucidate the underlying mechanisms by which MDM2 expression is regulated. We show that ATRA significantly upregulate the level of MDM2 mRNA by eightfold in U2OS cells, while MDM4 mRNA did not get affected by ATRA (Supplementary Figure S3). These data suggest that the induction of MDM2 by ATRA is at the mRNA level. However, which signaling pathway is involved in ATRA-induced MDM2 transcription is still unknown. For example, it is reported that MDM2 transcription is induced by p53 through its P2 promoter.[36] Moreover, MDM2 protein translation is also regulated by RNA-binding protein La, the hepatocyte growth factor receptor (Met) and the insulin-like growth factor 1 receptor.[37–39] In our study, although partial RARα is degraded, its transcriptional activity should be activated by its ligand ATRA. Importantly, sequence analysis showed that there exist a putative repeats of AGGTCA-related sequences (−456/−444) within MDM2 promoter sequence. Considering the induction of MDM2 mRNA occurs very rapidly (~2 h), it would be interesting to observe whether RARα is able to transcriptionally regulate MDM2.

In summary, we demonstrate for the first time that MDM2 functions as an ubiquitin E3 ligase for RARα. In addition, MDM2 impairs ATRA-induced osteogenic differentiation by promoting the ubiquitination-proteasome-pathway-mediated degradation of

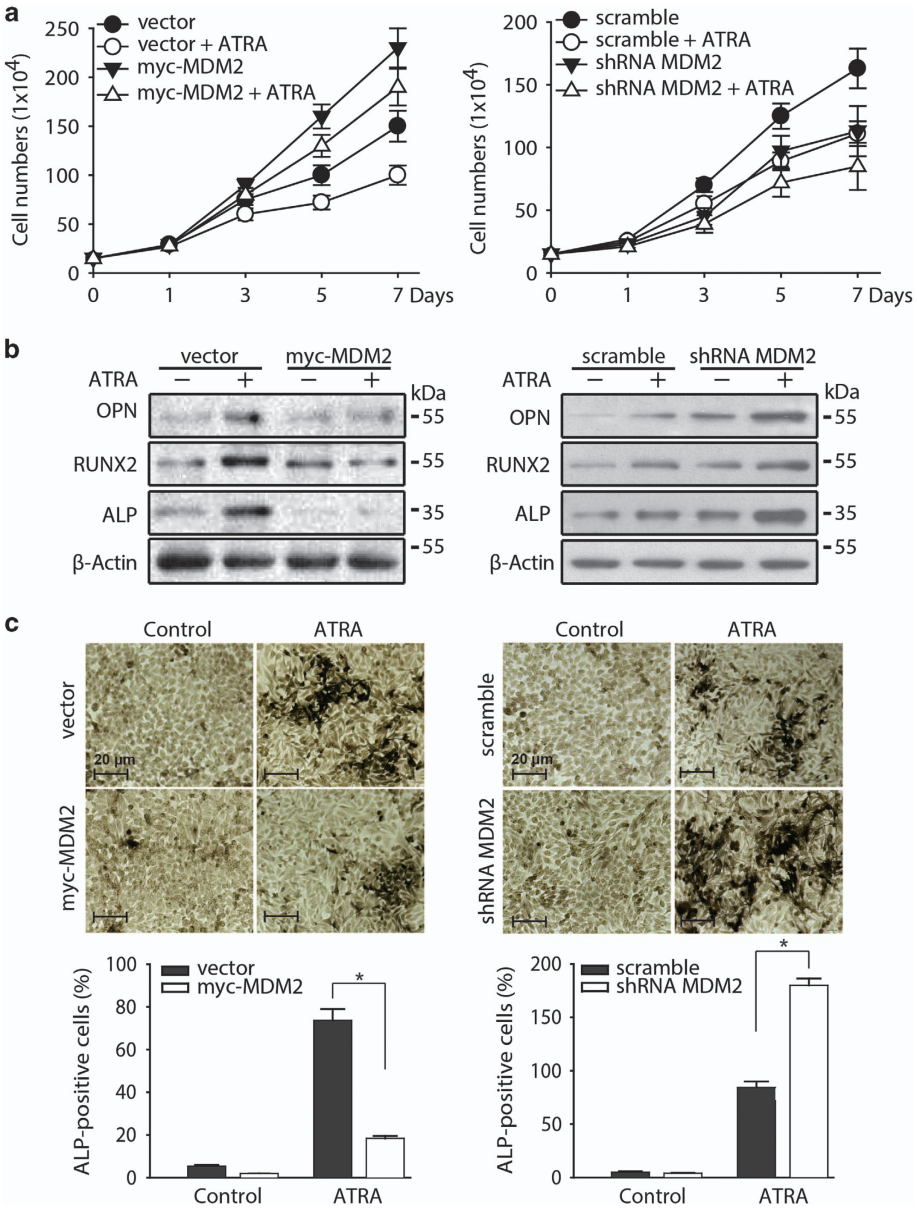

Figure 6. MDM2 impairs the ATRA-induced osteogenic differentiation of U2OS cells. (**a**) MDM2 modulates ATRA-inhibited cell proliferation. U2OS cells were transfected with MDM2 or shRNA MDM2 followed by ATRA treatment for the indicated times. The cell numbers in each group were counted every 2 days ($n = 3$). (**b**) MDM2 regulates ATRA-induced osteogenic differentiation protein expression. U2OS cells were transfected with MDM2 or shRNA MDM2 followed by ATRA treatment for 7 days. The protein levels of OPN, RUNX2 and ALP were determined. (**c**) MDM2 regulates the ATRA-mediated upregulation of ALP activity. U2OS cells were transfected with MDM2 or shRNA MDM2 followed by ATRA treatment for 7 days. The activity of ALP was evaluated by BCIP/NBT assay. Quantitative analysis of the average numbers of ALP-positive cells was from three independent experiments. $^*P < 0.05$.

RARα; therefore, reducing the expression of MDM2 by biological treatments may have great potential for potentiating the differentiation-inducing activity of ATRA. With these newly acquired mechanistic insights, this study not only directs us toward the future application of MDM2 inhibitors but also provides fresh insight into the design of new differentiation therapies directed against osteosarcoma.

MATERIALS AND METHODS

Cell culture and reagents

Human osteosarcoma U2OS and COS7 cells, which were from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China), were maintained in RPMI1640 and Dulbecco's modified Eagle's medium, respectively. All cells were routinely authenticated by short tandem repeat profiling and found to be mycoplasma free. All medium were supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) and 1% penicillin/streptomycin. All cells were incubated at 37 °C in a 5% CO_2 atmosphere.

ATRA, cycloheximide, nutlin-3 and MG132 were obtained from Sigma-Aldrich (St Louis, MO, USA). LMB solution was from Beyotime Institute of Biotechnology (Jiangsu, China). HLI373 was from Tocris Bioscience (Bristol, UK).

Primary antibodies against RARα (sc-551), MDM2 (SC-965), Ub (sc-8017), HA (sc-805), OPN (sc-73631), RUNX2 (sc-101145), ALP (sc-365765), p53 (sc-47698), β-Actin (sc-1615), glyceraldehyde 3-phosphate dehydrogenase (sc-25778) and Lamin B (sc-6216) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against myc (A00704-100) and Flag (A00187-100) were purchased from GenScript Co., Ltd (Nanjing, China). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

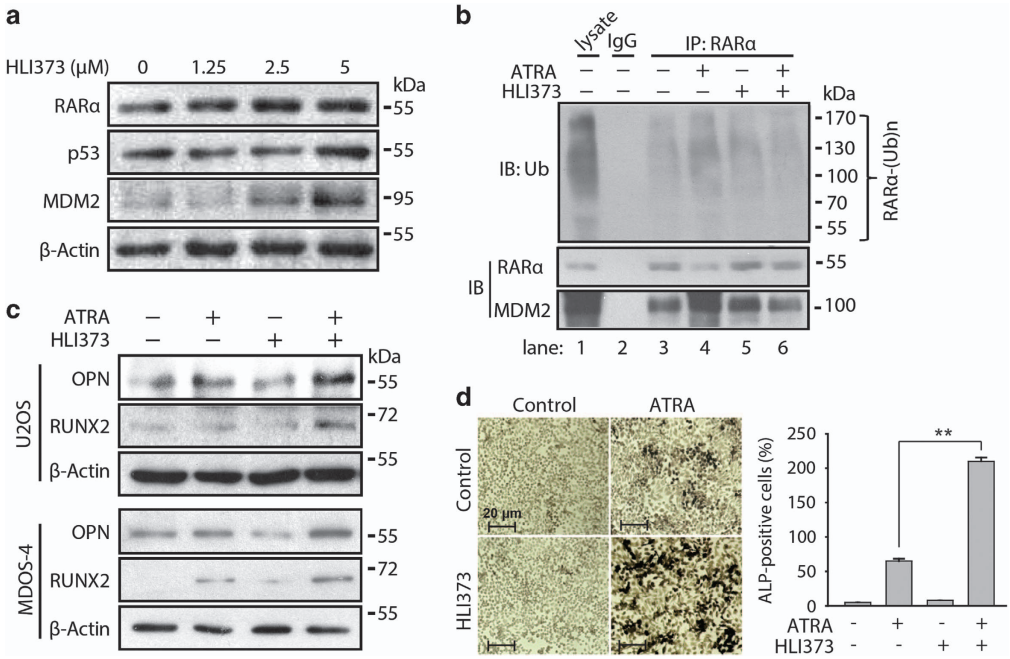

Figure 7. HLI373 synergizes with ATRA to promote the osteogenic differentiation of osteosarcoma cells. (**a**) HLI373 enhances the protein levels of RARα. U2OS cells were treated with serial concentrations of HLI373 for 24 h, and cell lysates were then immunoblotted with anti-RARα, anti-p53 and anti-MDM2 antibodies. (**b**) HLI373 inhibits the ubiquitination of RARα. U2OS cells were pretreated with HLI373 for 12 h followed by ATRA treatment for 6 h. Cell lysates were immunoprecipitated with anti-RARα antibody followed by immunoblotting with an anti-Ub antibody. (**c**) HLI373 synergizes with ATRA to promote the expression levels of OPN and RUNX2. U2OS cells and primary osteosarcoma blast MDOS4 cells were pretreated with HLI373 for 12 h followed by ATRA treatment for 7 days. The protein levels of OPN and RUNX2 were detected. (**d**) HLI373 synergizes with ATRA to promote the activity of ALP. U2OS cells were pretreated with HLI373 for 12 h followed by ATRA treatment for 7 days. The activity of ALP in U2OS cells was further evaluated by BCIP/NBT assay. Quantitative analysis of the average numbers of ALP-positive cells was from three independent experiments. $^{**}P < 0.01$.

Primary human osteosarcoma tissue samples

Tissue collection and analysis in this study were approved by the Ethics Committee of The Second Affiliated Hospital of Zhejiang University, Zhejiang University, and written informed consent to perform the biological studies was obtained from all participants. Human osteosarcoma tissues used for primary cell culture were obtained from fresh tissue sections from biopsies from osteosarcoma patients and maintained in Dulbecco's modified Eagle's medium/F12 medium as previously described.[9] All osteosarcoma specimens were histologically verified by two senior pathologists independently.

Immunofluorescence staining and quantitation

U2OS and COS7 cells cultured in 96-well plates were washed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The cells were then incubated overnight at 4 °C with the indicated antibodies diluted 1:60 in PBS containing 3% fetal bovine serum and 1% bovine serum albumin. After two PBS washes, the cells were incubated with fluorescein isothiocyanate- or rhodamine-conjugated secondary antibodies (1:100 dilution) for 1 h at room temperature. The cells were then incubated with 4′,6-diamidino-2-phenylindole for another 5 min. After three PBS washes, the probed cells were visualized with a confocal fluorescence microscope. Nuclear/cytoplasmic ratios were determined by calculating total pixel intensity in a circle 10 μm in diameter in the nucleus and the cytoplasm using the ImageJ software (NIH, Bethesda, MD, USA). The background was determined by calculating pixel intensity in a 10-μm circle in cell-free areas in each field and then subtracting this from the nuclear and cytoplasmic measurements for each cell in the field. Nuclear-to-cytoplasmic ratios were calculated as $\sum(\frac{\text{IntDen}}{\text{Area}})/n$.

Cell lysate preparation and western blotting analysis

U2OS and COS7 cells were lysed with IP buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid, 25 mM NaF, 25 mM β-glycerolphosphate, 0.1 mM sodium vanadate, 5μg/ml leupeptin, 0.1 mM phenylmethanesulfonylfluoride, 0.5% Triton X-100, 0.5% NP40) and incubated at 4 °C for 30 min. The lysate was then centrifuged at 14 000 *g* for 20 min to remove insoluble materials. Protein concentrations of whole-cell lysates were determined using the DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Equal aliquots of supernatants were incubated with 2 μg primary antibody at 4 °C overnight. The bound proteins were then analyzed by western blotting. Briefly, proteins were electrophoresed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After incubation with primary antibodies, proteins were visualized by incubation with horseradish peroxidase-conjugated secondary antibodies as appropriate, followed by enhanced chemiluminescence detection (Biological Industries, Beit Haemek, Israel). The separation of nuclear and cytosolic fractions was performed as previously reported.[10]

Immunoprecipitation

Prot Elut NHS-Activated Bead Kit (Elut-P012, Enriching Biotechnology, Shanghai, China) was used for IP. Briefly, beads were washed with PBS twice and then suspended in 50 μl coupling buffer. In all, 2 μg first antibodies or normal IgG for 2 h was added at room temperature with end-over-end mixing. After coupling, the same was centrifuged at 1000 *g* for 2 min and beads were saved; 500 μl blocking solutions for 30 min at room temperature was added, washed with 1ml PBS twice and the supernatants were discarded; and 500 μg cell lysates was added for overnight incubation at 4 °C with end-over-end mixing. Finally, the complex was washed with IP buffer for three times and 30 μl 0.1–0.2 M glycine–HCl was added for 5 min at room temperature and then neutralized with 1 M phosphate. The bound proteins were immunoblotted with the EasyBlot anti-Rabbit or anti-Mouse IgG Kit (GeneTex, Irvine, CA, USA).

Plasmids and small interfering RNA transfection

The pcmv-myc3-MDM2 plasmid was purchased from Addgene (Cambridge, MA, USA). Deletion mutants were constructed by

GenScript Co., Ltd. Scramble and MDM2 shRNA were synthesized by Shanghai GenePharma Co., Ltd (Shanghai, China). The sense strand of MDM2 shRNA was as follows: 5′-AATGCCTCAATTCACATAGAT-3′. Briefly, cells were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) or small interfering RNA using oligofectamine (Invitrogen).

In vitro ubiquitination assay

The reaction mixture (50 μl) contained RARα (500 ng), E1 (500 ng), E2 (500 ng), ubiquitin (10 ng) and MDM2 (500 ng), 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM ATP and 2 mM dithiothreitol. Reactions were incubated at 30 °C for 1 h. The reactions were terminated with sample loading buffer and run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels. A higher molecular ladder of ubiquitinated proteins was visualized by performing immunoblot analysis with the indicated antibodies.

Immunohistochemistry

Human osteosarcoma tissues were paraffin embedded. After deparaffinization, the slides were blocked with 3% hydrogen peroxide, preincubated in 20% normal goat serum and then probed with anti-MDM2 or anti-OPN followed by biotinylated secondary antibodies and horseradish peroxidase-conjugated avidin. Antibodies against MDM2 (BS1264) and OPN (BS1223) were from Bioworld Technology (Minneapolis, MN, USA). MDM2 and OPN were visualized with 3, 3′-diaminobenzidine.

Alkaline phosphatase assay

Alkaline phosphatase activity was assessed by colorimetric assays using the BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions.

Cell proliferation assay

Total cell number and viability were determined by trypan blue exclusion with manual counting in Burker chambers (VWR, West Chester, PA, USA).

Statistical analysis

Statistical analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). For all parameters measured, the values for all samples in different experimental conditions were averaged, and the s.d. of the mean was calculated. A Pearson correlation test was used to evaluate immunohistochemical analysis. Other assays were determined by analysis of variance, followed by Student's *t*-test. All measurements were carried out in triplicate. Significance was defined as $P < 0.05$.

ABBREVIATIONS

ALP, alkaline phosphatase; ATRA, all-*trans* retinoic acid; OPN, osteopontin; RARα, retinoic acid receptor alpha; RUNX2, runt-related transcription factor 2.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Natural Science Foundation of China (Nos. 81473227, 91029745 and 81273536), Zhejiang Provincial Program for Qianjiang Talents (2013R10025), Zhejiang Provincial Natural Science Foundation of China (No. Y14H160124) and the Fundamental Research Funds for the Central Universities.

REFERENCES

1 Niederreither K, Dolle P. Retinoic acid in development: towards an integrated view. *Nat Rev Genet* 2008; **9**: 541–553.
2 Hisada K, Hata K, Ichida F, Matsubara T, Orimo H, Nakano T *et al.* Retinoic acid regulates commitment of undifferentiated mesenchymal stem cells into osteoblasts and adipocytes. *J Bone Miner Metab* 2013; **31**: 53–63.
3 Dingwall M, Marchildon F, Gunanayagam A, Louis CS, Wiper-Bergeron N. Retinoic acid-induced Smad3 expression is required for the induction of osteoblastogenesis of mesenchymal stem cells. *Differentiation* 2011; **82**: 57–65.
4 Luo P, Yang X, Ying M, Chaudhry P, Wang A, Shimada H *et al.* Retinoid-suppressed phosphorylation of RARalpha mediates the differentiation pathway of osteosarcoma cells. *Oncogene* 2010; **29**: 2772–2783.
5 Haydon RC, Zhou L, Feng T, Breyer B, Cheng H, Jiang W *et al.* Nuclear receptor agonists as potential differentiation therapy agents for human osteosarcoma. *Clin Cancer Res* 2002; **8**: 1288–1294.
6 Haydon RC, Luu HH, He TC. Osteosarcoma and osteoblastic differentiation: a new perspective on oncogenesis. *Clin Orthop Relat Res* 2007; **454**: 237–246.
7 Dilworth FJ, Chambon P. Nuclear receptors coordinate the activities of chromatin remodeling complexes and coactivators to facilitate initiation of transcription. *Oncogene* 2001; **20**: 3047–3054.
8 He BC, Chen L, Zuo GW, Zhang W, Bi Y, Huang J *et al.* Synergistic antitumor effect of the activated PPARgamma and retinoid receptors on human osteosarcoma. *Clin Cancer Res* 2010; **16**: 2235–2245.
9 Zhang L, Zhou Q, Zhang N, Li W, Ying M, Ding W *et al.* E2F1 impairs all-trans retinoic acid-induced osteogenic differentiation of osteosarcoma via promoting ubiquitination-mediated degradation of RARalpha. *Cell Cycle* 2014; **13**: 1277–1287.
10 Ying M, Zhou X, Zhong L, Lin N, Jing H, Luo P *et al.* Bortezomib sensitizes human acute myeloid leukemia cells to all-trans-retinoic acid-induced differentiation by modifying the RARalpha/STAT1 axis. *Mol Cancer Ther* 2013; **12**: 195–206.
11 Zhu J, Gianni M, Kopf E, Honore N, Chelbi-Alix M, Koken M *et al.* Retinoic acid induces proteasome-dependent degradation of retinoic acid receptor alpha (RARalpha) and oncogenic RARalpha fusion proteins. *Proc Natl Acad Sci USA* 1999; **96**: 14807–14812.
12 Ladanyi M, Cha C, Lewis R, Jhanwar SC, Huvos AG, Healey JH. MDM2 gene amplification in metastatic osteosarcoma. *Cancer Res* 1993; **53**: 16–18.
13 Momand J, Jung D, Wilczynski S, Niland J. The MDM2 gene amplification database. *Nucleic Acids Res* 1998; **26**: 3453–3459.
14 Honda R, Tanaka H, Yasuda H. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett* 1997; **420**: 25–27.
15 Wallace M, Worrall E, Pettersson S, Hupp TR, Ball KL. Dual-site regulation of MDM2 E3-ubiquitin ligase activity. *Mol Cell* 2006; **23**: 251–263.
16 Lin HK, Wang L, Hu YC, Altuwaijri S, Chang C. Phosphorylation-dependent ubiquitylation and degradation of androgen receptor by Akt require Mdm2 E3 ligase. *EMBO J* 2002; **21**: 4037–4048.
17 Pan Y, Chen J. MDM2 promotes ubiquitination and degradation of MDMX. *Mol Cell Biol* 2003; **23**: 5113–5121.
18 Weber JD, Kuo ML, Bothner B, DiGiammarino EL, Kriwacki RW, Roussel MF *et al.* Cooperative signals governing ARF-mdm2 interaction and nucleolar localization of the complex. *Mol Cell Biol* 2000; **20**: 2517–2528.
19 Mayo LD, Dixon JE, Durden DL, Tonks NK, Donner DB. PTEN protects p53 from Mdm2 and sensitizes cancer cells to chemotherapy. *J Biol Chem* 2002; **277**: 5484–5489.
20 Sdek P, Ying H, Chang DL, Qiu W, Zheng H, Touitou R *et al.* MDM2 promotes proteasome-dependent ubiquitin-independent degradation of retinoblastoma protein. *Mol Cell* 2005; **20**: 699–708.
21 Florenes VA, Maelandsmo GM, Forus A, Andreassen A, Myklebost O, Fodstad O. MDM2 gene amplification and transcript levels in human sarcomas: relationship to TP53 gene status. *J Natl Cancer Inst* 1994; **86**: 1297–1302.
22 Berner JM, Forus A, Elkahloun A, Meltzer PS, Fodstad O, Myklebost O. Separate amplified regions encompassing CDK4 and MDM2 in human sarcomas. *Genes Chromosomes Cancer* 1996; **17**: 254–259.
23 Freemantle SJ, Spinella MJ, Dmitrovsky E. Retinoids in cancer therapy and chemoprevention: promise meets resistance. *Oncogene* 2003; **22**: 7305–7315.
24 Fang S, Jensen JP, Ludwig RL, Vousden KH, Weissman AM. Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. *J Biol Chem* 2000; **275**: 8945–8951.
25 Zhan C, Varney K, Yuan W, Zhao L, Lu W. Interrogation of MDM2 phosphorylation in p53 activation using native chemical ligation: the functional role of Ser17 phosphorylation in MDM2 reexamined. *J Am Chem Soc* 2012; **134**: 6855–6864.
26 Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z *et al.* In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* 2004; **303**: 844–848.
27 Poyurovsky MV, Katz C, Laptenko O, Beckerman R, Lokshin M, Ahn J *et al.* The C terminus of p53 binds the N-terminal domain of MDM2. *Nat Struct Mol Biol* 2010; **17**: 982–989.
28 Wang W, Hu Y. Small molecule agents targeting the p53-MDM2 pathway for cancer therapy. *Med Res Rev* 2012; **32**: 1159–1196.
29 Zhou Q, Zhang L, Chen Z, Zhao P, Ma Y, Yang B *et al.* Small ubiquitin-related modifier-1 modification regulates all-trans-retinoic acid-induced differentiation via stabilization of retinoic acid receptor alpha. *FEBS J* 2014; **281**: 3032–3047.
30 Gianni M, Boldetti A, Guarnaccia V, Rambaldi A, Parrella E, Raska I Jr *et al.* Inhibition of the peptidyl-prolyl-isomerase Pin1 enhances the responses

of acute myeloid leukemia cells to retinoic acid via stabilization of RARalpha and PML-RARalpha. *Cancer Res* 2009; **69**: 1016–1026.
31 Chai Z, Yang L, Yu B, He Q, Li WI, Zhou R *et al.* p38 mitogen-activated protein kinase-dependent regulation of SRC-3 and involvement in retinoic acid receptor alpha signaling in embryonic cortical neurons. *IUBMB Life* 2009; **61**: 670–678.
32 McCoy MA, Gesell JJ, Senior MM, Wyss DF. Flexible lid to the p53-binding domain of human Mdm2: implications for p53 regulation. *Proc Natl Acad Sci USA* 2003; **100**: 1645–1648.
33 Vassilev LT. MDM2 inhibitors for cancer therapy. *Trends Mol Med* 2007; **13**: 23–31.
34 Steinman HA, Burstein E, Lengner C, Gosselin J, Pihan G, Duckett CS *et al.* An alternative splice form of Mdm2 induces p53-independent cell growth and tumorigenesis. *J Biol Chem* 2004; **279**: 4877–4886.
35 Zhang Z, Zhang R. p53-independent activities of MDM2 and their relevance to cancer therapy. *Curr Cancer Drug Targets* 2005; **5**: 9–20.
36 Barak Y, Juven T, Haffner R, Oren M. mdm2 expression is induced by wild type p53 activity. *EMBO J* 1993; **12**: 461–468.
37 Trotta R, Vignudelli T, Candini O, Intine RV, Pecorari L, Guerzoni C *et al.* BCR/ABL activates mdm2 mRNA translation via the La antigen. *Cancer Cell* 2003; **3**: 145–160.
38 Moumen A, Patane S, Porras A, Dono R, Maina F. Met acts on Mdm2 via mTOR to signal cell survival during development. *Development* 2007; **134**: 1443–1451.
39 Xiong L, Kou F, Yang Y, Wu J. A novel role for IGF-1R in p53-mediated apoptosis through translational modulation of the p53-Mdm2 feedback loop. *J Cell Biol* 2007; **178**: 995–1007.

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