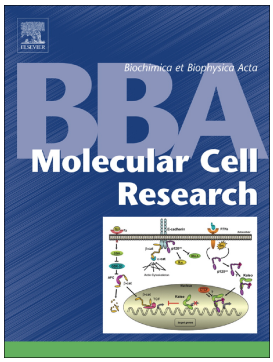


Long lasting inhibition of Mdm2-p53 interaction potentiates mesenchymal stem cell differentiation into osteoblasts

Simona Daniele, Chiara Giacomelli, Deborah Pietrobono, Elisabetta Barresi, Rebecca Piccarducci, Valeria La Pietra, Sabrina Taliani, Federico Da Settimo, Luciana Marinelli, Ettore Novellino, Claudia Martini, Maria Letizia Trincavelli

PII: S0167-4889(18)30228-3
DOI: https://doi.org/10.1016/j.bbamcr.2019.01.012
Reference: BBAMCR 18431

To appear in: *BBA - Molecular Cell Research*

Received date: 31 July 2018
Revised date: 3 December 2018
Accepted date: 24 January 2019

Please cite this article as: S. Daniele, C. Giacomelli, D. Pietrobono, et al., Long lasting inhibition of Mdm2-p53 interaction potentiates mesenchymal stem cell differentiation into osteoblasts, BBA - Molecular Cell Research, https://doi.org/10.1016/j.bbamcr.2019.01.012

Long lasting inhibition of Mdm2-p53 interaction potentiates mesenchymal stem cell differentiation into osteoblasts

Simona Daniele[a], Chiara Giacomelli[a], Deborah Pietrobono[a], Elisabetta Barresi[a], Rebecca Piccarducci[a], Valeria La Pietra[b], Sabrina Taliani[a], Federico Da Settimo[a], Luciana Marinelli[b], Ettore Novellino[b], Claudia Martini[a#], Maria Letizia Trincavelli[a].

[a]Department of Pharmacy, University of Pisa, 56126 Pisa, Italy.

[b]Department of Pharmacy, University of Naples Federico II, 80131 Naples, Italy.

Corresponding author at: Department of Pharmacy, University of Pisa. Via Bonanno 6, 56126 Pisa, Italy. Phone: +39-050-2219509/522; Fax: +39-050-2219609. *E-mail address:* claudia.martini@unipi.it.

Abbreviations: AC, adenylyl cyclase; ALP, Alkaline Phosphatase; cAMP, cyclic AMP; CREB, cAMP response element-binding protein; ERK, extracellular signal–regulated kinases; GPCRs, G protein-coupled receptors; GRK, G protein-coupled receptors kinase; Mdm2, Murine double minute 2; MSCs, Mesenchymal stem cells; NUT-3, Nutlin-3; Osx, Osterix; PFT-β, Pifithrin-β; Runx2, Runt-related transcription factor 2.

Abstract

The osteoblast generation from Mesenchymal stem cells (MSCs) is tightly coordinated by transcriptional networks and signalling pathways that control gene expression and protein stability of osteogenic "master transcription factors". Among these pathways, a great attention has been focused on p53 and its physiological negative regulator, the E3 ligase Murine double minute 2 (Mdm2). Nevertheless, the signalling that regulates Mdm2-p53 axis in osteoblasts remain to be elucidated, also considering that Mdm2 possesses numerous p53-independent activities and interacts with additional proteins.

Herein, the effects of Mdm2 modulation on MSC differentiation were examined by the use of short- and long-lasting inhibitors of the Mdm2-p53 complex. The long-lasting Mdm2-p53 dissociation was demonstrated to enhance the MSC differentiation into osteoblasts. The increase of Mdm2 levels promoted its association to G protein-coupled receptors kinase (GRK) 2, one of the most relevant kinases involved in the desensitisation of G protein-coupled receptors (GPCRs). In turn, the long-lasting Mdm2-p53 dissociation decreased GRK2 levels and favoured the functionality of A_{2B} Adenosine Receptors (A_{2B}ARs), a GPCR dictating MSC fate. EB148 facilitated cAMP accumulation, and mediated a sustained activation of extracellular signal–regulated kinases (ERKs) and cAMP response element-binding protein (CREB). Such pro-osteogenic effects were not detectable by using the reversible Mdm2-p53 complex inhibitor, suggesting the time course of Mdm2-p53 dissociation may impact on intracellular proteins involved in cell differentiation fate. These results suggest that the long-lasting Mdm2 binding plays a key role in the mobilization of intracellular proteins that regulate the final biological outcome of MSCs.

Keywords: Murine double minute 2; Mesenchymal stem cells; osteoblasts; G protein-coupled receptors kinase; long-lasting inhibitors; Murine double minute 2-p53 complex.

Introduction

Bone homeostasis results from the delicate balance between bone formation and resorption processes [1,2], refereed to mesenchymal stem cell derived osteoblasts and hematopoietic-derived osteoclasts, respectively [2,3].

Mesenchymal stem cells (MSCs) are multipotent stromal cells with self-renewal ability that are also capable of forming multiple cellular types [1,2], including chondrocytes and osteoblasts.

MSCs differentiation into osteoblasts is controlled by different "master transcription factors", including Runt-related transcription factor 2 (Runx2) and Osterix (Osx) [4] which in turn activate a repertoire of genes during differentiation of preosteoblasts into mature osteoblasts and osteocytes [5]. In order to assure normal osteogenesis, gene expression and protein stability of these transcription factors are controlled by transcriptional networks, signalling pathways, and epigenetic regulation [5]. Among these regulating factors, a great attention has been focused on p53 and on its physiological negative regulator Murine double minute 2 (Mdm2) [6]. In particular, p53 has been shown to suppress the expression of Runx2 and Osx [7,8] as well as to modulate the expression of several microRNAs negatively affecting osteoblast differentiation [9].

In contrast, Mdm2 has been shown to promote MSC differentiation into adipocytes [10] or osteoblasts [11,12]. In particular, Mdm2 gene expression, which is linked to enhanced osteogenesis, is modulated by the P2 promoter, and it is activated by 1,25-dihydroxy vitamin D3 in cells with wild type p53 [13].

However, the signalling that regulates p53-Mdm2 axis in osteoblast differentiation remains to be elucidated, also considering that the E3 ligase, Mdm2, actually possesses numerous p53-independent activities and interacts with additional proteins and/or ubiquitination substrates [6,14,15] which are involved in cell fate determination, differentiation and signalling [12,13].

Recently, indol-3ylglyoxyldipeptide derivatives have been rationally designed to dissociate the Mdm2-p53 complex [16,17]. One of these derivatives has been chemically modified to produce a long-lasting binding to Mdm2, namely EB148 (Fig. 1A) [18]. In human glioblastoma cells, EB148,

produced a peculiar induction of Mdm2 gene transcription, and a different kinetic pattern in the regulation of p53 gene targets [18].

In the present paper, the effects of the modulation of Mdm2-p53 complex were evaluated on MSC differentiation program towards osteoblast phenotype. In particular, the differential effects of short-term versus long-lasting inhibitors of Mdm2-p53 complex in driving MSC fate were compared, exploring the contribution of Mdm2 in the regulation of intracellular signalling pathways involved in MSC differentiation.

Materials and Methods

Compounds. The Mdm2 inhibitor, Nutlin-3 (NUT-3, Sigma Aldrich, Milan, Italy, Fig. 1A), was used as reference compound [19]. The indol-3-ylglyoxyldipeptide derivative EB54 (2-phenylindol-3-yl)glyoxyl-L-leucine-L-phenylalanine ethylester, Fig. 1A [16], and its isothiocyanato derivative EB148 (5-[*N*-(5-isothiocyanato-2-phenylindol-3-yl)glyoxyl-L-leucine-L-phenylalanine ethyl ester (Fig. 1A) [18] were tested in parallel. EB54 and EB148 were synthesised as previously reported [16,18]. Based on the compounds' efficacy to dissociate the Mmd2-p53 complex [16], the derivatives were tested hereby at a concentration corresponding to 10- or 100-fold their IC_{50} values. When indicated, a higher range of concentrations was chosen for EB54 and EB148.

When indicated, Pifithrin-β (PFT-β, Sigma Aldrich, Italy) was used as p53 inhibitor [18, 20]. PD184352 (2-(2-Chloro-4-iodophenylamino)-N-cyclopropylmethoxy-3,4-difluorobenzamide, Sigma-Aldrich, Milan, Italy), and SQ22536 (9-(Tetrahydro-2-furanyl)-9*H*-purin-6-amine, Sigma-Aldrich, Milan, Italy) [21, 22] were used as inhibitors of Extracellular Regulated Kinase (ERK) and adenylyl cyclase (AC), respectively.

Cell cultures. Human bone marrow MSCs and cell culture medium were from Lonza (Milan, Italy). The cell line was monitored for DNA profiling and used until passage 5, for fewer than 6 months after resuscitation [23,24]. Cells were sub-cultured in normal growth medium (MSCGM) and plated ($5x10^3$ cells/cm^2). The medium was changed every 3 to 4 days to remove non-adherent cells. In osteoblast differentiation studies, cells were seeded ($9x10^3$ cells/cm^2) and switched to specific osteogenic medium, as previously described [23].

Cell proliferation assay. MSCs were seeded in 96-well microplates ($5x10^3$ cells/well) and cultured in proliferation or in osteogenic medium for 48 h or 7 days, in the absence or presence of EB148 (ranging from 100 nM to 100 μM), EB54 (10-100 μM), NUT-3 (1-10 μM), or medium alone (control). For long term experiments, treatments were repeated every three days, and cell

proliferation was evaluated by MTS assay following the manufacturer's instruction (Promega, Milan, Italy) [23,24].

Mineralization assay. MSCs ($9x10^3$ cells/cm^2) were cultured in osteogenic medium in the absence (control) or in the presence of EB148 (100 nM-100 µM), EB54 (1 µM) or NUT-3 (10 µM) for 7, 14 or 21 days. Following treatments, cells were fixed in 2% formaldehyde, and the rate of mineralization was quantified using OsteoImage™ Staining Reagent (Lonza, Milan, Italy), which specifically binds to the hydroxyapatite portion of the bone-like nodules deposited by cells [23]. The mineralization process was confirmed by alizarin red staining [24,25]. Photographs were taken analysing three different wells for each condition (20 × objective lens).

MSCs ($1x10^4$ cells/well) were also cultured for 7, 14 or 21 days in osteogenic medium in the absence (control) or in the presence of EB148 (1 µM), EB54 (1 µM), NUT-3 (10 µM). To unveil the contribute of p53, AC or ERKs in compounds-elicited effects, MSCs were challenged in osteogenic medium for 21 days with EB148, EB54 and NUT-3, in the absence or presence of 1 µM PFT-β (which blocks p53, [20]) or 1 µM PD184352 (which blocks ERKs, [21]) or 1 µM SQ22536 (which blocks AC, [22]). Following treatments, cells were fixed in 2% formaldehyde, and the rate of mineralization was quantified by alizarin red staining [24,25]. For quantitative analysis, absorbance was read at 530 nm.

Real-time RT-PCR analysis. MSCs were cultured in osteogenic medium in the absence (control) or presence of EB148 (1 µM), EB54 (1 µM) or NUT-3 (10 µM) for 2, 7 or 14 days. Following treatments, mRNA was extracted, and the expression levels of MDM2, p53, p21 and PUMA were quantified by real-time RT-PCR, as previously described [23,26]. In parallel, the expression levels of A_{2B} adenosine receptor (A_{2B}AR), GRK_2 and osteogenic markers (Runx2, Osx, alkaline phosphatase or ALP, and Osteocalcin) were quantified by real-time RT-PCR in 14-days treated

cells [23,26]. Primer sequences, annealing temperatures and product sizes have been reported elsewhere [23-26].

Western blotting analysis of p53 and A_{2B} adenosine receptor. MSCs were treated with DMSO (control) or with EB148 (1 μM) for 2 or 14 days, and then lysed by the addition of RIPA buffer containing proteases' inhibitors (100 μL). Equal amounts of the cell extracts (30 μg of proteins) were diluted in Laemmli solution, resolved by SDS-PAGE (7.5%), transferred to PVDF membranes and probed overnight at 4°C with primary antibody anti-p53 (diluted 1:500; 70R-31561, Filtzgerald) or anti-A_{2B}AR (1:200; sc-28266, Santa Cruz) or GAPDH (diluted 1:5000; G9545, Sigma-Aldrich). The primary antibody was detected using anti-rabbit IgG light chains conjugated to peroxidase (1:10,000; Sigma-Aldrich). The peroxidase was detected using a chemioluminescent substrate (ECL, PerkinElmer). Densitometric analysis of immunoreactive bands was performed using ChemiDoc^{TM} XRS+ System (BioRad, Hercules, California, USA).

GRK2 association to Mouse Double Minute 2 (Mdm2) or to A_{2B}AR. To test Mdm2-GRK2 association, a co-immunoprecipitation assay was performed. MSCs were incubated in osteogenic medium alone (control), or in the presence of EB148 (1 μM), EB54 (1 μM) or NUT-3 (10 μM) for 2 or 7 days. For the determination of GRK2-Mdm2 heterocomplexes, cells were lysed following treatments, and incubated with anti-Mdm2 antibody (sc-5304, Santa Cruz Biotechnology; 3 μg/sample) overnight at 4 °C under constant rotation, and then immunoprecipitated with protein A-Sepharose (2 h at 4 °C) [16,26-28]. Immunocomplexes were resolved by SDS-PAGE (7.5%), transferred into PVDF membranes and incubated with an anti-GRK2 (sc-562, Santa Cruz Biotechnology; 1:200) or an anti-Mdm2 antibody (input samples). Signals were detected using a chemioluminescent substrate (ECL, Perkin Elmer, Waltham, MA, USA) [16,26,28]. Densitometric

analysis of immunoreactive bands was performed using ImageJ Software (version 1.41; Bethesda, MD, USA).

For GRK2-A_{2B}AR heterocomplexes, cells were lysed following treatments and incubated with anti-A_{2B}AR antibody (Santa Cruz Biotechnology, sc-28996; 3 µg/sample) overnight at 4 °C under constant rotation, and then immunoprecipitated with protein A-Sepharose (2 h at 4 °C) [16,26-28]. Immunocomplexes were resolved by SDS-PAGE (7.5%), transferred into PVDF membranes and incubated with an anti-GRK2 (sc-562, Santa Cruz Biotechnology; 1:200) or an anti-A_{2B}AR antibody (input samples). Signals were detected using a chemioluminescent substrate (ECL, Perkin Elmer, Waltham, MA, USA) [16,26,28]. Densitometric analysis of immunoreactive bands was performed using ChemiDoc^{TM} XRS+ System (BioRad, Hercules, California, USA).

ERK and CREB phosphorylation assays. MSCs ($5x10^3$ cells/well) were cultured in osteogenic medium in the absence (control) or in the presence of EB148 (1 µM), EB54 (1 µM) or NUT-3 (10 µM) for 30 min, 120 min or 7 days. To determie the contribute of p53 and AC on ERK activation, MSCs were challenged in osteogenic medium with EB148 or EB54, in the absence or presence of 1 µM PFT-β (which blocks p53) or 1 µM SQ22536 (which blocks AC). In contrast, to unveil the contribute of ERK on CREB activation, MSCs were incubated in osteogenic medium with 1 µM PD184352 (which blocks ERKs).

Following incubations, cells were fixed with 4% formaldehyde, and the levels of ERK1/2 (total and phosphorylated) or CREB (phosphorylated) were determined by immunoenzymatic assays [29,30]. Briefly, after washing, the cells were incubated with quenching buffer (1% H_2O_2; 0.1% sodium azide in wash buffer) and then with a blocking solution (1% BSA; 0.1% Triton X-100 in PBS) for 60 min [29,30]. After blocking, cells were washed and challenged with the specific primary antibody (anti-phospho ERK1/2, 1:500, sc-7383 Santa Cruz Biotechnology; anti-ERK1/2, 1:500, #4695 Cell Signaling Technology; anti-phospho CREB, 1:300, sc-81486 Santa Cruz

Biotechnology; anti-CREB, 1:500, sc-377154 Santa Cruz Biotechnology) overnight at 4 °C. Then, the samples were incubated with secondary HRP-conjugated antibodies and later with a developing solution, allowing a colorimetric quantification of total and phosphorylated levels (absorbance read at 450 nm). Blanks were obtained processing wells without cells in the absence of the primary antibody. The Crystal Violet solution was used to determine the relative number of cells in each well and to normalise the reading absorbance. The results were calculated by subtracting the mean background from the values obtained from each test condition; values were expressed as the percentage of the control (untreated cells) [29,30].

Quantitation of ERK-Mdm2 complex. MSCs ($2x10^5$ cells/well) were cultured in 6-well plates in osteogenic medium and challenged with EB148 (1 μM), EB54 (1 μM) or NUT-3 (10 μM) for 120 min or 7 days. At the end of treatments, cells were collected, and suspended in lysis buffer containing 1% of a protease inhibitor cocktail [18]. An immunoenzymatic assay was used to quantify the levels of ERK/Mdm2 complexes [16-18,31,32]. Briefly, cell lysates (20 μg in a final volume of 100 μl) were transferred in wells pre-coated with an anti-Mdm2 antibody; following 60 min, each well was washed, incubated for 15 min with 1% BSA to block non-specific sites, and then challenged for 1.5 h at room temperature with an antibody specific for ERK (sc-7383 SantaCruz Biotechnology, 1:500). Following extensive washes, the samples were challenged with an anti-rabbit HRP-conjugate antibody, and washed again. The absorbance was read at 450 nm following the addition of a developing solution (TMB substrate kit, Thermo Fisher Scientific). Blanks were obtained processing cell lysates in the absence of the primary anti-ERK antibody [16,18].

Measurement of cAMP levels during MSC differentiation into osteoblasts. MSCs ($2x10^4$ cells/well) were cultured in 24-well plates in osteogenic medium and treated with EB148 (1 μM), EB54 (1 μM) or NUT-3 (10 μM) for 2, 7, 14 or 21 days. At the end of treatments, cells were lysed,

and the intracellular cyclic AMP (cAMP) levels were quantified using a competitive protein binding method [23,24,33].

A_{2B}AR desensitization kinetics. MSC cells ($4x10^4$ cells/well) were seeded in a black, clear bottom PerkinElmer 96-well CellCarrier™ microplate (#6005550). After 24 h, the desensitization experiments were performed incubating the cells with BAY60-6583 (10 nM) in the absence or presence of EB148 (10 µM) for 5, 30 and 120 min. At the end of pre-incubation, the medium was changed and cells were treated with the phosphodiesterase inhibitor Ro201724 and stimulated again with BAY 60-6583 (10 nM) for 15 min [23,24,27,34] Successively, cells were lysed with 100 µl of 1X lysis buffer (PerkinElmer, #AL003C). Lysates were either tested immediately in AlphaLISA assays or frozen at –20°C for later testing. cAMP levels were quantified using cAMP AlphaLISA kit (PerkinElmer, #AL315) following manufacturer's instructions. Standard curves for each AlphaLISA immunoassay were performed in the same diluent as the samples being tested (1X lysis buffer) using the recombinant standards provided in each kit. Cells were imaged with the EnSightTM multimode plate reader [34]. Curves were plotted with a sigmoidal concentration-response curve with variable slope. Quantitation of protein levels in cellular assays were interpolated off their respective standard curves.

Statistical analysis. GraphPad was used for graphical presentation. Statistical analyses were performed using a one-way ANOVA study followed by the Bonferroni test for repeated measurements [23,24]. Differences were considered statistically significant when $P < 0.05$.

Results

Mdm2-p53 inhibitors. In previous papers [16,18], EB54 and EB148 (Fig. 1A) have been demonstrated to efficaciously dissociate the Mdm2-p53 complex with comparable IC_{50} values about 10 nM. By the introduction of an isothiocyanate group, the indol-3-ylglyoxyldipeptides compound EB148 has been shown to bind Mdm2 with long-lasting properties and to slow down new complex formation [18]. Herein, the derivatives were tested hereby at a concentration corresponding to 10 or 100 fold their IC_{50} values. When indicated, a higher range of concentrations was chosen for EB54 and EB148. NUT-3 (Fig. 1A), displaying an IC_{50} value of about 100 nM, was used as a reference standard.

Short- and long-lasting Mdm2 ligands exerted differential effects on MSCs proliferation/viability. As a first step, the ability of the compounds to affect MSC proliferation/viability was assessed (Fig. 1B and C). To verify the compounds' toxicity, a 48 h treatment in proliferating medium or 7 days in osteogenic medium were chosen. The latter treatment protocol was chosen considering 7 days as the required time to achieve the first step of MSC *in vitro* differentiation [23,24].

The reference compound NUT-3, tested at 10 µM concentration, significantly decreased MSC proliferation at both 48 h in proliferating medium (Fig. 1B) and 7 days in osteogenic medium (Fig. 1C). Similar results were obtained with high concentrations (i.e, 10 µM and 100 µM) of the reversible analogue EB54 (Fig. 1B and C), as previously reported [17]. These data are consistent with the anti-proliferative effects of p53 reactivators. In contrast, the long-lasting Mdm2 blocker, EB148, did not significantly alter MSC proliferation/viability up to 100 µM concentration (Fig. 1B and C). These results suggest that the differences between the reversible and long-lasting compounds on Mdm2 binding kinetic and time-course of p53 reactivation caused different effects on the viability of MSCs.

Figure 1

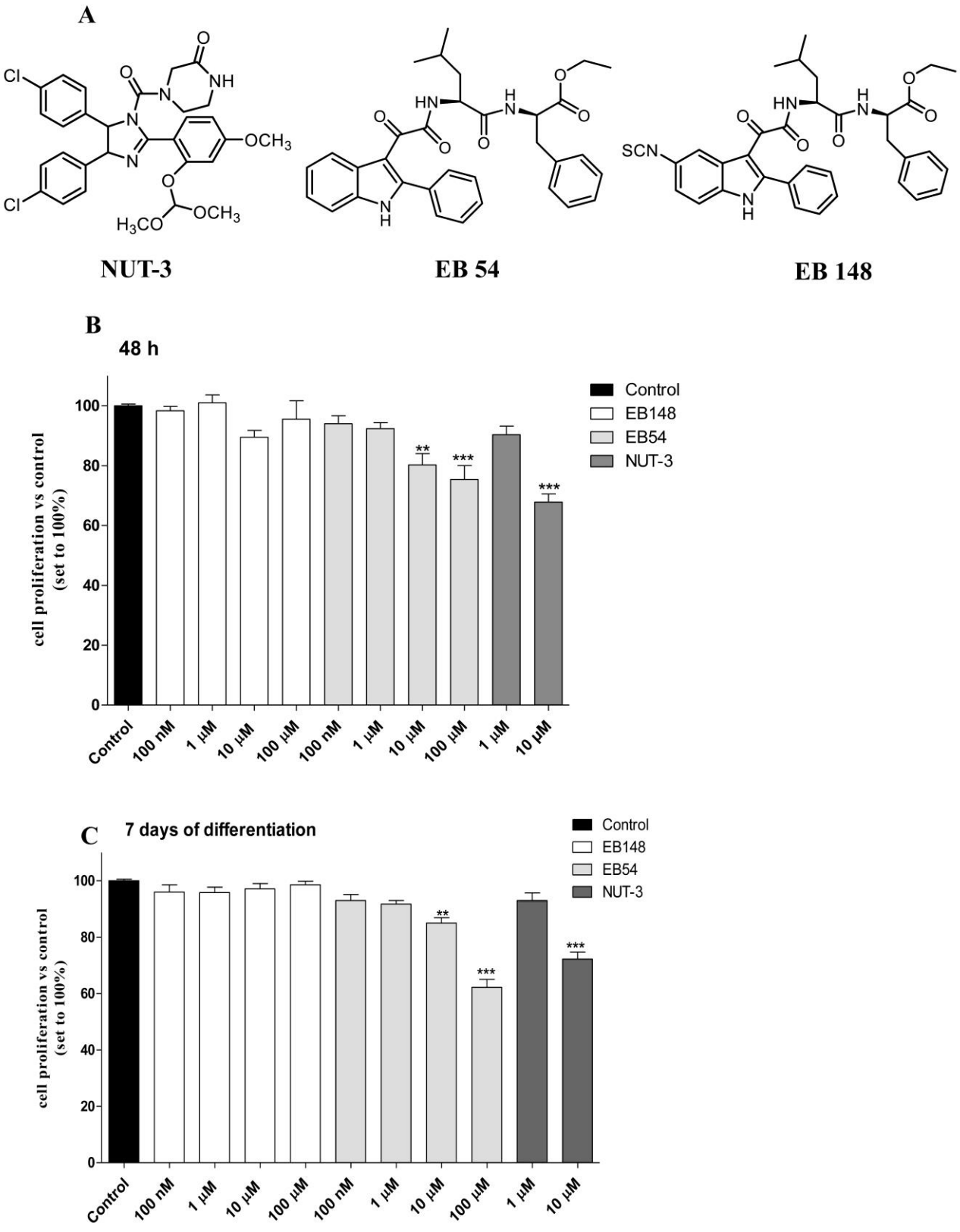


Fig. 1. Short-lasting, but not long-lasting Mdm2 ligands, caused a reduction in proliferation of MSCs. (A) Chemical structures of the tested compounds. (B, C) MSCs were cultured in

proliferation medium for 48 h (B) or in osteogenic medium for 7 days (C), in the absence (control) or in the presence of the indicated concentrations of EB148, EB54 or NUT-3. Following treatments, cell proliferation was estimated as reported in the Methods section. The data were expressed as percentage with respect to untreated control cells (mean values ± SEM, N=3). The significance of the differences was determined with a one-way ANOVA with Bonferroni post-test: ** $p<0.01$, *** $p<0.001$ vs. control.

Short- and long-lasting Mdm2 ligands exerted differential effect on MSC differentiation. Once established the toxic concentrations of Mdm2 ligands, MSCs were cultured in osteogenic medium and challenged with stated compound concentrations for different days, in order to determine if the inhibition of Mdm2-p53 complex may potentiate cellular differentiation. MSC differentiation into osteoblasts was verified by quantitative mineralization assay (Fig. 2A-C), by monitoring the induction of osteogenic gene expression (Fig. 2D), and calcium deposits by Alizarin Red staining (Supplementary Fig. 1).

As depicted in Fig. 2, neither EB54 nor NUT-3 enhanced the differentiation induced by culturing MSCs in osteogenic medium for 7 days (Fig. 2A) and 14 days (Fig. 2B). Similar results were obtained using NUT-3 in differentiating medium following 21 days (Fig. 2C and Supplementary Fig. 1). Surprisingly, EB54 significantly enhanced the percentage of mineralization with respect to control cells in osteogenic medium following 21 days (i.e., Fig. 2C and Supplementary Fig. 1), when cells reached terminal maturation into osteoblasts.

EB148 significantly enhanced cell differentiation in osteogenic medium at 7-day treatment yet (Fig. 2A). The effects elicited by the long-lasting compound (1 μM) were time-dependent up to 14-day treatment and reached a peak at the terminal maturation of MSCs (Fig. 2B). Such temporal differences in potentiating osteogenesis shown by EB148 and EB54 may be ascribed to differences in Mdm2 binding residence time and in the recycling kinetics of the target protein [16,18] that may occur in 21 days of differentiation.

A real time RT-PCR analysis on MSC differentiating markers was assessed to confirm the data obtained by the mineralization assay. As depicted in Fig. 2D, EB148 treatment significantly enhanced the mRNA levels of Runx2, the key transcription factor involved in MSC differentiation [35]. Furthermore, a modest but significant induction in ALP and Osx genes was evidenced (Fig. 2D), thus confirming the pro-differentiating effect of EB148. The lack of induction of osteocalcin mRNA, following EB148 treatment, may be ascribed to the late onset of this gene during osteoblast formation [35].

Of note, a weak induction in ALP and Runx2 mRNA was noticed challenging MSCs with EB54 (Fig. 2D), without any significant effects on Osx and osteocalcin mRNA levels. These data are in accordance with those obtained in the mineralization assay and suggest that EB54 induces only a slow and weak MSC differentiation.

Figure 2

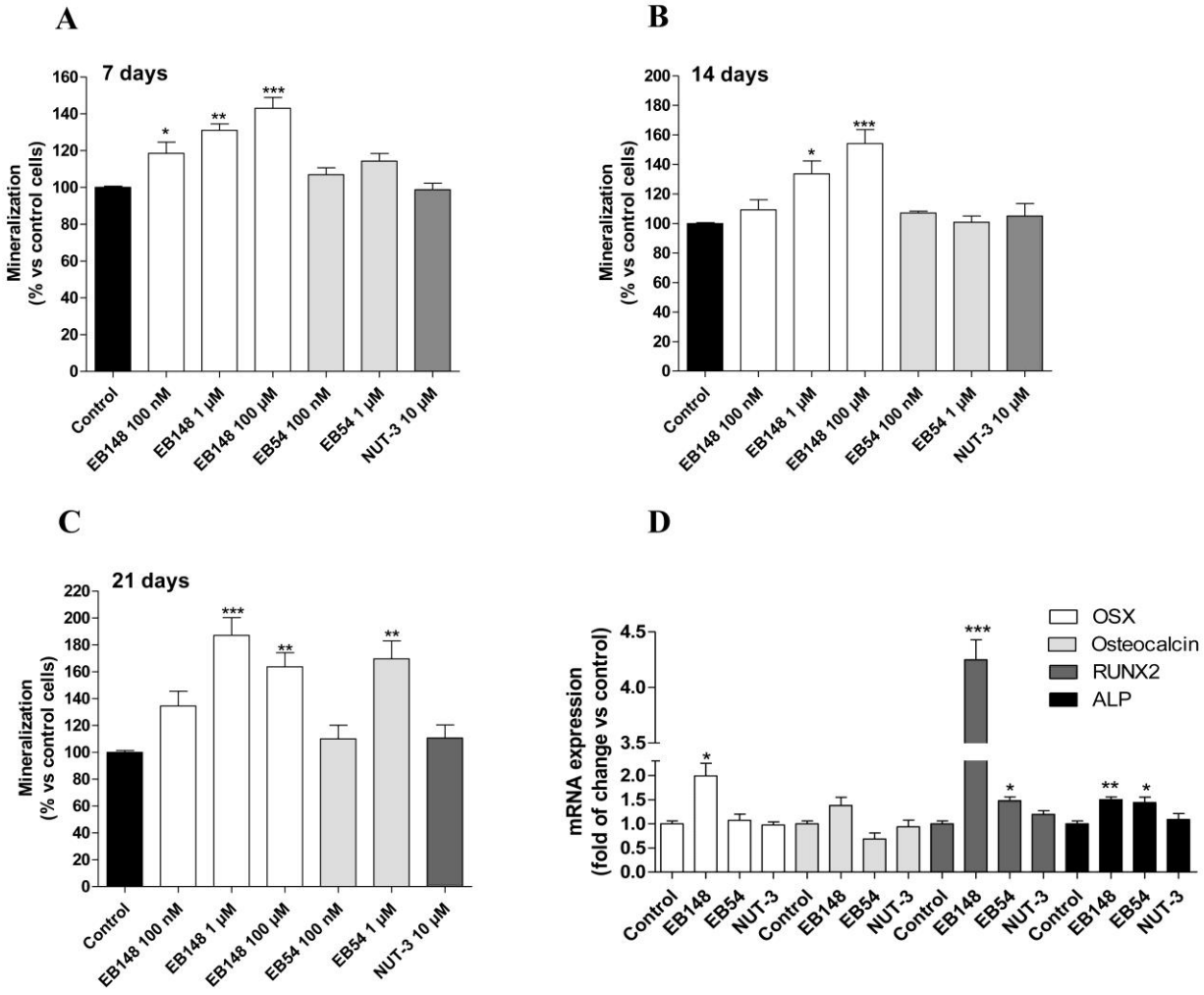


Fig. 2. Long-lasting, but not short-lasting Mdm2 ligands, promoted MSC differentiation into osteoblasts. (A-C) MSCs were cultured in osteogenic medium for 7 (A), 14 (B) or 21 (C) days, in the absence (control) or in the presence of EB148 (100 nM, 1 µM, 100 µM), EB54 (100 nM, 1 µM) or NUT-3 (10 µM). At each time point, calcium deposits were quantified as described in the Methods section. The data were expressed as percentage of MSC mineralization with respect to the untreated cells (mean values ± SEM, N=3). (D) Cells were treated as in (B). mRNA expression levels of the osteogenic markers OSX, osteocalcin, Runx2 and ALP were determined by real time RT-PCR. The data were expressed as fold of changes with respect to basal value (set to 1, mean values ± SEM, N=3). The significance of the differences was determined with a one-way ANOVA with Bonferroni post-test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control.

The long-lasting Mdm2-p53 dissociation promoted MSC differentiation despite p53 reactivation. The osteogenic induction was evaluated upon p53 blockade to understand the extent of p53-dependent effects on EB148-differentiating capability. For this purpose, MSCs were challenged with EB148, EB54, or NUT-3, in the presence of PTF-β, which blocks p53 nuclear activities [20], and osteogenic differentiation was quantified by Alizarin Red staining.

The results (Fig. 3A) showed that blocking p53 "per se" induced a significant enhancement of MSC differentiation, thus confirming that p53 inactivation can augment osteogenic differentiation [7-9,36]. The pro-osteogenic effects elicited by EB54 and EB148 were potentiated in the presence of the p53 inhibitor (Fig. 3A), thus suggesting the pro-differentiating effects are mainly ascribed to Mdm2 following dissociation from p53, rather than a p53 reactivation.

Having confirmed that p53 decreases MSC differentiation into osteoblasts, we investigated the extent of p53 reactivation in response to the long-lasting ligand EB148. In particular, the accumulation of p53 protein and p53-target gene mRNA levels were evaluated upon cell treatment with EB148.

As a first step, a real-time RT-PCR analysis was performed to evaluate the kinetics of p53 transcriptional activity, i.e, the transcription levels of p53 target genes (ie., Mdm2, p53 itself, PUMA and p21) following 2, 7 and 14 days of treatment.

Challenging cells with EB148 for 2 days caused a statistically significant increase in the mRNA levels of p53 target genes (Fig. 3B). In particular, a 3.0- and 4.4- and 7.4-fold induction of Mdm2, PUMA and p21 mRNA, respectively, was observed. Similar results were detected following 7 and 14 days of treatment (Fig. 3B), observing just slightly lower increases with respect to those observed following 2 days of treatment. Of note, PUMA mRNA was not detectable following 14 days of MSC differentiation *in vitro* and was not included in the graph (Fig. 3B).

Notable, a lower but significant induction of Mdm2 transcription was observed upon cell incubation with EB54 (Supplementary Fig. 2). In contrast, NUT-3 did not induce long-term induction of Mdm2 transcription, consistent with previous data [16,26,37]. These results are consistent with an

activation of p53 transcriptional activity upon EB148 treatment and are consistent with our data obtained in glioma cells [18].

Conversely, mRNA level of p53 raised up significantly following 7 days of EB148-treatment only (Fig. 3B). The p53 gene transcription is tightly regulated by feed-back mechanisms involving p53 itself and Mdm2. In this respect, it is not surprising that the p53 and its target genes present different kinetic of gene expression [16,26].

As depicted in Fig. 3C-D, EB148 induced a significant accumulation of p53 protein following 2 days of treatment. A still significant but moderate increase of the protein was detected following 14 days of MSC differentiation (Fig. 3C-D). These results indicate that challenging MSCs with the long-lasting inhibitor of Mdm2-p53 complex determined p53 activation, as demonstrated by the increase of p53 target gene transcription, and accumulation of p53 protein, up to 14 days of treatment.

Overall, the results demonstrate that, upon MSC treatment with EB148, p53 is still activated to some extent, but the p53-independent effects of Mdm2 overcome the p53 dependent effects in our experimental model.

Figure 3

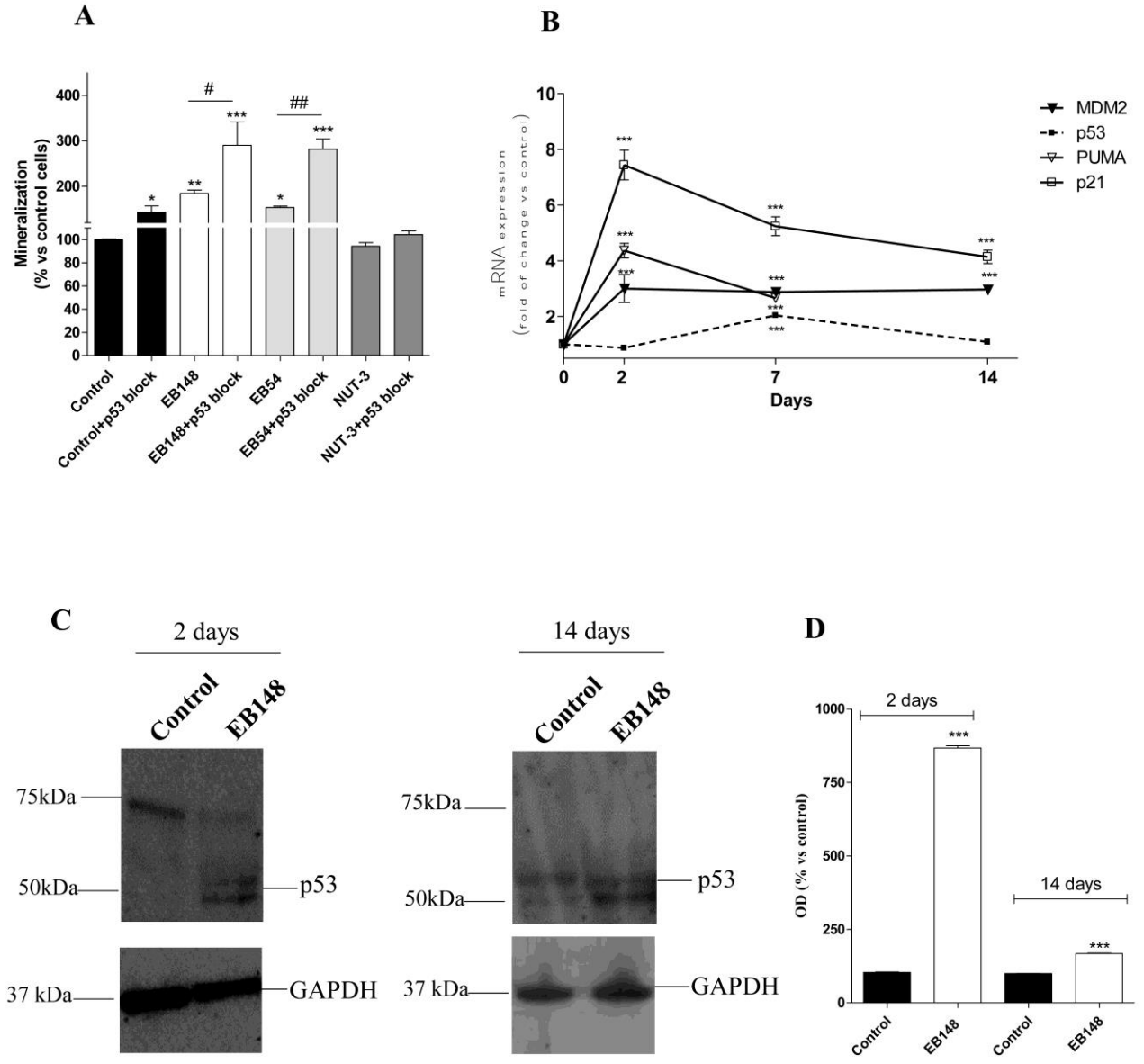


Fig. 3. The long-lasting Mdm2 ligand promoted MSC differentiation despite p53 reactivation. (A) MSCs were cultured in osteogenic medium for 21 days with EB148 (1 µM), EB54 (1 µM) or NUT-3 (1 µM), in the absence or in the presence of the p53 inhibitor PTB-β (1 µM). Following treatments, calcium deposits were quantified as described in the Methods section. The data were expressed as percentage of mineralization with respect to the untreated cells (mean values ± SEM, N=3). (B) MSCs were cultured in proliferation medium for 2 days and in osteogenic medium for 7 days or 14 days, in the absence (control) or in the presence of EB148 (1 µM). mRNA expression levels of Mdm2, p53, PUMA and p21 were determined by real time RT-PCR. The data were expressed as fold of changes with respect to basal value (set to 1, mean values ± SEM, N=3). (C, D)

p53 proteins levels were determined by western blotting assays in MSCs treated for 2 or 14 days with EB148. A representative immunoblot (C) and the relative densitometric analysis (D) were shown. The significance of the differences was determined with a one-way ANOVA with Bonferroni post-test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control; # $p<0.05$, ## $p<0.01$ vs. EB148.

The long-lasting, but not short-lasting Mdm2 ligands induced a Mdm2-dependent degradation of GRK2. Once demonstrated the p53-independent effects of Mdm2 overcome the dependent ones in our experimental model, we investigated the molecular mechanisms at the basis of Mdm2 actions in MSCs. Mdm2 has been demonstrated to act as a E3-ubiquitin ligase on several substrates different from p53 [6,14,15]. In particular, it seems to play a key role in the regulation of stability and activity of isoform 2 of GRK [38], one of the most relevant kinases involved in the phosphorylation and desensitisation of GPCRs [39]. Specifically, Mdm2 association to GRK2 may enhance the kinase ubiquitination, thus promoting its degradation [38].

In MSCs, we demonstrated EB148 induced a significant decrease in GRK2 levels following 7-day treatment (Fig. 4A and B). Lower and not significant effects on GRK2 levels were shown, following EB54 or NUT-3 addition (Fig. 4A and B).

Since several proteins targeted to the proteasome pathway go through poly-ubiquitination [24,33], the potential role of ubiquitination in GRK2 degradation was examined by assessing the kinase association to the Mdm2 ubiquitin ligase [24,33].

EB148 and EB54 induced a significant GRK2 association to Mdm2 following 7-day treatment (Fig. 4C and D); the highest percentage of association was obtained following incubation with the long-lasting Mdm2 inhibitor. Taken together, these data suggest the long-lasting Mdm2 inhibition favoured GRK2 kinase degradation. In contrast, EB54 promoted Mdm2 association to GRK2 without any significant effect on kinase degradation (Fig. 4C and D), thus suggesting that additional mechanisms may occur for this Mdm2 inhibitor. Challenging MSCs with the reference compound, NUT-3, did not modify the levels of Mdm2-GRK2 heterocomplexes.

Nevertheless, a real-time PCR analysis was performed in order to determine if the observed regulation in GRK2 levels could involve, at least in part, a transcriptional mechanism. The results showed that challenging MSCs with EB148 and EB54 significantly reduced the expression of GRK2 mRNA after 7 days of differentiation (Fig. 4E). Based on such data, a late transcriptional mechanism could be hypothesised for EB148 and EB54 (see discussion section). Moreover, in the case of EB148 a post-transcriptional regulation of GRK2 was also assumed, considering the involvement of the proteasome pathway, which has been identified as a major mechanism for modulating GRK2 expression levels [24].

Figure 4

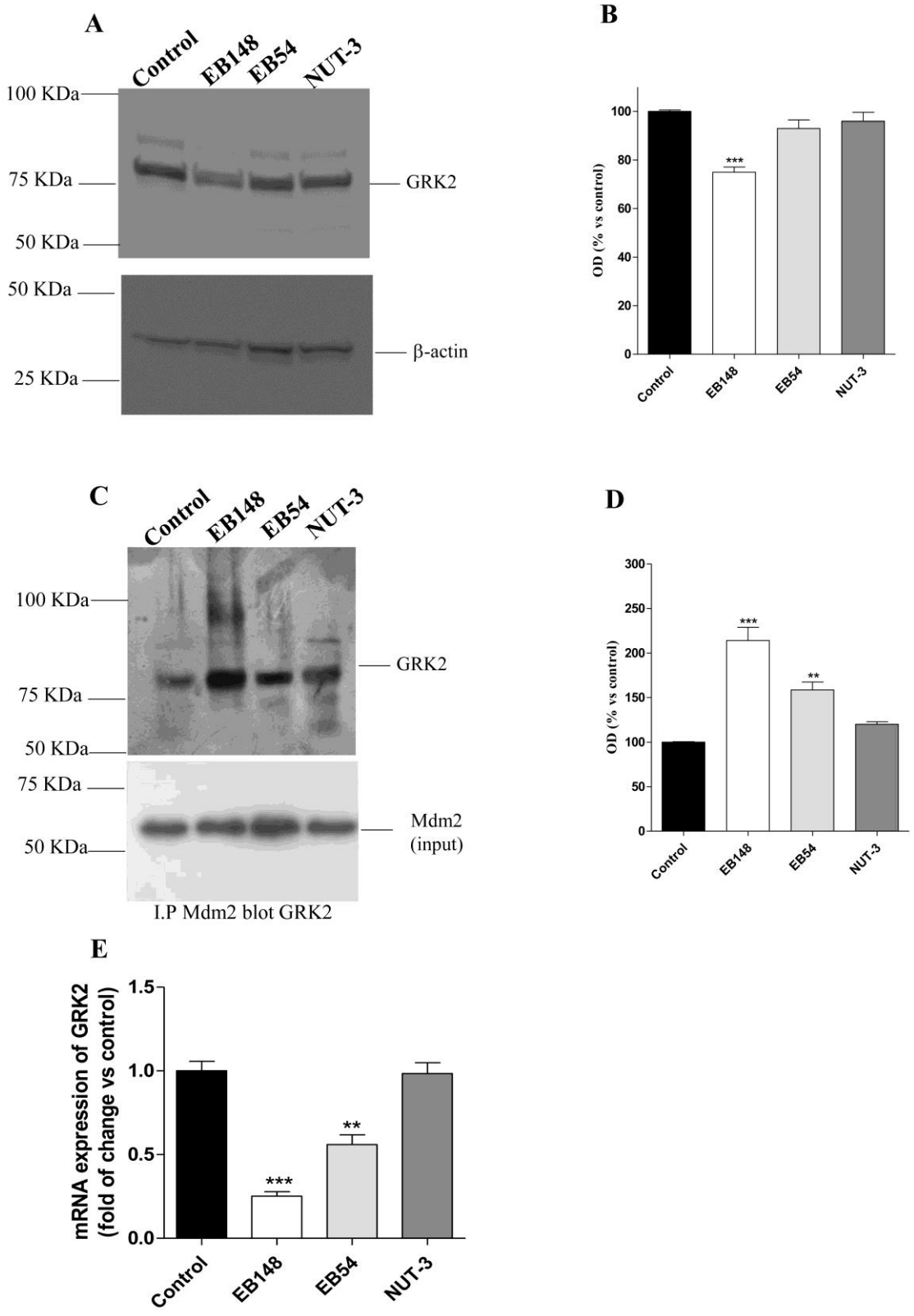


Fig. 4. Long-lasting, but not short-lasting Mdm2 ligands, regulated GRK2 turnover. (A-E) MSCs were cultured in osteogenic medium for 7 days, in the absence (control) or in the presence of EB148 (1 µM), EB54 (1 µM) or NUT-3 (1 µM). (A, B) GRK2 levels and (C, D) Mmd2 association

to GRK2 were determined by co-immunoprecipitation/western blotting assays. An immunoblot for each condition (A, C) and the relative densitometric analyses (B, D) were shown. (E) mRNA expression levels of GRK2 were determined by real-time PCR. The data were expressed as fold of changes with respect to basal value (set to 1, mean values ± SEM, N=3). The significance of the differences was determined with a one-way ANOVA with Bonferroni post-test: ** $p<0.01$, *** $p<0.001$ vs. control.

The Long-lasting Mdm2 ligand regulated the functionality of A_{2B} adenosine receptors. GRK proteins play a crucial role in regulating the functional responses of a plethora of GPCRs. Among these membrane receptors, the A_{2B}AR for purine nucleosides have been recently demonstrated to be involved in osteogenic processes [23]. Considering the decrease in GRK2 levels mediated by EB148, we investigated the effects of the different modulators of Mdm2-p53 axis on mRNA expression of A_{2B}AR. Consistent with this hypothesis and with our previous data [24], challenging MSCs with EB148 or EB54 led to an enhancement in the transcriptional levels of A_{2B}AR (Fig. 5A), strengthening the hypothesis that the modulation of Mdm2-p53 axis put forwards the osteogenic process [23,24,40,41].

Western blot analysis was used to confirm the observed data at a post-transcriptional level. The A_{2B}AR antibody recognized a specific immunoreactive band at 45 kDa (Fig. 5B and C). A_{2B}AR expression decreased along with MSC differentiation into osteoblasts (Fig. 5B and C), consistent with previous reports [41]. EB148 did not induce a significant enhancement of A_{2B}AR protein levels in MSCs treated for 2 or 14 days (Fig. 5B and C). These data demonstrate that the long-lasting inhibitor did not affect A_{2B}AR protein expression despite its regulation on the A_{2B}AR mRNA levels.

Nevertheless, when MSCs were challenged with EB148, a significant decrease of A_{2B}AR association to GRK2 was evidenced (Fig. 5D and E). Globally, these data demonstrate that the long-lasting inhibitor caused a decrease of GRK2 levels (Fig. 4C and D) and a reduction of its association rate to A_{2B}AR.

Considering the pivotal role of GRK2 association to a GPCR in determining its functionality, functional experiments were performed to disclose if EB148 can modify A_{2B}AR responsiveness.

In cAMP assays, A_{2B}AR was confirmed to undergo rapid desensitization upon pre-challenging with its selective agonist BAY606583 (Fig. 5F), as reported previously [23,24]. EB148 was demonstrated to prevent partially the loss of responsiveness induced by the GPCR agonist, especially at the shorter time of pre-incubation (Fig. 5F). In accordance, the desensitization rate in the presence of EB148 was significantly reduced ($t_{1/2}$ –EB148: 3.67±0.68 min^{-1}; $t_{1/2}$ +EB148: 7.68±0.91 min^{-1}). All in all, these data demonstrate that the long-lasting Mdm2-p53 inhibitor was able to slow-down the kinetics of A_{2B}AR desensitization, and are consistent with the decrease in GRK2 levels and A_{2B}AR-GRK2 association.

Figure 5

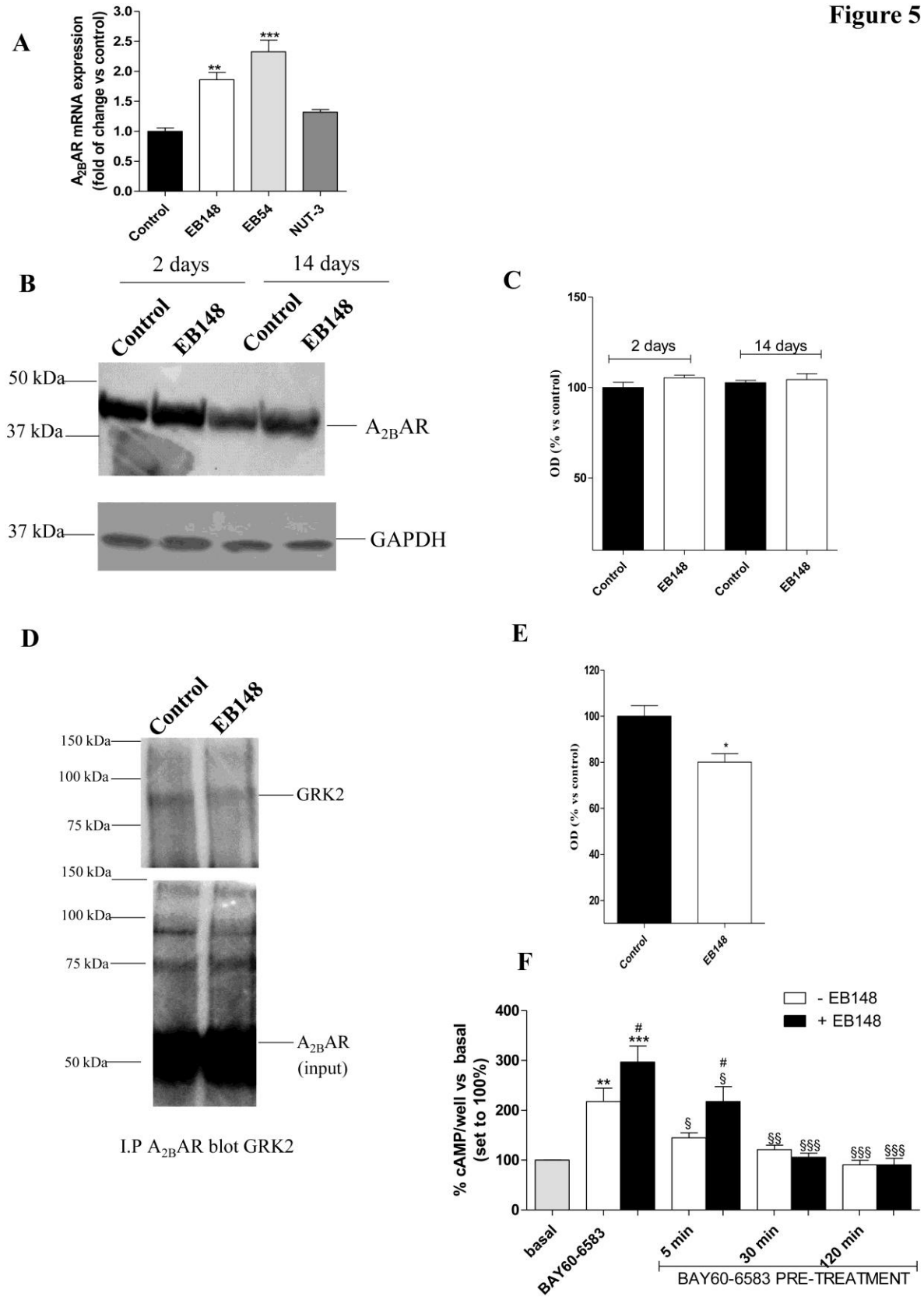


Fig. 5. The long-lasting Mdm2 ligand regulated the functionality of A_{2B} adenosine receptors. (A-C) MSCs were cultured in osteogenic medium for 7 days, in the absence (control) or in the presence of EB148 (1 µM), EB54 (1 µM) or NUT-3 (1 µM). mRNA expression levels of A_{2B}AR

were determined by real-time RT-PCR. The data were expressed as fold of changes with respect to basal value (set to 1, mean values ± SEM, N=3). (B, C) MSCs were cultured in the absence (control) or in the presence of EB148 (1 μM) for the indicated times. A_{2B}AR levels were determined by western blotting assay. The immunoblot (B) and the relative densitometric analyses (C) were shown. (D, E) MSCs were treated as in (B). A_{2B}AR association to GRK2 was determined by co-immunoprecipitation assays, as described in the Methods section. The immunoblot (D) and the relative densitometric analyses (E) were shown. (F) MSCs were cultured in proliferation medium in the absence or presence of 10 μM EB148 for 2 h. At each time point, MSCs were incubated with 10 nM BAY60-6583 for different times (5 to 120 min). After extensive washing, cells were treated with 10 nM BAY60-6583. Intracellular cAMP levels were evaluated as reported in the Methods section. The data were expressed as cAMP percentages with respect to the level for untreated cells (basal), which was set to 100% (mean values ± SEM; n = 3). The significance of the differences was determined with a one-way ANOVA with Bonferroni post-test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control; # $p<0.05$, vs. EB148; § $p<0.05$, §§ $p<0.01$, §§§ $p<0.001$ vs. respective BAY60-6583 10 nM.

The long-lasting Mdm2 ligands increased cAMP accumulation and an adenylyl cyclase-dependent MSC differentiation. The intracellular pathways putatively linked to MSC differentiation upon the dissociation of Mdm2-p53 complex were analysed. Among these signals, the cAMP accumulation in MSCs was investigated as the major intracellular mediator that is linked to GPCR and to GRK activities. As depicted in Fig. 6A, the cAMP levels of unstimulated MSCs (i.e., control cells), grown in osteogenic medium, gradually raised up during the process of cellular differentiation, reaching a peak following 14 days in culture. Following this peak, the basal cAMP levels partially decreased in the final part of the differentiation process (i.e., 21 days). These data are consistent with current literature [42] demonstrating that duration, rather than the strength, of the signal, is involved in the differentiating properties of cAMP [43].

Neither EB54 nor NUT-3 modified the kinetic pattern of cAMP accumulation during MSC differentiation in culture (Fig. 6A). Interestingly, EB148 potentiated cAMP accumulation with respect to control cells, up to 14-day stimulation (Fig. 6A). Such effect was not noticed following

21 days in culture, suggesting that the long-lasting Mdm2 inhibitor facilitates cAMP accumulation until such time as it has to remain switched-on during MSC differentiation process.

To reveal the contribute of cAMP signalling in MSC fate, osteoblast differentiation was assessed in the presence of EB148 upon AC inhibition by SQ22536. AC inhibition induced "per se" a significant decrease in osteogenic differentiation (Supplementary Fig. 3). Consistent with our data, activation of cAMP signalling alters the lineage commitment of MSCs, favoring osteogenesis [44]. Challenging cells with EB148 upon AC inhibition significantly decreased the compound-elicited effects on MSC differentiation, demonstrating the involvement of cAMP.

The long-lasting Mdm2 ligands caused a sustained activation of ERK and CREB. The kinetic pattern of ERK activation can regulate the final biological outcomes of these kinases. In particular, a transient kinase activation is commonly associated with a proliferative effect, whereas their sustained phosphorylation over time triggers differentiation signalling pathways [45-47]. In this respect, the kinetics of ERK activation in response to the Mdm2 modulators were evaluated in MSCs. EB148 induced a significant enhancement of phosphorylated/total ERK ratio following 30 min of treatment (Fig. 6B). This signal remained switched-on up to 7 days after cell treatment (Fig. 6B). In contrast, NUT-3 did not induce a significant ERK activation (Fig. 6B). Of note, in the case of EB54, a change in the p-/total ERK ratio was noticed only after 7 days of incubation (Fig. 6B).

Consistent with the data observed for ERK kinetics of activation, the ratio of phosphorylated/total CREB was significantly higher than control cells upon MSCs incubation with EB148 only (Supplementary Fig. 4A). CREB activation observed in the presence of EB148 was significantly reduced upon ERK inhibition with PD184352 (Supplementary Fig. 4B), suggesting that CREB activation is a cAMP-ERK dependent mechanism.

All together, these data suggest that the cAMP-ERK-CREB axis might play a role in the effects of the long-lasting Mdm2 inhibitor.

The long-lasting Mdm2 ligand promoted an ERK-dependent MSC differentiation. Since ERK signalling has been shown to interact with and to control Mdm2 ubiquitin ligase activity [17,48,49], the association between ERKs and Mdm2 in response to cell treatment with the different Mdm2 ligands was investigated. The results showed a significant enhancement in the ERK-Mdm2 association upon MSC incubation with EB148 for 7 days (Fig. 6C). No significant variation of ERK-Mdm2 complexes was noticed in the presence of EB54 or NUT-3. These data are consistent with those obtained in ERK phosphorylation assay (Fig. 6B), and suggest that the prolonged ERK phosphorylation, induced by EB148, can further regulate Mdm2 stability/activity.

To disclose if ERK activation may be independent to the increase in cAMP levels, immunoenzymatic assays were repeated upon AC inhibition. The results showed that EB148-elicited ERK phosphorylation was significantly and completely reduced in the presence of the AC inhibitor SQ22536 (Fig. 6D). These data suggest that cAMP signalling is mainly involved in the compound's ability to activate ERKs. Similar results were obtained when cells were challenged with EB54 (Fig. 6D), despite the compound showed a weaker capability to phosphorylate ERKs.

Surprisingly, PTF-β, which blocks p53 transcriptional activity, partially prevented ERK activation following 7 days of cellular treatment with the Mdm2 ligands EB54 and EB148 (Fig. 6D). These data suggest that ERK activation is only partially a p53-independent mechanism.

Furthermore, to investigate if a direct connection between ERK and MSC differentiation can exist, osteoblast differentiation was assessed in the presence of Mdm2 ligands upon ERK inhibition by PD184352. ERK inhibition induced "per se" a significant decrease in osteogenic differentiation, confirming that ERK signalling is required for MSC driving to osteoblasts [50-52]. Challenging cells with EB148 in the presence of the ERK inhibitor significantly but not completely decreased the compound-elicited effects on MSC differentiation (Fig. 6E). Similar results were observed when cells were treated with EB54 (Fig. 6E), suggesting that the basal ERK signalling supports EB54-mediated effects.

Figure 6

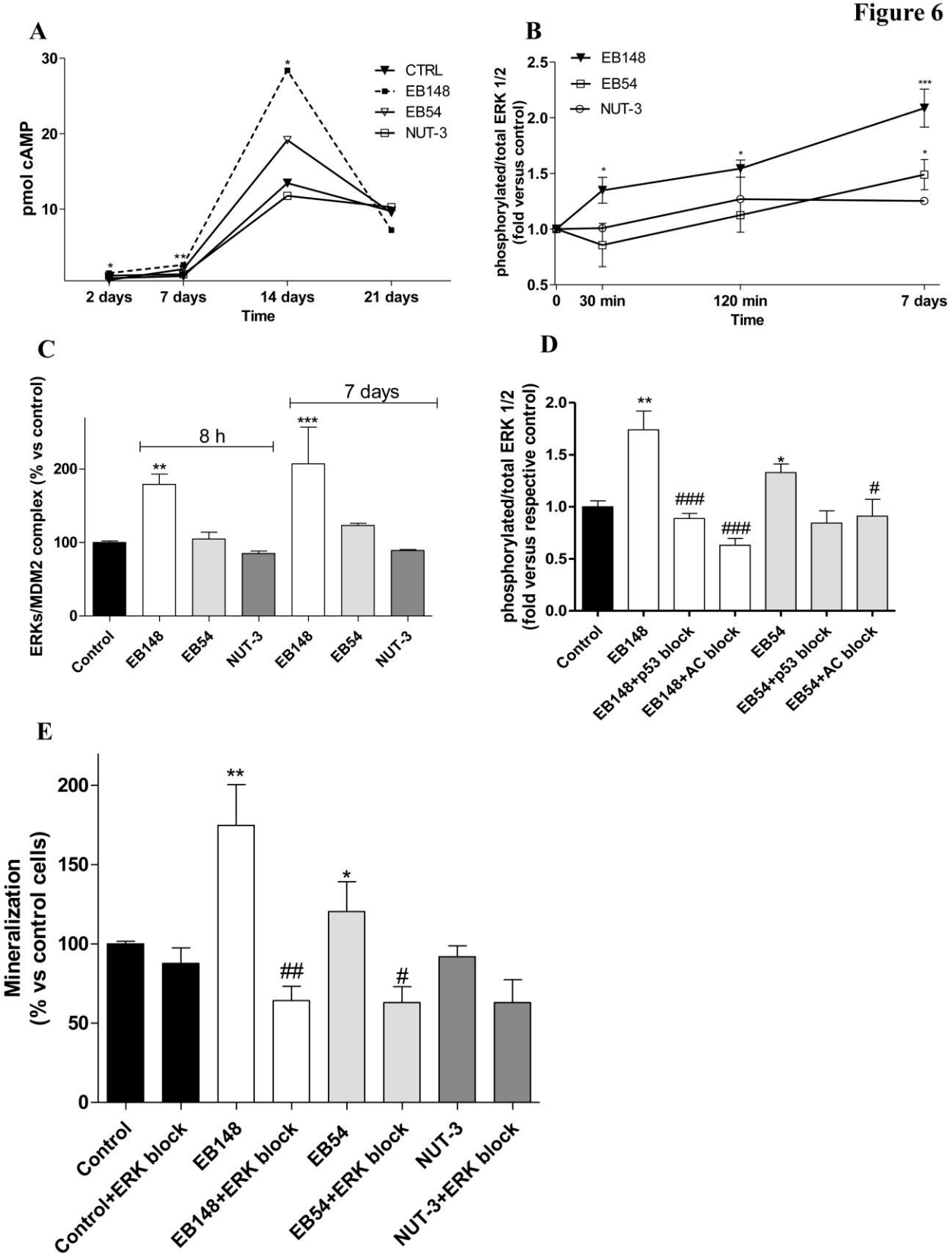


Fig. 6. The long-lasting Mdm2 ligand promoted a sustained ERK activation and an ERK-dependent MSC differentiation. (A) MSCs were cultured for the indicated times in the absence

(control) or in the presence of EB148 (1 μM), EB54 (1 μM) or NUT-3 (10 μM). At each time point, cells were lysed, and the cAMP levels were quantified as reported in the Method section. The data were expressed as pmol/well (mean values ± SEM, N=3). (B) MSCs were treated with EB148 (1 μM), EB54 (1 μM) or NUT-3 (1 μM) for 30 min, 120 min or 7 days and the levels of total and phosphorylated ERK1/2 were determined by immunoenzymatic assays. The data are expressed as ratio between phosphorylated and total protein levels versus that of untreated (control) cells (mean values ± SEM, N=3). (C) MSCs were challenged as in (A) for 8h or 7 days. Following treatments, the cells were collected and suspended in lysis buffer. Equal amounts of cell lysates were captured on wells pre-coated with Mdm2 antibody. After extensive washing, the levels of the ERK-Mdm2 complex were quantified using an antibody specific for ERKs, as described in the Method section. The data are expressed as percentage of control set to 100% (mean ± SEM, N=3). (D) MSCs were treated with EB148 (1 μM) or EB54 (1 μM) for 7 days in the absence or in the presence of the p53 inhibitor, PFT-β (1 μM), or the AC inhibitor, SQ22536 (1 μM). Following treatment, phosphorylated and total ERK were measured as in (B). (E) MSCs were cultured for 21 days in osteogenic medium with EB148 (1 μM), EB54 (1 μM) or NUT-3 (1 μM) in the absence or in the presence of ERK inhibitor, PD184352 (1 μM). Following treatments, calcium deposits were quantified as described in the Methods section. The data were expressed as percentage of MSC mineralization with respect to the untreated cells (mean values ± SEM, N=3). The significance of the differences was determined with a one-way ANOVA with Bonferroni post-test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ vs. EB148.

Discussion

In the present study, we demonstrated that the regulation of Mdm2-p53 axis in MSCs by the means of a long-lasting inhibition is an unexplored and effective strategy to direct these cells towards a differentiation program. Long-lasting dissociation of Mdm2-p53 complex caused a significant increase in Mdm2-mediated degradation of GRK2 protein. As a consequence, the drop in the levels of this kinase in an active form reduces the physiological desensitization of Gs-coupled receptors, such as A_{2B}ARs, and increases intracellular signalling molecules, such as cAMP, ERK and CREB, so prompting up the MSC differentiation to an osteoblastic phenotype (Fig. 7). Globally, our data shed light on the intracellular pathways related to Mdm2-mediated osteogenesis in human MSCs. Moreover, these results suggest that the long-lasting inhibition of Mdm2-p53 complex increased the interaction with other intracellular proteins that in turn regulated the final biological outcome of MSCs.

Mdm2 has been characterized as the key negative regulator of p53; actually, recent studies have shown a p53-independent role of Mdm2 in the ubiquitination and regulation of several proteins that contribute to main aspects of cell proliferation, apoptosis, and differentiation [6,12-15]. In addition, Mdm2 has been demonstrated to play distinct biological effects depending on the tissue and cell type that is examined [13,53].

The role of Mdm2-p53 axis in MSC differentiation has been extensively investigated both *in vitro* and in animal models. In particular, p53 signalling pathways are known to negatively regulate bone formation and MSCs lineage commitment [2,7-9,12,54-56]. On the other hand, Mdm2 knocking out in osteoblasts blocked murine bone formation, indicating that Mdm2 modulates p53 during *in vivo* bone development [11,12]. Moreover, in a recent study, the stress-responsive gene Bre has been shown to negatively affect p53 protein stability by promoting Mdm2-mediated ubiquitination, thus suggesting it as a novel p53 modulator during osteogenesis [2].

Herein, by the use of different disruptors of the Mdm2-p53 complex, we investigated the effects of Mdm2 modulation on MSC differentiation into osteoblasts, focusing in particular on the Mdm2-

regulated modulation of intracellular signalling pathways involved in MSC differentiation, such as ERK, cAMP and MAPK. Specifically, we compared reversible and long-lasting disruptors of Mdm2-p53 complex, which have been demonstrated in human glioblastoma cells to induce peculiar kinetic pattern in the regulation of p53 target genes, Mdm2 protein, and ERK signalling [18].

As first step, MSC proliferation/viability was examined following incubation with the Mdm2 ligands. The standard Mdm2 ligand, NUT-3, tested at 10 μM, significantly decreased MSC viability, consistent with the data reported for the drug in $CD34^{+}CD38^{-}$ cells [48], which are similar to hematopoietic stem progenitors. Similar data were obtained with the reversible Mdm2 ligand, EB54. In contrast, the long-lasting Mdm2 ligand, EB148, did not significantly alter MSC proliferation rate. These results suggest that the differences exerted by the reversible and long-lasting compounds, on Mdm2 binding kinetics and possibly on the time-course of p53 reactivation, caused different effects on the viability of these staminal non-cancer cells. In this respect, we showed that the tested compounds differently modulated the p53 transcriptional activity in human MSCs, as well as in glioblastoma cells [16,22,32]. Consistently, bi-phasic kinetics for nuclear accumulation of p53 following ionizing radiation has been evidenced in embryonic stem cells [49] in which the p53 target genes, i.e. Mdm2, p21 and PUMA, are transcribed in the first wave of nuclear accumulation [49].

When MSCs were grown in osteogenic medium, a significant enhancement of osteogenesis was evidenced in the case of the long-lasting inhibitor EB148. A slightly increase in mineralization and in the transcription of osteogenic genes were evidenced for its analogue EB54, but not for the standard drug NUT-3.

The Mdm2 ligands, and in particular the long-lasting one, were demonstrated to reactivate effectively p53 transcriptional activity. Nevertheless, upon MSC treatment with EB148, the p53-independent effects of Mdm2 overcame the p53-dependent effects promoting osteogenesis. Actually, the induction of the osteogenic process elicited by EB148 was enhanced significantly upon blockage of p53 nuclear activity.

According to our evidences, Mdm2 has been shown to play a key role in MSC differentiation into adipocytes [10] or osteoblasts [11-13]. According to this line of evidences, p53 has been reported to negatively regulate the differentiation of MSCs by downregulating the expression levels of key transcription factor genes involved in the early phases of osteogenesis, such as Osx and Runx2 [59]. Moreover, Bre, a stress-responsive protein interacting with p53, enhances Mdm2-mediated ubiquitination of p53 protein, promoting bone formation [2].

Based on these evidences, we focused our attention on the putative mechanisms that could be linked to Mdm2 actions in MSCs. In particular, looking for Mdm2-substrates different from p53 [14-16], the attention was focused on the isoform 2 of GRK [38], one of the most relevant kinases involved in the phosphorylation and desensitisation of GPCRs [39].

The long-lasting Mdm2 ligand only induced a significant decrease in GRK2 levels; consistent with literature data [38], such effect was ascribed to an enhancement of Mdm2 association to GRK2 that promote the kinase ubiquitination and degradation. Furthermore, a decrease in GRK2 transcriptional levels was evidenced, too. In contrast, GRK2 levels were not modified in the presence of EB54 or NUT-3, proposing that the binding kinetics influence the cytoplasmic Mdm2 availability for substrates' ubiquitination.

The Mdm2-mediated decrease of GRK2 levels has been linked to an enhancement of GPCR availability/functionality, including β–adrenergic receptors [60]. Consistent with this hypothesis, challenging MSCs with EB148 decreased GRK2 association to A_{2B}AR, one of the main GPCR involved in dictating MSC fate [23,24,40,41]. Despite an enhancement in the transcriptional levels of A_{2B}AR elicited by EB148, no significant changes in the receptor protein expression was evidenced. Nevertheless, the rate of GRK2-A_{2B}AR association diminished upon EB148 incubation, and the compound decreased significantly the desensitisation rate of the GPCR, finally increasing A_{2B}AR functionality.

Likewise, GRK2 has been shown to interact with a variety of signalling proteins and to have 'effector' functions beyond receptor desensitization [60,61], which would constitute interesting deal to think about in future studies.

Besides the nature of GPCR involved in such regulation, cAMP accumulation in MSCs was investigated as the major intracellular mediator linked to GPCR and to GRK activities. The cAMP levels of unstimulated MSCs (i.e., control cells), grown in osteogenic medium, gradually raised up during the process of cellular differentiation, reaching a peak following 14 days in culture decreasing in the final part of the differentiation process (i.e., 21 days). Our data support the hypothesis that duration, rather than the strength, of the signal, is involved in the differentiating properties of cAMP [43]. Interestingly, EB148 potentiated cAMP accumulation with respect to control cells, up to 14 days of stimulation, but not following 21 days in culture. These data suggest that the long-lasting Mdm2 inhibitor facilitates cAMP accumulation until such time as it has to remain switched-on during MSC differentiation process. Similar results have been demonstrated for inductors of the cAMP pathway or for positive A_{2B}AR allosteric modulators [23,44], and confirm that cAMP signalling is crucial to prime lineage commitment of MSC towards osteoblast phenotype, despite it is not involved in the terminal phase of osteoblast maturation.

Moreover, consistent with the hypothesis that activation of cAMP signalling alters the lineage commitment of MSCs, promoting osteogenesis [44], the block of AC was found to induce "per se" a significant decrease in osteogenic differentiation. cAMP was demonstrated to be crucial in determining EB148-elicited enhancement of MSC differentiation, proving that EB148 effects can be ascribed, at least partially, to an indirect involvement of the cAMP pathway.

As Mdm2-mediated regulation of GRK2 has been suggested to influence the GPCR-mediated MAPK cascade [62,63], ERK activation was monitored in MSCs upon addition of the different Mdm2 ligands. Consistent with the data obtained on the regulation of GRK2 levels, the long-lasting modulator induced a sustained activation of ERKs. Accordingly, a transient kinase activation is

commonly associated with a proliferative effect, whereas ERK sustained phosphorylation over time triggers differentiation signalling pathways [45-47].

Co-immunoprecipitation assays demonstrated that ERK regulation was ascribed to a direct interaction between ERKs and Mdm2, as reported previously [18,48,49]. Consistently, this association was increased in the presence of EB148. Nevertheless, a direct effect of MAPK on GRK2 turnover can-not be excluded, as previously suggested [64].

The MAPK activation elicited by EB148 was demonstrated to be linked to cAMP signalling, and, surprisingly, to be partially dependent from p53. The latter contribution may be ascribed to p53-dependent Mdm2 transcription that may enhance AC activation.

Supporting the key role of ERKs in osteogenesis [50-52], basal MSC differentiation was found to decrease significantly upon ERK blockade and challenging cells with EB148 in the presence of the ERK inhibitor significantly decreased the compound-elicited effects on MSC differentiation.

Finally, EB148 induced an ERK-dependent CREB phosphorylation, confirming that the CREB pathway is involved in osteogenesis [65].

Figure 7

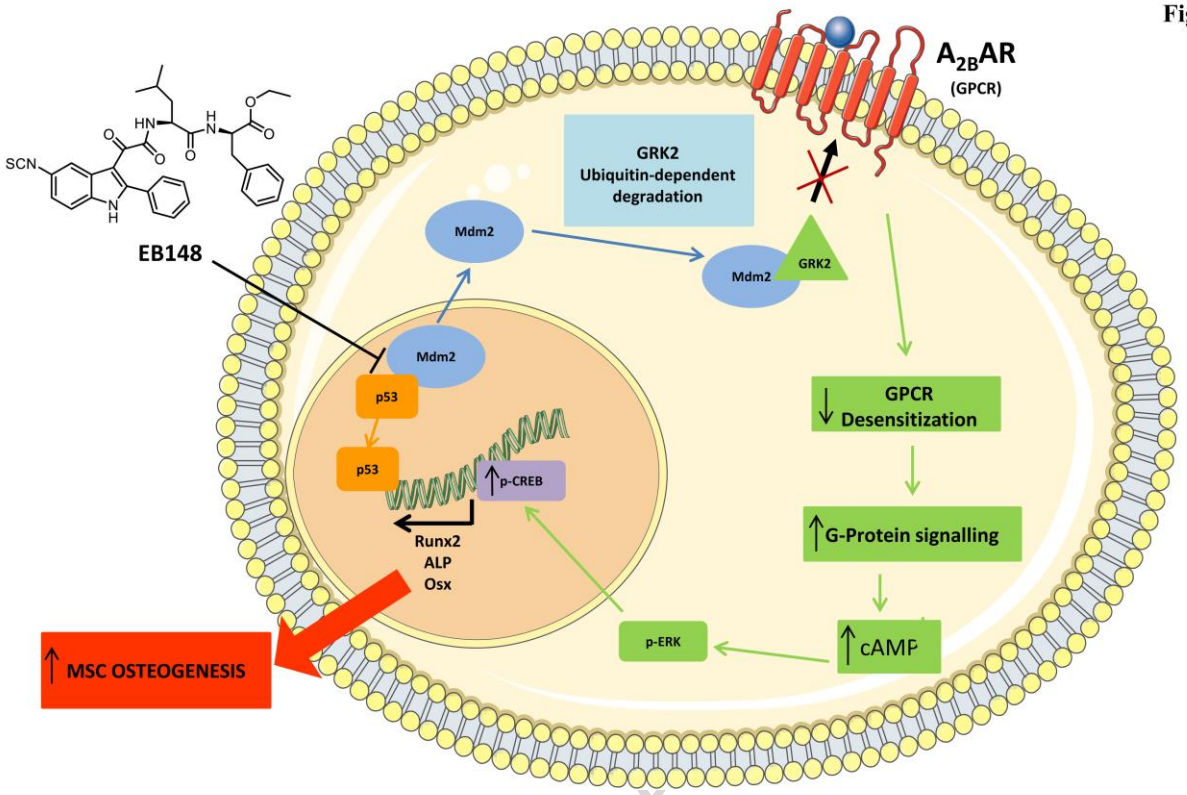


Fig. 7. A cartoon illustrating the signalling pathways involved in MSC differentiation into osteoblasts upon long-lasting dissociation of p53-Mdm2 complex. EB148 mediated a long-lasting releasing of Mdm2 from its complex with p53 and increased Mdm2 binding to GRK2, favouring in turn a Mdm2-degradation of the kinase. The decrease in GRK2 levels caused a decrease in GRK2 association to A_{2B} Adenosine Receptor, a GPCR involved in dictating MSC fate. These events mediated a reduction in GPCR desensitization and an increase in G protein-related signals, including cAMP accumulation and a sustained activation of ERK and CREB. At a cellular level, the long-lasting Mdm2-p53 dissociation promoted the transcription of the "osteogenic master regulators" Runx2, Osx and ALP, causing in turn an enhancement in MSC differentiation into osteoblasts. Dashed lines indicate putative mechanisms.

Conclusions

In this paper, it was demonstrated that the long-lasting dissociation of Mdm2 from p53 potentiated MSC differentiation into osteoblasts, with the involvement of the following intracellular mechanisms (Fig. 7): i) reduction of the intracellular concentration of GRK2; ii) reduction of GRK2 association to A_{2B}AR and increase in the GPCR functionality; iii) enhancement of cAMP

accumulation, probably due to a major availability of Gs coupled receptors; iv) sustained activation of ERKs and CREB. Globally, our data shed light on the intracellular pathways related to Mdm2-mediated osteogenesis in human MSCs.

Of note, the experiments showed that, despite a reactivation of p53 functionality, the pro-osteogenic effects of the long-lasting ligand were mainly ascribed to Mdm2 actions, independent from p53. Thus, these results highlight the importance of the target residence time in the recruitment of signalling proteins dictating the final outcome of MSCs.

Conflict of interest

The authors declare no conflict of interest.

Funding information

This work was supported by the Italian Ministry of Education (Project of National Research Interest PRIN 2015, 2015E8EMCM_007).

Authors' contributions

SD, MLT, LM, EN and CM were involved with the conception, design, and interpretation of data. SD, CG, DP, RP performed the experiments. EB, VLP, ST, FDS synthetized compounds and were involved with data analysis. CM and LM acquired funding. CM, LM, FDS, EN and MLT provided general overall supervision of the study. All authors contributed to the drafting and critical revision of the manuscript and gave final approval of the version to be published.

References

[1] L.J. Raggatt, N.C. Partridge, Cellular and molecular mechanisms of bone remodelling, J Biol Chem 285 (2010) 25103-8. doi: 10.1074/jbc.R109.041087.

[2] F. Jin, Y. Wang, X. Wang, Y. Wu, X. Wang, Q. Liu, Y. Zhu, E. Liu, J. Fan, Y. Wang, Bre Enhances Osteoblastic Differentiation by Promoting the Mdm2-Mediated Degradation of p53, Stem Cells 35(2017)1760-1772. doi: 10.1002/stem.2620.

[3] N.A. Sims, T.J. Martin, Coupling the activities of bone formation and resorption: a multitude of signals within the basic multicellular unit, Bonekey Rep 3 (2014) 481. doi:10.1038/bonekey.2013.215.

[4] G. Karsenty, H.M. Kronenberg, C. Settembre, Genetic control of bone formation, Annu Rev Cell Dev Biol, 25 (2009) 629-48. doi: 10.1146/annurev.cellbio.042308.113308.

[5] K.M. Sinha, X. Zhou, Genetic and molecular control of osterix in skeletal formation, J Cell Biochem 114 (2013) 975-84. doi: 10.1002/jcb.24439.

[6] M. Wienken, A. Dickmanns, A. Nemajerova, D. Kramer, Z. Najafova, M. Weiss, O. Karpiuk, M. Kassem, Y. Zhang, G. Lozano, S.A. Johnsen, U.M. Moll, X. Zhang, M. Dobbelstein, MDM2 Associates with Polycomb Repressor Complex 2 and Enhances Stemness-Promoting Chromatin Modifications Independent of p53, Mol Cell 61 (2016) 68-83. doi: 10.1016/j.molcel.2015.12.008.

[7] M. Tataria, N. Quarto, M.T. Longaker, K.G. Sylvester, Absence of the p53 tumor suppressor gene promotes osteogenesis in mesenchymal stem cells, J Pediatr Surg 41 (2006) 624-32. doi: 10.1016/j.jpedsurg.2005.12.001.

[8] A. Molchadsky, I. Shats, N. Goldfinger, M. Pevsner-Fischer, M. Olson, A. Rinon, E. Tzahor, G. Lozano, D. Zipori, R. Sarig, V. Rotter, p53 plays a role in mesenchymal differentiation programs, in a cell fate dependent manner, PLoS One 3 (2008) e3707. doi: 10.1371/journal.pone.0003707.

[9] Y. He, L.F. de Castro, M.H. Shin, W. Dubois, H.H. Yang, S. Jiang, P.J. Mishra, L. Ren, H. Gou, A. Lal, C. Khanna, G. Merlino, M. Lee, P.G. Robey, J. Huang, p53 loss increases the osteogenic differentiation of bone marrow stromal cells, Stem Cells 33 (2015) 1304-19. doi: 10.1002/stem.1925.

[10] P. Hallenborg, S. Feddersen, S. Francoz, I. Murano, U. Sundekilde, R.K. Petersen, V. Akimov, M.V. Olson, G. Lozano, S. Cinti, B.T. Gjertsen, L. Madsen, J.C. Marine, B. Blagoev, K. Kristiansen, Mdm2 controls CREB-dependent transactivation and initiation of adipocyte differentiation, Cell Death Differ 19 (2012) 1381-9. doi: 10.1038/cdd.2012.15.

[11] H. Chen, K. Kolman, N. Lanciloti, M. Nerney, E. Hays, C. Robson, N. Chandar, p53 and MDM2 are involved in the regulation of osteocalcin gene expression, Exp Cell Res 318 (2012) 867-76. doi: 10.1016/j.yexcr.2012.02.022.

[12] C.J. Lengner, H.A. Steinman, J. Gagnon, T.W. Smith, J.E. Henderson, B.E. Kream, G.S. Stein, J.B. Lian, S.N. Jones, Osteoblast differentiation and skeletal development are regulated by Mdm2-p53 signaling, J Cell Biol 172 (2006) 909-21. doi: 10.1083/jcb.200508130.

[13] H. Chen, G. Reed, J. Guardia, S. Lakhan, O. Couture, E. Hays, N. Chandar, Vitamin D directly regulates Mdm2 gene expression in osteoblasts, Biochem Biophys Res Commun 430 (2013) 370-4. doi: 10.1016/j.bbrc.2012.11.003.

[14] S. Bohlman, J.J. Manfredi, p53-independent effects of Mdm2, Subcell Biochem 85(2014) 235-46. doi: 10.1007/978-94-017-9211-0_13.

[15] J.C. Marine, G. Lozano, Mdm2-mediated ubiquitylation: p53 and beyond Cell Death Differ 17 (2010) 93-102. doi: 10.1038/cdd.2009.68.

[16] S. Daniele, S. Taliani, E. Da Pozzo, C. Giacomelli, B. Costa, M.L. Trincavelli, L. Rossi, V. La Pietra, E. Barresi, A. Carotenuto, A. Limatola, A. Lamberti, L. Marinelli, E. Novellino, F. Da Settimo, C. Martini, Apoptosis therapy in cancer: the first single-molecule co-activating p53 and the translocator protein in glioblastoma, Sci Rep 4 (2014) 4749. doi: 10.1038/srep04749.

[17] S. Daniele, V. La Pietra, E. Barresi, S. Di Maro, E. Da Pozzo, M. Robello, C. La Motta, S. Cosconati, S. Taliani, L. Marinelli, E. Novellino, C. Martini, F. Da Settimo, Lead Optimization of 2-Phenylindolylglyoxylyldipeptide Murine Double Minute(MDM)2/Translocator Protein (TSPO) Dual Inhibitors for the Treatment of Gliomas, J Med Chem 59 (2016) 4526-38. doi: 10.1021/acs.jmedchem.5b01767.

[18] S. Daniele, E. Barresi, E. Zappelli, L. Marinelli, E. Novellino, F. Da Settimo, S. Taliani, M.L. Trincavelli, C. Martini, Long lasting MDM2/Translocator protein modulator: a new strategy for irreversible apoptosis of human glioblastoma cells, Oncotarget 7 (2016) 7866-84. doi: 10.18632/oncotarget.6872.

[19] P. Secchiero, R. Bosco, C. Celeghini, G. Zauli, Recent advances in the therapeutic perspectives of Nutlin-3, Curr Pharm Des, 17 (2011) 569-77. doi: 10.2174/138161211795222586.

[20] E. Da Pozzo, V. La Pietra, B. Cosimelli, F. Da Settimo, C. Giacomelli, L. Marinelli, C. Martini, E. Novellino, S. Taliani, G. Greco, p53 functional inhibitors behaving like pifithrin-β counteract the Alzheimer

peptide non-β-amyloid component effects in human SH-SY5Y cells, ACS Chem Neurosci, 5 (2014) 390-399. doi: 10.1021/cn4002208.

[21] M.S. Squires, P.M. Nixon, S.J. Cook, Cell-cycle arrest by PD184352 requires inhibition of extracellular signal-regulated kinases (ERK) 1/2 but not ERK5/BMK1, Biochem J, 366(Pt 2) (2002) 673-80. doi: 10.1042/BJ20020372

[22] H.J. Kim, Y.R. Cho, S.H. Kim, D.W. Seo, TIMP-2-derived 18-mer peptide inhibits endothelial cell proliferation and migration through cAMP/PKA-dependent mechanism, Cancer Lett, 343(2) (2014) 210-6. doi:10.1016/j.canlet.2013.10.037.

[23] M.L. Trincavelli, S. Daniele, C. Giacomelli, S. Taliani, F. Da Settimo, B. Cosimelli, G. Greco, E. Novellino, C. Martini, Osteoblast differentiation and survival: A role for A2B adenosine receptor allosteric modulators, Biochim Biophys Acta 1843 (2014) 2957-66. doi: 10.1016/j.bbamcr.2014.09.013.

[24] S. Daniele, L. Natali, C. Giacomelli, P. Campiglia, E. Novellino, C. Martini, M.L. Trincavelli, Osteogenesis Is Improved by Low Tumor Necrosis Factor Alpha Concentration through the Modulation of Gs-Coupled Receptor Signals, Mol Cell Biol 37 (2017) e00442-16. doi: 10.1128/MCB.00442-16.

[25] B. Gharibi, A.A. Abraham, J. Ham, B.A. Evans, Adenosine receptor subtype expression and activation influence the differentiation of mesenchymal stem cells to osteoblasts and adipocytes, J Bone Miner Res 26 (2011) 2112-24. doi: 10.1002/jbmr.424.

[26] B. Costa, S. Bendinelli, P. Gabelloni, E. Da Pozzo, S. Daniele, F. Scatena, R. Vanacore, P. Campiglia, A. Bertamino, I. Gomez-Monterrey, D. Sorriento, C. Del Giudice, G. Iaccarino, E. Novellino, C. Martini, Human glioblastoma multiforme: p53 reactivation by a novel MDM2 inhibitor, PLoS One 8 (2013) e72281. doi: 10.1371/journal.pone.0072281.

[27] M. Fumagalli, E. Bonfanti, S. Daniele, E. Zappelli, D. Lecca, C. Martini, M.L. Trincavelli, M.P. Abbracchio, The ubiquitin ligase Mdm2 controls oligodendrocyte maturation by intertwining mTOR with G protein-coupled receptor kinase 2 in the regulation of GPR17 receptor desensitization, Glia 63 (2015) 2327-39. doi: 10.1002/glia.22896.

[28] M.L. Trincavelli, S. Daniele, E. Orlandini, G. Navarro, V. Casadó, C. Giacomelli, S. Nencetti, E. Nuti, M. Macchia, H. Huebner, P. Gmeiner, A. Rossello, C. Lluís, C. Martini, A new D_2 dopamine receptor agonist allosterically modulates A(2A) adenosine receptor signalling by interacting with the A(2A)/D_2 receptor heteromer, Cell Signal 24 (2012) 951-60. doi: 10.1016/j.cellsig.2011.12.018.

[29] S. Daniele, E. Da Pozzo, E. Zappelli, C. Martini, Trazodone treatment protects neuronal-like cells from inflammatory insult by inhibiting NF-κB, p38 and JNK, Cell Signal 27 (2015) 1609-29. doi: 10.1016/j.cellsig.2015.04.006.

[30] S. Daniele, D. Lecca, M.L. Trincavelli, O. Ciampi, M.P. Abbracchio, C. Martini, Regulation of PC12 cell survival and differentiation by the new P2Y-like receptor GPR17, Cell Signal 22 (2010) 697-706. doi: 10.1016/j.cellsig.2009.12.006.

[31] F. Merlino, S. Daniele, V. La Pietra, S. Di Maro, F.S. Di Leva, D. Brancaccio, S. Tomassi, S. Giuntini, L. Cerofolini, M. Fragai, C. Luchinat, F. Reichart, C. Cavallini, B. Costa, R. Piccarducci, S. Taliani, F. Da Settimo, C. Martini, H. Kessler, E. Novellino, L. Marinelli, Simultaneous Targeting of RGD-Integrins and Dual Murine Double Minute Proteins in Glioblastoma Multiforme, J Med Chem 61 (2018) 4791-4809. doi: 10.1021/acs.jmedchem.8b00004.

[32] M. Giustiniano, S. Daniele, S. Pelliccia, V. La Pietra, D. Pietrobono, D. Brancaccio, S. Cosconati, A. Messere, S. Giuntini, L. Cerofolini, M. Fragai, C. Luchinat, S. Taliani, G. La Regina, F. Da Settimo, R. Silvestri, C. Martini, E. Novellino, L. Marinelli, Computer-Aided Identification and Lead Optimization of Dual Murine Double Minute 2 and 4 Binders: Structure-Activity Relationship Studies and Pharmacological Activity, J Med Chem 60 (2017) 8115-8130. doi: 10.1021/acs.jmedchem.7b00912.

[33] S. Daniele, M.L. Trincavelli, M. Fumagalli, E. Zappelli, D. Lecca, E. Bonfanti, P. Campiglia, M.P. Abbracchio, C. Martini, Does GRK-β arrestin machinery work as a "switch on" for GPR17-mediated activation of intracellular signaling pathways?, Cell Signal 26 (2014) 1310-25. doi: 10.1016/j.cellsig.2014.02.016.

[34] C. Giacomelli, S. Daniele, C. Romei, L. Tavanti, T. Neri, I. Piano, A. Celi, C. Martini, M.L. Trincavelli, The A(2B) Adenosine Receptor Modulates the Epithelial-Mesenchymal Transition through the Balance of cAMP/PKA and MAPK/ERK Pathway Activation in Human Epithelial Lung Cells, Front Pharmacol, 9 (2018) 54. doi: 10.3389/fphar.2018.00054.

[35] J. Xu, Z. Li, Y. Hou, W. Fang, Potential mechanisms underlying the Runx2 induced osteogenesis of bone marrow mesenchymal stem cells, Am J Transl Res 7 (2015) 2527-35.

[36] L. Bezu, A. Sauvat, J. Humeau, L.C. Gomes-da-Silva, K. Iribarren, S. Forveille, P. Garcia, L. Zhao, P. Liu, L. Zitvogel, L. Senovilla, O. Kepp, G. Kroemer, eIF2α phosphorylation is pathognomonic for immunogenic cell death, Cell Death Differ, 25 (2018) 1375-1393. doi: 10.1038/s41418-017-0044-9.

[37] S. Daniele, B. Costa, E. Zappelli, E. Da Pozzo, S. Sestito, G. Nesi, P. Campiglia, L. Marinelli, E. Novellino, S. Rapposelli, C. Martini, Combined inhibition of AKT/mTOR and MDM2 enhances Glioblastoma Multiforme cell apoptosis and differentiation of cancer stem cells, Sci Rep 5 (2015) 9956. doi: 10.1038/srep09956.

[38] A. Salcedo, F. Jr. Mayor, P. Penela, Mdm2 is involved in the ubiquitination and degradation of G-protein-coupled receptor kinase 2, EMBO J 25 (2006) 4752-62. doi: 10.1038/sj.emboj.7601351.

[39] C. Ribas, P. Penela, C. Murga, A. Salcedo, C. García-Hoz, M. Jurado-Pueyo, I. Aymerich, F. Jr. Mayor, The G protein-coupled receptor kinase (GRK) interactome: role of GRKs in GPCR regulation and signaling, Biochim Biophys Acta 1768 (2007) 913-22. doi: 10.1016/j.bbamem.2006.09.019

[40] C. Corciulo, T. Wilder, B.N. Cronstein, Adenosine A2B receptors play an important role in bone homeostasis, Purinergic Signal 12 (2016) 537-47. doi: 10.1007/s11302-016-9519-2.

[41] S.H. Carroll, N.A. Wigner, N. Kulkarni, H. Johnston-Cox, L.C. Gerstenfeld, K. Ravid, A2B adenosine receptor promotes mesenchymal stem cell differentiation to osteoblasts and bone formation in vivo, J Biol Chem 287 (2012) 15718-27. doi: 10.1074/jbc.M112.344994.

[42] R. Siddappa, J. Doorn, J. Liu, E. Langerwerf, R. Arends, C. van Blitterswijk, J. de Boer, Timing, rather than the concentration of cyclic AMP, correlates to osteogenic differentiation of human mesenchymal stem cells, J Tissue Eng Regen Med 4 (2010) 356-65. doi: 10.1002/term.246.

[43] R. Siddappa, A. Martens, J. Doorn, A. Leusink, C. Olivo, R. Licht, L. van Rijn, C. Gaspar, R. Fodde, F. Janssen, C. van Blitterswijk, J. de Boer, cAMP/PKA pathway activation in human mesenchymal stem cells in vitro results in robust bone formation in vivo, Proc Natl Acad Sci U S A 105 (2008) 7281-6. doi: 10.1073/pnas.0711190105.

[44] R. Kao, W. Lu, A. Louie, R. Nissenson, Cyclic AMP signaling in bone marrow stromal cells has reciprocal effects on the ability of mesenchymal stem cells to differentiate into mature osteoblasts versus mature adipocytes, Endocrine 42 (2012) 622-36. doi: 10.1007/s12020-012-9717-9.

[45] W. Kolch, M. Calder, D. Gilbert, When kinases meet mathematics: the systems biology of MAPK signaling, FEBS Lett. 579 (2005) 1891-5. doi: 10.1016/j.febslet.2005.02.002

[46] A. Herrero, B. Casar, P. Colón-Bolea, L. Agudo-Ibáñez, P. Crespo, Defined spatiotemporal features of RAS-ERK signals dictate cell fate in MCF-7 mammary epithelial cells, Mol Biol Cell 27 (2016) 1958-68. doi: 10.1091/mbc.E15-02-0118.

[47] S. Traverse, N. Gomez, H. Paterson, C. Marshall, P. Cohen, Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor, Biochem J 288 (1992) 351-5. doi: 10.1042/bj2880351.

[48] M. Phelps, A. Phillips, M. Darley, J.P. Blaydes, MEK-ERK signaling controls Hdm2 oncoprotein expression by regulating hdm2 mRNA export to the cytoplasm, J Biol Chem 280 (2005) 16651-8. doi: 10.1074/jbc.M412334200.

[49] A. Phillips, C.J. Jones, J.P. Blaydes, The mechanisms of regulation of Hdm2 protein level by serum growth factors, FEBS Lett. 580 (2006) 300-4. doi: 10.1016/j.febslet.2005.12.026.

[50] R.M. Salasznyk, R.F. Klees, M.K. Hughlock, G.E. Plopper, ERK signaling pathways regulate the osteogenic differentiation of human mesenchymal stem cells on collagen I and vitronectin, Cell Commun Adhes, 11 (2004) 137-53. doi: 10.1080/15419060500242836.

[51] C. Ge, G. Xiao, D. Jiang, R.T. Franceschi, Critical role of the extracellular signal-regulated kinase-MAPK pathway in osteoblast differentiation and skeletal development, J Cell Biol, 176 (2007) 709-18. doi: 10.1083/jcb.200610046.

[52] C.F. Lai, L. Chaudhary, A. Fausto, L.R. Halstead, D.S. Ory, L.V. Avioli, S.L. Cheng, Erk is essential for growth, differentiation, integrin expression, and cell function in human osteoblastic cells, J Biol Chem, 276 (2001) 14443-50. doi: 10.1074/jbc.M010021200.

[53] J.D. Grier, S. Xiong, A.C. Elizondo-Fraire, J.M. Parant, G. Lozano, Tissue-specific differences of p53 inhibition by Mdm2 and Mdm4, Mol Cell Biol 26 (2006) 192-8. doi: 10.1128/MCB.26.1.192-198.2006.

[54] H. Liu, B. Li, p53 control of bone remodelling, J Cell Biochem 111 (2010) 529-34. doi: 10.1002/jcb.22749.

[55] X. Wang, H.Y. Kua, Y. Hu, K. Guo, Q. Zeng, Q. Wu, H.H. Ng, G. Karsenty, B. de Crombrugghe, J. Yeh, B. Li, p53 functions as a negative regulator of osteoblastogenesis, osteoblast-dependent osteoclastogenesis, and bone remodelling, J Cell Biol 172 (2006) 115-25. doi: 10.1083/jcb.200507106.

[56] W. Liu, M. Qi, A. Konermann, L. Zhang, F. Jin, Y. Jin, The p53/miR-17/Smurf1 pathway mediates skeletal deformities in an age-related model via inhibiting the function of mesenchymal stem cells, Aging (Albany NY) 7 (2015) 205-18. doi: 10.18632/aging.100728.

[57] B.Z. Carter, P.Y. Mak, D.H. Mak, V.R. Ruvolo, W. Schober, T. McQueen, J. Cortes, H.M. Kantarjian, R.E. Champlin, M. Konopleva, M. Andreeff, Synergistic effects of p53 activation via MDM2 inhibition in combination with inhibition of Bcl-2 or Bcr-Abl in CD34+ proliferating and quiescent chronic myeloid leukemia blast crisis cells, Oncotarget 6 (2015) 30487-99. doi: 10.18632/oncotarget.5890.

[58] V. Solozobova, A. Rolletschek, C. Blattner, Nuclear accumulation and activation of p53 in embryonic stem cells after DNA damage, BMC Cell Biol 10 (2009) 46. doi: 10.1186/1471-2121-10-46.

[59] T. Velletri, N. Xie, Y. Wang, Y. Huang, Q. Yang, X. Chen, Q. Chen, P. Shou, Y. Gan, G. Cao, G. Melino, Y. Shi, P53 functional abnormality in mesenchymal stem cells promotes osteosarcoma development, Cell Death Dis. 7 (2016) e2015. doi: 10.1038/cddis.2015.367.

[60] P. Penela, A. Ruiz-Gómez, J.G. Castaño, F. Jr. Mayor, Degradation of the G protein-coupled receptor kinase 2 by the proteasome pathway, J Biol Chem 273 (1998) 35238-44. doi: 10.1074/jbc.273.52.35238.

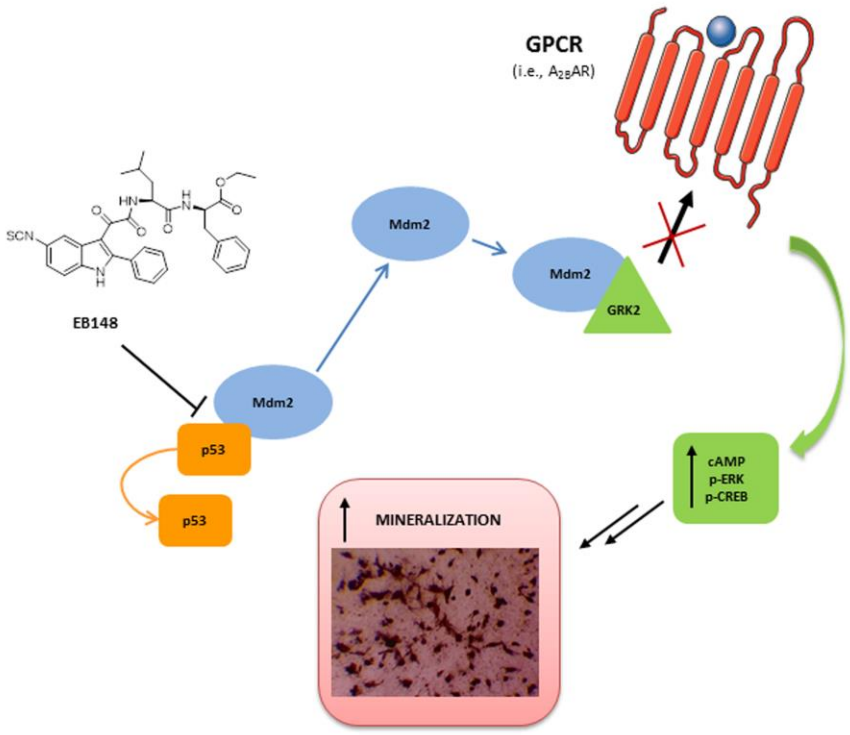
[61] P. Penela, C. Murga, C. Ribas, V. Lafarga, F. Jr. Mayor, The complex G protein-coupled receptor kinase 2 (GRK2) interactome unveils new physiopathological targets, Br J Pharmacol 160 (2010) 821-32. doi: 10.1111/j.1476-5381.2010.00727.x.

[62] S.K. Shenoy, P.H. McDonald, T.A. Kohout, R.J. Lefkowitz, Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin, Science 294 (2001) 1307-13. doi: 10.1126/science.1063866.

[63] Y. Daaka, L.M. Luttrell, S. Ahn, G.J. Della Rocca, S.S. Ferguson, M.G. Caron, R.J. Lefkowitz, Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase, J Biol Chem 273 (1998) 685-8. doi: 10.1074/jbc.273.2.685.

[64] A. Elorza, P. Penela, S. Sarnago, F. Jr. Mayor, MAPK-dependent degradation of G protein-coupled receptor kinase 2, J Biol Chem 278 (2003) 29164-73. doi: 10.1074/jbc.M304314200.

[65] H. Zhang, L. Li, Q. Dong, Y. Wang, Q. Feng, X. Ou, P. Zhou, T. He, J. Luo Activation of PKA/CREB Signaling is Involved in BMP9-Induced Osteogenic Differentiation of Mesenchymal Stem Cells, Cell Physiol Biochem 37 (2015) 548-62. doi: 10.1159/000430376.



Graphical abstract

Highlights:

- Long-lasting dissociation of Mdm2-p53 induces mesenchymal stem cell differentiation
- Long-lasting dissociation of Mdm2-p53 decreases GRK2 levels
- Long-lasting dissociation of Mdm2-p53 induced sustained ERK and CREB activation