Anti-oncogenic and pro-myogenic action of the MKK6/p38/AKT axis induced by targeting MEK/ERK in embryonal rhabdomyosarcoma

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Received February 24, 2022; Accepted June 16, 2022

DOI: 10.3892/or.2022.8363

Abstract. Insights into the molecular and cellular biology of embryonal rhabdomyosarcoma (ERMS), an aggressive paediatric tumour, are required in order to identify new targets for novel treatments that may benefit patients with this disease. The present study examined the functional effects of MKK3 and MKK6, two upstream kinases of p38, and found that the ectopic expression of MKK6 led to rapid p38 activation and the myogenic differentiation of ERMS cells, whereas MKK3 failed to induce differentiation, while maintaining the proliferation state. Myogenin and myosin heavy chain were induced in MKK6-overexpressing ERMS cells and were inhibited by the p38 inhibitor, SB203580. The expression of Myc and ERK-PO4 increased under the effect of SB203580, whereas it decreased in MKK6-overexpressing cells. AKT activation was part of the myogenic program triggered by MKK6 overexpression alone. To the best of our knowledge, the present study demonstrates, for the first time, that the endogenous MKK6 pathway may be recovered by MEK/ERK inhibition (U0126 and trametinib) and that it concomitantly induces the reversal of the oncogenic pattern and the induction of the myogenic differentiation of ERMS cell lines. The effects of MEK/ERK inhibitors markedly increase the potential clinical applications in ERMS, particularly on account of the MEK inhibitor-induced early MKK6/p38 axis activation and of their anti-oncogenic effects. The findings presented herein lend further support to the antitumour effects of MKK6; MKK6 may thus represent a novel target for advanced personalised treatments against ERMS.

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Key words: embryonal rhabdomyosarcoma, MEK/ERK inhibitors, MKK6, p38, Myc

Introduction

Rhabdomyosarcoma (RMS) is an extremely aggressive paediatric tumour that can occur in any part of the body. The embryonal (ERMS) and alveolar (ARMS) histological subtypes of RMS are differentiated on the basis of distinct genetic alterations that may play a role in the pathogenesis of these tumours (1,2). The most common alterations are represented by p53 and K-Ras or N-Ras mutations (3-5). Ras is a small GTP-binding protein that lies upstream of several signalling pathways, including Raf/MEKs/ERKs and PI3K/AKT, both of which play the role of mediators in cell survival and proliferation (5-8). The abnormal expression of Ras leads to the upregulation of these pathways and, consequently, to tumorigenesis (9). The constitutive activation of ERKs, which results from Ras mutations in RMS cells, leads to the reduced capacity of myodifferentiation due to the inhibition of p38 signalling (10), which is known to regulate the expression of specific skeletal muscle genes (11,12). Indeed, MEK/ERK inhibition in RD cells has been shown to induce the p38-dependent rescue of the myogenic program that is also related to a marked c-Myc downregulation (13,14). The deregulated expression

of Myc family proteins (c-, L-, N-) has been found to play a pivotal role in the transformation from the normal phenotype to the malignant phenotype. Indeed, Myc family members are aberrantly activated in a wide range of human haematological malignancies and solid tumours (15). In particular, the c-Myc transcription factor controls numerous cellular functions, including cell cycle progression, cell growth, genomic instability, angiogenesis and apoptosis (16), and exerts a positive and negative effect on differentiation (17,18), thereby proving to be at a crossroads of several signalling pathways.

The functional interaction between Ras and Myc has long been known to enhance the accumulation of transcriptionally active Myc (19). Since MEK/ERK inhibition in ERMS plays an anti-oncogenic and pro-myogenic role (14,20) and MEK/ERK inhibitors affect p38 MAPK (13), the present study aimed to investigate whether p38 reverses the transformed phenotype by inducing Myc downregulation in the ERMS cell system. The upstream activators of p38 are MKK6 and the closely related MKK3 (21). It is noteworthy that all p38 MAPKs are common substrates of MKK6 and MKK3 kinases. However, p38 activation by one or more MKK kinases is dependent on the type of stimulus, specific cell type and strength of the stimulus (22). Notably, both MKK3 and MKK6 play a dual role by either promoting or suppressing cancer (23).

Given the potential involvement of MKK3 and MKK6 in myogenic fate, the ambiguity of their specific roles, the balance between MKK3 and MKK6, as well as the interplay with mutant p53 in directing the final biological outcome, the present study aimed to investigate the effects of MKK6 and MKK3 kinases in the anti-oncogenic function and myogenic differentiation induced by MEK/ERK inhibition in ERMS cellular models. Herein, it is demonstrated that MEK/ERK inhibitors concomitantly decrease Myc expression and induce myogenic differentiation through MKK6/p38/AKT pathways. The specific AKT1 isoform is essential for initiation of differentiation and myoblast mobility, while AKT2 has been proven to be essential for myotube maturation (24). Of note, it has been demonstrated that during myogenesis, the p38 pathway activation involves the concurrent activation of AKT. For all these reasons, the present study investigated whether AKT activation is induced during the pathological myogenesis of ERMS-derived cells and examined its association with p38 activation (25). More importantly, the present study demonstrates that MEK/ERK inhibitors restore MKK6/p38 and AKT pathway activation, which is a novel finding in RMS tumours and, as it occurs in normal myogenesis and in later stages of myotubes development, AKT is activated (24). Lastly, these results are in accordance with previous findings on the mechanisms leading to myogenic differentiation in embryonic development and in adult muscle (26).

Materials and methods

Cell culture and treatments. The ERMS RD (cat. no. CCL-136™; ATCC) cell line was tested and authenticated by ATCC for the expression of myoglobin and myosin ATPase cellular products. The ERMS TE671 (cat. no. HTL97021) cell line was obtained from the Interlab Cell Line Collection in 2006. The TE671 cells, hereafter indicated as TE cells, were tested and authenticated by the Interlab Cell Line Collection for the expression of nicotinic acetylcholine receptor, acetylcholine receptor and peripheral type benzodiazepine receptor. The cells were cultured in Dulbeccos' modified Eagles' medium (DMEM), supplemented with glutamine, gentamicin (Gibco; Thermo Fisher Scientific, Inc.) and 10% heat-inactivated foetal bovine serum (HyClone; Cytiva). All cell lines were maintained at 37˚C in 5% CO_2.

At 1 day after plating, the cells were treated with 5 μM SB203580 (cat. no. S8307; MilliporeSigma) for 1 day, 10 μM U0126 MEK inhibitor for 3 h, overnight (O/N), 1 day or 3 days (cat. no. V1121; Promega Corporation) or 10 nM trametinib for 3 h, overnight (O/N) or 3 days (cat. no. SC-364639; Santa Cruz Biotechnology, Inc.).

Plasmid transfection. Cells were seeded at 1,5x10^6 cells/well in 6-well plates. At 1 day after plating, the RD or TE cells were transfected with 4 μg/well of specific plasmid using Lipofectamine 2000® (cat. no. 11668019; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The following plasmids were used: CMV (cat. no. 16440; Addgene, Inc.), MKK3-Ala (dnMKK3, cat. no. 14669; Addgene, Inc.), MKK3-Glu (caMKK3, cat. no. 14670; Addgene, Inc.), expressing dominant negative and constitutively active MKK3 isoforms, respectively; the MKK6-EE (caMKK6-EE) constitutively active form of MKK6 was a gift from Professor Puri Pier Lorenzo (Development, Aging and Regeneration Program, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, USA) (10). Plasmids expressing shRNA specific for p38α knockdown and puromycin resistance for transfected cell selection were obtained from OriGene Technologies, Inc. shRNAs were cloned in the pRS plasmid under the U6 promoter, with puromycin as selectable marker and ampicillin as bacterial resistance. A combination of two different shRNAs for p38 (shp38) was used (cat. no. TR320309), specifically 'C' with the sequence, 5'-CAGTGACTTTACAGG AGGTTGTGGATGCT-3', and 'D' with the sequence, 5'-CCA GTAGTCAGAAGCAGGTTCTTGATGTC-3'. As a negative control, non-effective shRNA was used [scramble (SCR), cat. no. TR30012] with the sequence, 5'-GCACTACCAGAG CTAACTCAGATAGTACT-3'. To enrich the cell populations in transfected cells, p-BABE-puro (cat. no. 1764-DNA.cg; Addgene, Inc.) was co-transfected at a 1:3 ratio with plasmids lacking puromycin resistance in order to perform 2-3 days of selection to remove untransfected cells. Following 6-8 h of transfection, the cells were cultured in complete medium for one night before medium containing 2.5 μg/ml of puromycin (cat. no. P7225-25MG; MilliporeSigma) was added for selection purposes. Semi-stable cell lines were obtained at the end of the puromycin treatment.

Total lysate preparation and western blot analysis. Total lysates were obtained after scraping the RD or TE cells in RIPA buffer, modified as follows: 20 mM Tris HCl pH 7.6, 140 mM NaCl, 0.5% IGEPAL (NP40), 2 mM EDTA, 0.5% DOC and 0.5% SDS supplemented with protease and phosphatase inhibitor (cat. no. 11836153001 and cat. no. 04906845001, respectively; Roche Diagnostics), sonicated for 30 sec. Following Lowry or Bradford quantification, 50-100 μg of lysates were processed for western blot analysis. Total proteins were separated on 8, 10 or 12% SDS PAGE and

blotted onto nitrocellulose (cat. no. 16533; Schleicher & Schuell GmbH) or PVDF membranes (cat. no. 10600029; Amersham; Cytiva). Filters were blocked with 5% non-fat dry milk or 3% BSA for 1 h at room temperature and incubated overnight at 4˚C with the following primary antibodies: Anti-Myc (cat. no. sc-40; 1:300), anti-ERK-PO4 E-4 (cat. no. sc-7383; 1:500), anti-ERK1/2 C-9 (cat. no. sc-514302; 1:500), anti-p38-PO4 (cat. no. sc-166182; 1:1,000), anti-p38 (cat. no. sc-535; 1:500), anti-MKK3 (cat. no. sc-961; 1:500), anti-cyclin D1 (cat. no. sc-20044; 1:1,000), anti-p21 (cat. no. sc-6246; 1:200), anti-GAPDH (cat. no. sc-47724; 1:500) and anti-tubulin (cat. no. sc-5286; 1:500) (all from Santa Cruz Biotechnology, Inc.); anti MKK6-PO4 D8E9 (MEK3/6) (cat. no. 12280; 1:1,000), anti-MKK6 D31D1 (cat. no. 8550; 1:1,000), anti-AKT-PO4 Thr308 (cat. no. 4056; 1:1,000), anti-AKT-PO4 Ser473 (cat. no. 9271; 1:1,000) and AKT (cat. no. 9272; 1:1,000) (all from Cell Signalling Technology, Inc.); anti-myosin heavy chain (MHC; cat. no. MF20; 1:300) and anti-myogenin (cat. no. F5D; 1:300) were monoclonal from hybridoma supernatant (all from Developmental Studies Hybridoma Bank). Filters were then incubated for 1 h at room temperature in 2% non-fat dry milk or 3% BSA with the following secondary antibodies: peroxidase-conjugate sheep anti-mouse (cat. no. A90-146B; 1:2,000) or donkey anti-rabbit IgG (cat. no. A120-108B; 1:2,000) (both from Bethyl Laboratories, Inc.). Immunocomplexes were detected by means of ECL Chemidoc XRS+ acquisition (Bio-Rad Laboratories, Inc.). All experiments were performed three times unless otherwise indicated. Representative western blot images are shown and densitometric analysis was performed using ImageJ software version 1.53k (National Institutes of Health) and the results are reported in each respective figure as the mean ± standard deviation (SD).

Cell proliferation assay. The changes in the proliferative potential of RD cells (overexpressing CMV, caMKK3 or caMKK6, or transfected with shp38 or SCR shRNA) were analysed. Specifically, RD cells overexpressing caMKK6 were treated with or without SB203580, whilst p38-silenced (shp38) RD cells were treated with or without U0126. At the end of puromycin selection of transfected cells or the specific treatments, cells were counted using the trypan blue (cat. no. T10282; Invitrogen; Thermo Fisher Scientific, Inc.) exclusion method according to the manufacturer's instructions. Briefly, the cell suspension was added to trypan blue stain in a 1:1 mixture and incubated for 2 min at room temperature. The results are plotted as the mean ± SD of three independent transfections.

Cell morphology and immunofluorescence. To observe the morphological changes in RD cells under the different experimental conditions [overexpressing caMKK3 or caMKK6 genes, overexpressing caMKK6, and treated with or without SB203580, p38-silenced (shp38) treated with or without U0126], the specific samples were photographed under a phase contrast microscope (Nikon Eclipse TS100; Nikon Corporation) at x20 magnification.

For immunofluorescence assays, the cells were fixed in 4% paraformaldehyde for 10 min at room temperature and washed; non-specific binding sites were blocked with 3% BSA in PBS for 20 min at room temperature. The cells were then incubated for 1 h at room temperature with a 1:100 dilution of the anti-MHC (cat. no. MF20), or anti-Myc monoclonal antibody (cat. no. sc-40; 1:100). After rinsing with PBS, the cells were incubated with anti-mouse IgG-Cy3 (cat. no. A90-516C3) or anti-mouse IgG-Cy2 (cat. no. A90-516C2) antibodies (all from Bethyl Laboratories, Inc.) and DAPI (MilliporeSigma). Staining was visualised on a Zeiss Axioskop 2 Plus microscope (Carl Zeiss AG). The experiments were performed twice.

Bioinformatics analysis. MKK3 and MKK6 gene expression analysis across different public databases of RMS primary biopsies was performed by interrogating the R2-Genomics Analysis and Visualization Platform (http://r2.amc.nl). Specifically, to compare the expression levels of MKK3 and MKK6 in a large cohort of RMS tumours and normal skeletal muscle (NSM), five different datasets were analysed: Barr (RMS, GSE66533), Davicioni (RMS) (27), Schafer Welle (RMS and NSM), Assmann (NSM, GSE9103) and Hofman (NSM, GSE3307), for a total of 100 RMS tumour biopsies and 187 NSM tissues. Based on the specific characteristics of each dataset, a different approach of analysis was used as specified below.

The MegaSampler algorithm (http://r2.amc.nl) was used to compare the expression levels of MKK3 and MKK6 genes from multiple datasets, which are on the same chip type and are normalised by the same algorithm. In particular, the Barr-MAS5.0-u133p2 dataset (n=25), filtered for the exclusion of PAX-FOXO fusion positive tumour samples, was compared with the Assmann-MAS5.0-u133p2 dataset (n=40), whilst the Daviconi-MAS5.0-u133a dataset (n=60), filtered for the exclusion of RMS subtypes other than ERMS, was compared with the Hofman-MAS5.0-u133a dataset (n=121).

As regards the Schafer Welle-MAS5.0-u133a dataset, MKK3 and MKK6 gene expression levels were extracted and correlated only in the ERMS subtype and NSM samples, by excluding ARMS biopsies.

The box dot plot of MKK3 or MKK6 expression in ERMS biopsies and NSM generated from R2-Genomics Analysis and Visualization Platform was downloaded and formatted for publication. To calculate whether the means of the expression levels between the selected datasets differed significantly, one-way ANOVA was performed using the R2 platform.

To evaluate the relative expression of MKK3 and MKK6 in each RMS dataset, the ‘multiple gene’ option was used by interrogating the Barr, Davicioni or Schafer Welle datasets.

Statistical analyses. Statistical analyses were performed using the Student's t-test, one-way ANOVA or two-way ANOVA. Dunnett's post hoc test or Tukey's post hoc test were applied for multiple comparisons. A probability value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

MKK6, but not MKK3 induces p38 activation and the myogenic differentiation of RD cells. Taking into account the data available on the promyogenic role of MKK6 (10), the present study aimed to investigate the possible differential roles played by MKK3 and MKK6 in the ERMS reversal of

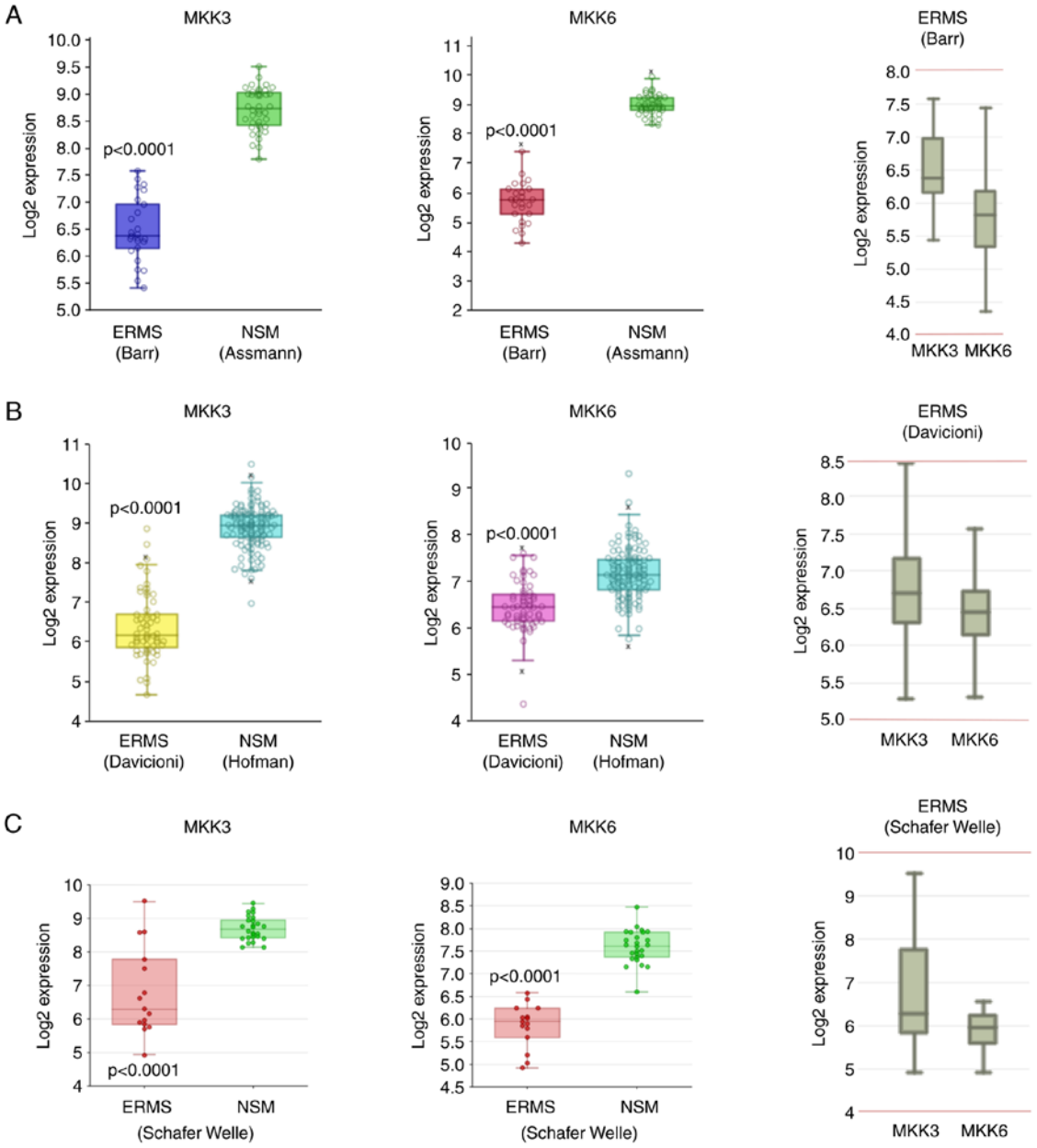

Figure 1. MKK3 and MKK6 gene expression levels in ERMS and NSM samples. Using the R2-Genomics Analysis and Visualization Platform, MKK3 or MKK6 gene expression was assessed in ERMS primary tumours or NSM across different datasets: (A) Barr and Assmann datasets, (B) Davicioni and Hofman datasets, (C) Schafer Welle dataset. Statistical analysis was performed using one-way ANOVA. (A-C) Right panels illustrate the relative expression of MKK3 and MKK6 in ERMS samples. ERMS, embryonal rhabdomyosarcoma; NSM, normal skeletal muscle.

the transformed phenotype. MKK3 and MKK6 expression levels in ERMS primary biopsies compared to NSM were obtained by interrogating three different public transcriptomic datasets deposited on the R2-Genomics Analysis and Visualization Platform (http://r2.amc.nl). The bioinformatics analysis highlighted that MKK3 and MKK6 expression was lower in patients with ERMS compared to NSM (Fig. 1). Moreover, the relative expression of MKK3 and MKK6 differed, with the MKK3 levels being higher than the MKK6 levels in ERMS primary biopsies (Fig. 1, right panels). With the purpose of studying a possible distinct role of MKK3 and MKK6 kinases, the RD cells were co-transfected with CMV, dnMKK3 (MKK3-Ala), caMKK3 (MKK3-Glu) or caMKK6 (MKK6-EE) expression plasmids together with a puromycin-positive vector (as described in the 'Materials and methods' section) so as to allow transfected cells to be specifically selected and enriched by puromycin treatment. The transfection efficiency was assessed by analysing the MKK3 and MKK6 expression levels using western blot assays, as shown in Fig. 2A. While caMKK6 induced Myc (P<0.001) and ERK-PO4 downregulation, caMKK3 led to an increase in Myc (P<0.001) and ERK-PO4 (P<0.001) expression levels, whereas dnMKK3 did not (Fig. 2A). Notably, the reduced proliferative potential of RD cells induced by caMKK6 was demonstrated by the decrease in cyclin D1 expression (P=0.001), which was markedly upregulated in caMKK3-transfected cells (P<0.001), and the concomitant marked increase in p21 protein levels (P<0.001) only in caMKK6-transfected cells (Fig. 2A).

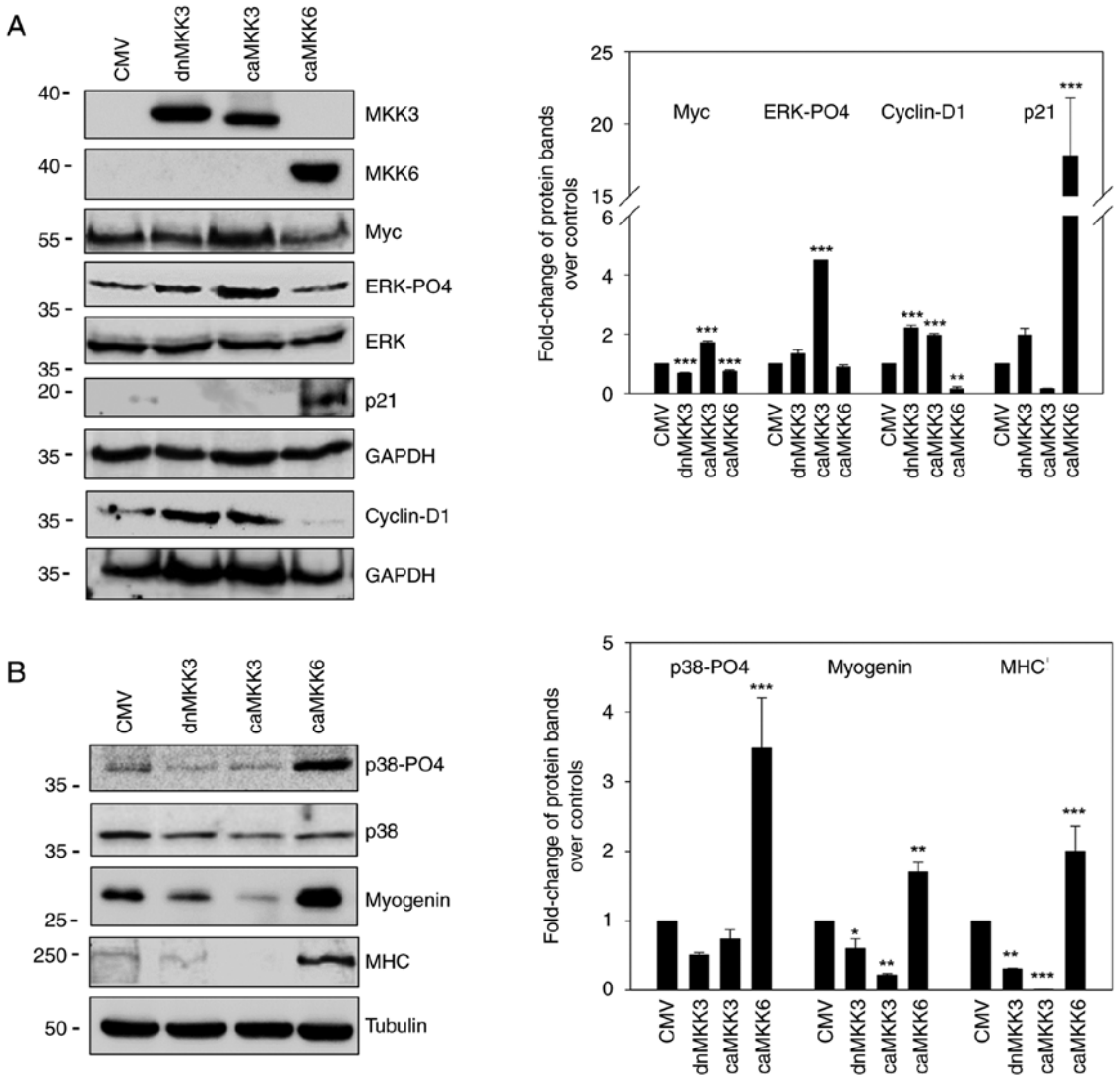

Figure 2. Role of MKK3 and MKK6 in the control of proliferation and differentiation of RD cells. (A) MKK3 and MKK6 western blots are shown as the control of the transfection with dnMKK3, caMKK3 or caMKK6 vectors; western blot analysis of Myc, ERK-PO4 and ERK, cyclin D1 and p21 in RD cells transfected with caMKK6, dnMKK3 or caMKK3. GAPDH was used as a loading control. (B) Western blot analysis of myogenic differentiation markers, MHC, myogenin and p38-PO4 using the same samples as in (A). Tubulin was used as a loading control. Phospho-kinases were also normalised for unphosphorylated isoforms. The numbers on the left of the blots indicate the protein size (kDa). Experiments were performed three times. (A and B) Right panels illustrate the quantitative evaluations of the different western blots performed, expressed as the mean ± SD. Statistical analyses were performed using one-way ANOVA with Dunnett's post hoc test: $^{***}P<0.001$; $^{**}P<0.01$ vs. CMV. MHC, myosin heavy chain.

To assess the distinct role of MKK3 and MKK6 in the induction of myogenic differentiation, specific markers were examined using western blot analysis. As shown in Fig. 2B, p38-PO4 ($P<0.001$), myogenin ($P=0.005$) and MHC ($P<0.001$) expression levels were markedly induced by caMKK6; however, this was not observed with dnMKK3 or caMKK3 transfection.

The evident contrasting role played by MKK3 and MKK6 in RD cells was also confirmed by measuring the growth potential of CMV-MKK3- and MKK6-transfected cells at 4 days following transfection. In accordance with the observed alteration in the cyclin D1 and p21 expression levels, trypan blue dye exclusion assay revealed a consistent decrease in cell proliferation only in RD cells transfected with caMKK6 ($P=0.002$), whilst caMKK3-transfected cells maintained a high proliferative state compared to the mock-transfected control cells ($P=0.01$) (Fig. 3B). The transfection efficiency was confirmed by analysing the MKK3 and MKK6 protein levels using western blot analysis (Fig. 3A). Moreover, morphological investigations in caMKK6- or caMKK3-transfected RD cells confirmed that caMKK6 reduced the number of cells and induced an elongated myogenic-like morphology, whereas caMKK3 increased the number of cells without inducing any morphological changes when compared with the empty vector-transfected cells (Fig. 3C). Finally, immunofluorescence analyses detected decreased staining for Myc in caMKK6-transfected cells whereas staining in caMKK3-transfected cells was increased (Fig. 3C). By contrast, the expression of the myogenic marker MHC, was markedly induced in caMKK6-, but not in caMKK3-transfected cells. Taken together these results indicate that the MKK6/p38 cascade, but not MKK3, triggers growth arrest and induces myogenic differentiation at the morphological and biochemical level by reducing both ERK-PO4 and Myc expression.

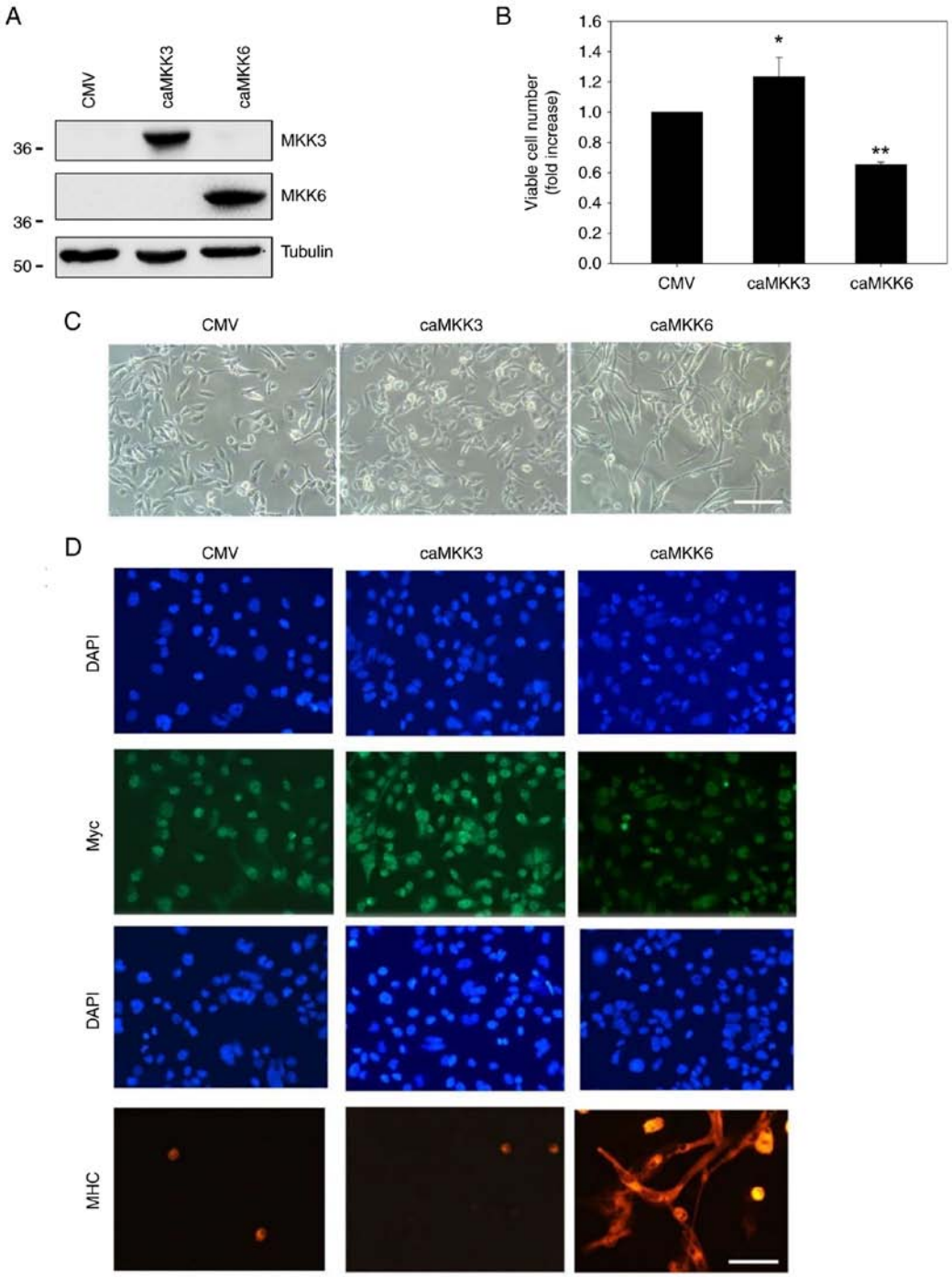

Figure 3. Morphological and functional changes induced by MKK6 or MKK3 overexpression in RD cells. (A) Western blots showing MKK3 and MKK6 expression as the control of the transfection with the specific vectors. Tubulin was used as a loading control. The numbers on the left of the blots indicate the protein size (kDa). (B) Differences in viable RD cell number in cells transfected with empty vector (CMV), caMKK3 or caMKK6 assessed using the trypan blue exclusion assay. Histograms represent the mean value ± SD of three independent experiments. Statistical analyses were performed using one-way ANOVA with Dunnett's post hoc test: $^{**}P<0.01$; $^{*}P<0.05$ vs. CMV. (C) Phase contrast images of RD cells transfected with CMV, caMKK3 or caMKK6. MKK6 induces typical elongated myogenic morphology not present in caMKK3 transfected RD cells. (D) CMV-, caMKK3- or caMKK6-transfected cells after immunofluorescence staining with Myc or MHC antibodies. DAPI was used for nuclear staining. Scale bars, 50 μm. Experiments were performed twice. MHC, myosin heavy chain.

Effects of p38 on the pro-myogenic and anti-oncogenic action of MKK6 and MEK inhibitor. To demonstrate the function of p38 in mediating the anti-oncogenic and pro-myogenic action of MKK6, caMKK6-transcted RD cells were treated with or without SB203580 (Fig. 4). The investigation of the effects of p38 inhibition on the proliferative potential and morphology of RD cells revealed that 5 μM SB203580 exposure alone did not induce significant changes in cell proliferation and morphology (Fig. 4A and B). By contrast, the p38 inhibitor was able to counteract the decrease in cell proliferation observed in RD cells transfected with caMKK6 by ~20% (P=0.04), as indicated by the trypan blue dye exclusion assay, and to revert the MKK6-induced myogenic differentiation. Indeed, MKK6-overexpressing RD cells

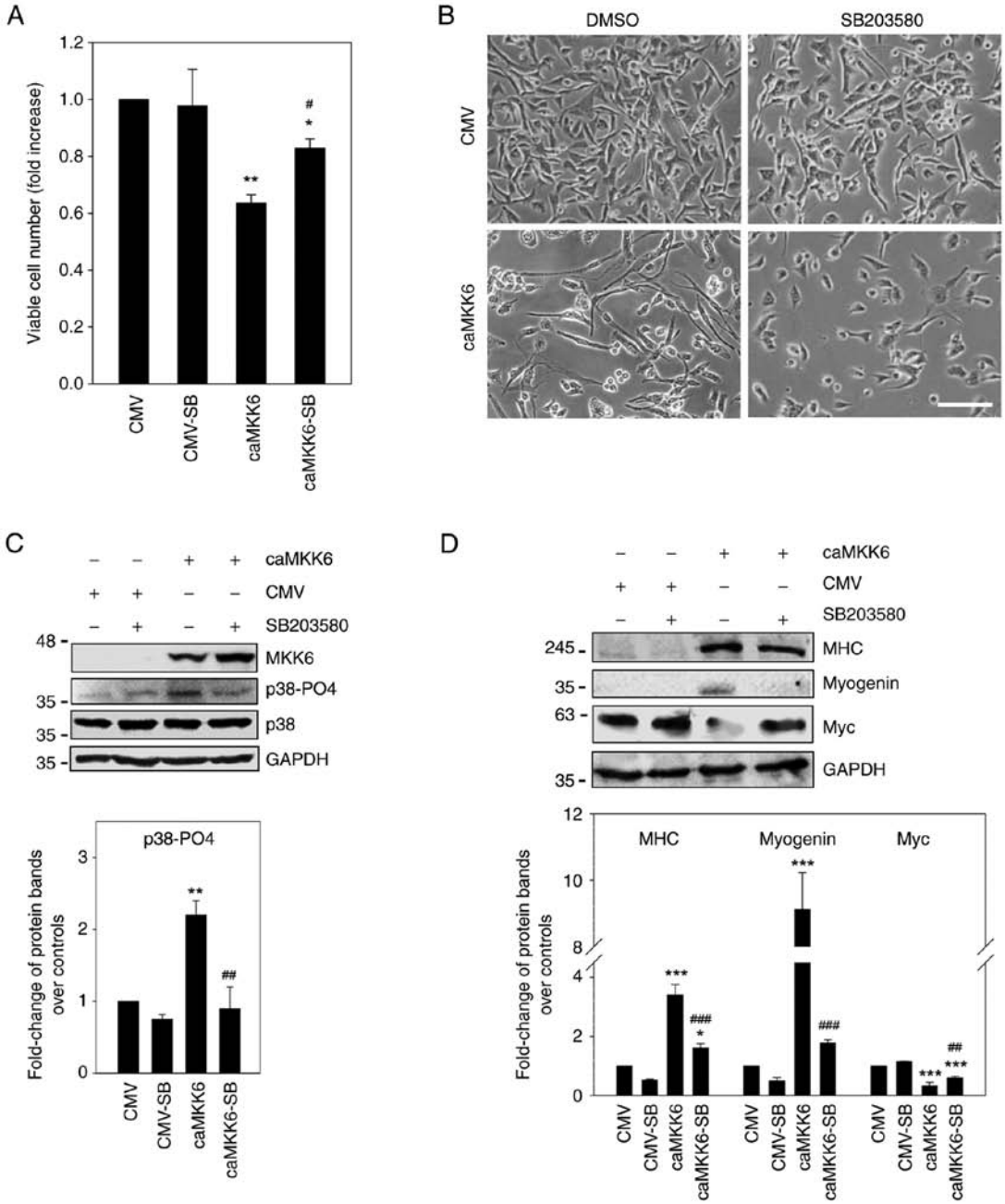

Figure 4. Anti-oncogenic and pro-myogenic signals are mediated by p38 activation in RD cells. (A) Proliferation of RD cells overexpressing MKK6 treated with or without SB203580 (5 μM) assessed using trypan blue exclusion assay. Histograms represent the mean value ± SD of two independent experiments. Statistical analyses were performed using two-way ANOVA with Tukey's post hoc test: **P<0.01; *P<0.05 vs. CMV; $^{\#}$P<0.05 vs. caMKK6. (B) Morphological evaluation of RD cells transfected with caMKK6 treated with or without SB203580 (5 μM). Scale bars, 50 μm. Experiments were performed twice. (C) RD cells transfected with empty vector (CMV) or caMKK6 and treated with or without the SB203580 (5 μM) p38 inhibitor were analysed for phospho-active p38 expression level. GAPDH was used for protein quantification. Phospho-p38 was also normalised for total unphosphorylated isoform. MKK6 expression is shown as a transfection control. (D) Western blots of MHC, myogenin and Myc in RD cells transfected with CMV or caMKK6 and treated with or without SB203580 (5 μM) p38 inhibitor. (C and D) The numbers on the left of the blots indicate the protein size (kDa). Lower panels represent quantitative evaluations of the western blots expressed as the mean ± SD. Statistical analyses were performed using two-way ANOVA with Tukey's post hoc test: ***P<0.001; **P<0.01; *P<0.05 vs. CMV; $^{\#\#\#}$P<0.001; $^{\#\#}$P<0.01 vs. caMKK6. Experiments were performed three times. MHC, myosin heavy chain.

treated with SB203580 exhibited less elongated cellular bodies compared to MKK6-overexpressing cells not treated with the inhibitor (Fig. 4A and B). In agreement with these results, it was found that the p38-PO4 levels were markedly increased in caMKK6-transfected cells (P=0.002) and were markedly downregulated by SB203580 treatment (P=0.002) (Fig. 4C). On the other hand, CMV mock-transfected RD cells exhibited a barely detectable p38-PO4 basal level, thus making it difficult to observe alterations in this kinase. When caMKK6-transfected cells were treated with 5 μM of the p38 inhibitor, SB203580, the expression levels of MHC (P<0.001) and myogenin (P<0.001) significantly decreased and were not detected in the CMV-transfected cells (Fig. 4D).

Moreover, the Myc expression levels were significantly decreased in the caMKK6-transfected cells in comparison to the CMV mock-transfected control cells (P<0.001)

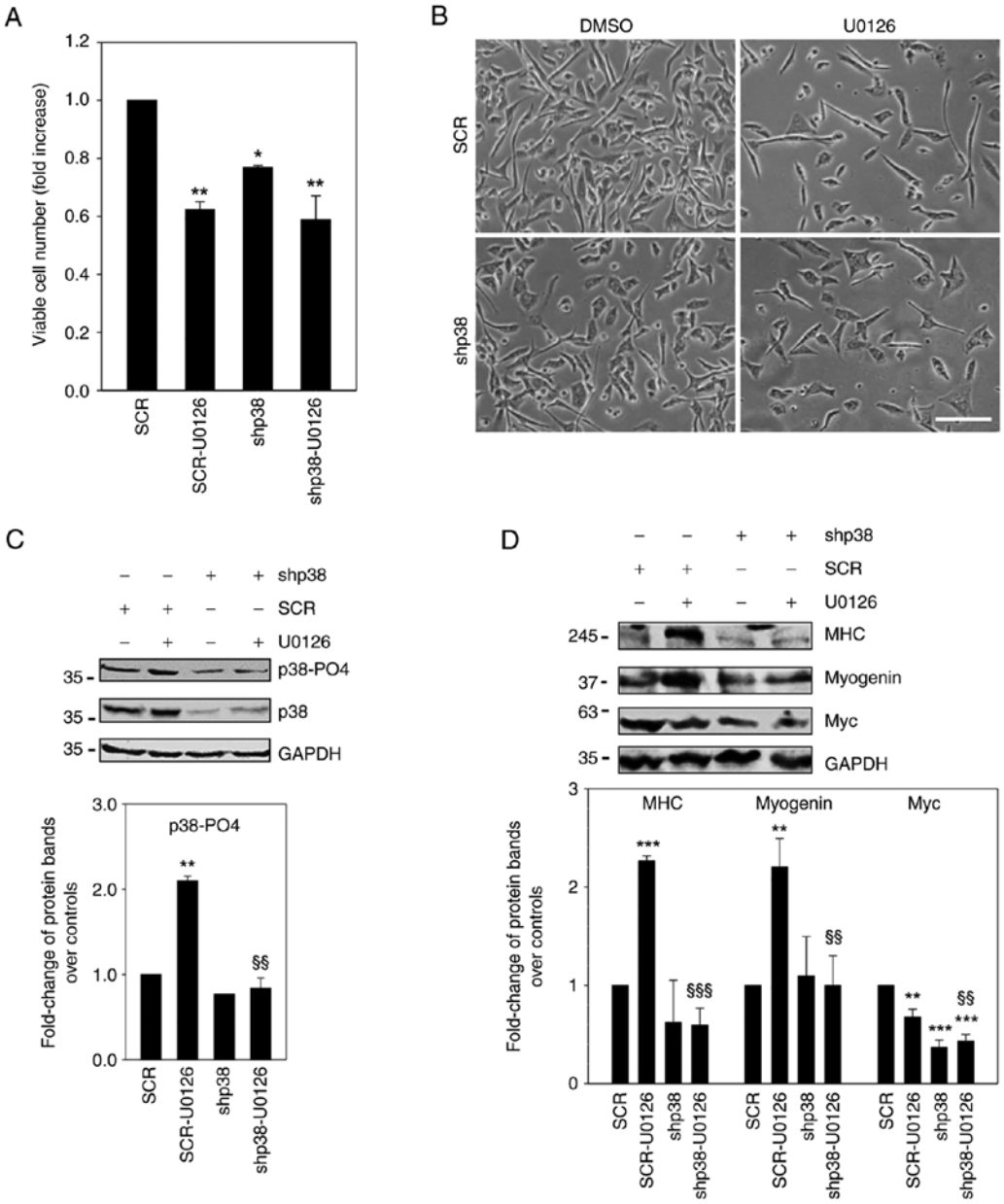

Figure 5. Anti-oncogenic and pro-myogenic effects induced by MEK/ERK inhibitor U0126 in RD cells are mediated by p38. (A) Cell proliferation of p38-silenced RD cells treated with or without U0126 (10 μM) assessed using trypan blue exclusion assay. Histograms represent the mean value ± SD of two independent experiments. Statistical analyses were performed using two-way ANOVA with Tukey's post hoc test: **P<0.01; *P<0.05 vs. CMV. (B) Morphological evaluation of p38-silenced RD cells treated with or without U0126 (10 μM). Scale bars, 50 μm. Experiments were performed twice. (C) RD cells silenced with a combination of two p38 shRNA or scramble (SCR) shRNA and treated or not with 10 μM U0126 were analysed for phospho-active p38 expression level. GAPDH was used for protein quantification. (D) Western blots of MHC, myogenin and Myc in RD cells silenced as in (C) treated with 10 μM U0126 or left untreated. GAPDH was used as a loading control. (C and D) The numbers on the left of the blots indicate the protein size (kDa). Lower panels represent the quantitative evaluations of the western blots expressed as the mean ± SD. Statistical analyses were performed using two-way ANOVA with Tukey's post hoc test: ***P<0.001; **P<0.01 vs. SCR; §§§P<0.001; §§P<0.01 vs. SCR-U0126. Experiments were performed three times. MHC, myosin heavy chain.

(Fig. 4D); however, the decrease in Myc expression was counteracted by treatment with SB203580 (P<0.001 vs. CMV; P=0.004 vs. caMKK6) (Fig. 4D), whilst in the CMV mock-transfected control cells, the effect of p38 inhibitor on Myc expression was minimal (Fig. 4D). The effects of the p38 inhibitor on the ERK-PO4 and Myc levels were also examined in untransfected RD cells and in cells treated with the MEK inhibitor (Fig. S1A).

To verify whether the absence of p38 affects the anti-oncogenic action of MEK inhibitor, p38-silenced (shp38) RD cells were treated with or without U0126 (Fig. 5). By investigating the proliferative potential, it was found that p38 silencing reduced the proliferation rate of RD cells by ~20% (Fig. 5A, shp38 vs. SCR, P=0.02), whilst U0126 treatment reduced this by ~40% (Fig. 5A, SCR-U0126 vs. SCR, P=0.003). The effect of U0126 on the proliferation of p38-silenced cells was visibly diminished (shp38-U0126 vs. shp38). By analysing the morphology, it was observed that U0126 induced a more elongated shape in the SCR-treated cells, whilst these same effects were attenuated in p38-silenced cells (Fig. 5B). As shown in Fig. 5C, the U0126-mediated upregulation of phospho-active p38 was reduced in p38-silenced cells (shp38-U0126 vs.

SCR-U0126, P=0.004) but not in SCR-U0126 cells (Fig. 5C), since the total p38 expression was markedly downregulated by its specific silencing. In p38-silenced cells, U0126 treatment led to barely visible MHC (P<0.001 vs. SCR-U0126) and myogenin (P=0.004 vs. SCR-U0126) expression levels (Fig. 5D), whereas it induced the high expression of both proteins in SCR-transfected cells (Fig. 5D), as well as in untransfected cells (Fig. S1B). Moreover, the U0126-mediated-Myc downregulation was visibly attenuated in p38-silenced cells compared to untreated cells (Fig. 5D).

Taken together, these different approaches on RD cells (MKK6 enforced expression with or without SB203580 treatment and p38 silencing with or without U0126 exposure), indicate the contribution of MKK6 kinase in the activation of p38, which in turn is crucial in inducing the expression of myogenic markers and in counteracting the ERMS oncogenic phenotype. Notably, the p38-silenced RD cells were less responsive in processing anti-oncogenic signals induced by U0126, thereby revealing a contribution of p38 in orchestrating myogenic phenotype expression, including growth arrest.

MKK6 induces pro-myogenic and anti-oncogenic effects mediated by the p38 pathway in the TE ERMS-derived cell line. The present study then examined the effects of caMKK6 in another embryonal RMS cell line that the authors had previously used (28) due to its sensitivity to the differentiating effects of MEK inhibitor in both *in vitro* and *in vivo* assays. The enforced expression of MKK6 in TE cells induced MAPK-p38 activation (P=0.04), whilst both ERK-PO4 (P<0.001) and Myc (P<0.001) levels decreased (Fig. 6). The reduction in ERK-PO4 and Myc levels was reversed by SB203580 (P<0.001 and P=0.02 vs. caMKK6). MHC (P<0.001) and myogenin (P<0.001) expression levels were also induced by MKK6 overexpression, and abolished by p38 silencing (P<0.001 and P<0.001 vs. caMKK6). These data and those aforementioned suggest that the MKK6/p38 axis transmits anti-oncogenic and pro-myogenic specific signals in ERMS-derived cell lines.

MEK/ERK inhibitors in ERMS cell lines mimic MKK6-induced differentiation pathways. A growing body of evidence suggests that the MEK/ERK pathways play a positive role in the differentiation of certain cell types (29,30). However, in pathological myogenic differentiation, the MEK/ERK pathways have been found to be involved in myogenic differentiation induced by Myc inactivation (14). To date, and to the best of our knowledge, no data are yet available on the mechanisms through which MEK/ERK inhibitors induce Myc downregulation, growth arrest and myogenic differentiation in ERMS cells. The present study thus investigated whether the treatment of RD cells with the MEK inhibitors induces MKK6 kinase activation and, as a consequence, p38 activation. Trametinib, a second generation MEK inhibitor with nanomolar activity, was also used, which specifically inhibits ERK and affects the p38 pathways in the same manner as RD cells were affected by U0126 treatment (Fig. S2). The results of this experiment confirmed that the U0126 data were not due to off-target effects. However, since most of the data collected were based on experiments that included U0126 treatment, the U0126 inhibitor was used in the majority of the experiments presented herein.

It was hypothesised that MEK inhibitors mimic the myodifferentiation pattern mediated by caMKK6 by transmitting anti-oncogenic signals. The treatment of RD and TE cells with U0126 or trametinib induced the MKK6/p38 pathways (Figs. 7 and S3). The results demonstrated that MKK6-PO4 was detectable after short (3 h), as well as prolonged (O/N) treatments with both MEK inhibitors in RD cells (P=0.04 and P<0.001; P=0.02 and P<0.001) (Fig. 7) and particularly after U0126 exposure in TE cells (P<0.001; P=0.009) (Fig. S3). Moreover, p38-PO4 expression was markedly increased in U0126- and trametinib-treated cells following the same time course as MKK6, particularly in RD cells (P<0.001 and P<0.001; P<0.001 and P<0.001) (Fig. 7).

Myogenin and MHC proteins were detected following the use of both inhibitors only after prolonged treatments (3 days) in both RD (P=0.02 and P=0.007; P=0.04 and P<0.001) (Fig. 7) and TE (P=0.004 and P=0.002; P=0.04 and P=0.001) cells (Fig. S3).

These results indicate, for the first time, to the best of our knowledge, that MEK/ERK inhibition causes the concomitant activation of the MKK6/p38 cascade and myogenic program, a finding that has not previously been reported in ERMS cells.

AKT is part of the myogenic pathway induced by the MKK6/p38 axis. The deregulated expression of Myc has been reported to affect PI3K/AKT activation by enhancing p38 in cells treated with toxic agents (31). In addition, the activation of the p38 pathway affects AKT at the transcriptional and protein levels (25). Thus, the present study investigated whether the MEK inhibitor-mediated downregulation of Myc in the ERMS system was linked to the concomitant variation of AKT-PO4 expression and whether p38 activation may contribute to the phospho-AKT modulation. For this purpose, RD and TE cells were treated for different periods of time with 10 μM U0126, and AKT-PO4 Ser473 and Thr308 levels were analysed (Fig. 8).

While the expression levels of the two isoforms of phospho-AKT were very low in the control cells, particularly AKT-PO4 Thr308, these levels significantly increased when the MEK inhibitor was added to both ERMS lines at the early and late treatment times [AKT-PO4 Ser473: P=0.04, P=0.004 and P=0.008 (RD cells); P=0.04, P=0.03 and P=0.01 (TE cells); AKT-PO4 Thr308: P=0.009, P=0.001 and P=0.02 (RD cells); P=0.009, P<0.001 and P=0.001 (TE cells)]. AKT activation was also confirmed by treating the RD cells with 10 nM trametinib (data not shown). Notably, the AKT-PO4 levels increased only in cells overexpressing MKK6 (P<0.001 and P<0.001), but not with the caMKK3 vector, similarly to p38-PO4 levels (P<0.001) (Fig. 9A); this suggested that MKK6-induced AKT activation may be mediated by p38.

Indeed, caMKK6 induced the phosphorylated form of AKT in both Ser473 and Thr308 (Fig. 9A and B) and the reduction in AKT activation following treatment with the p38 inhibitor, SB203580 (P<0.001 and P<0.001 vs. caMKK6), demonstrated the positive contribution of MAPK p38 to the activation of both AKT isoforms in MKK6 overexpressing cells (Fig. 9B).

Likewise, the silencing of p38 prevented the activation of AKT mediated by U0126-induced p38 activation (AKT-PO4 Ser473 P<0.001 vs. SCR-U0126), which was present in the SCR control-transfected cells following U0126 treatment

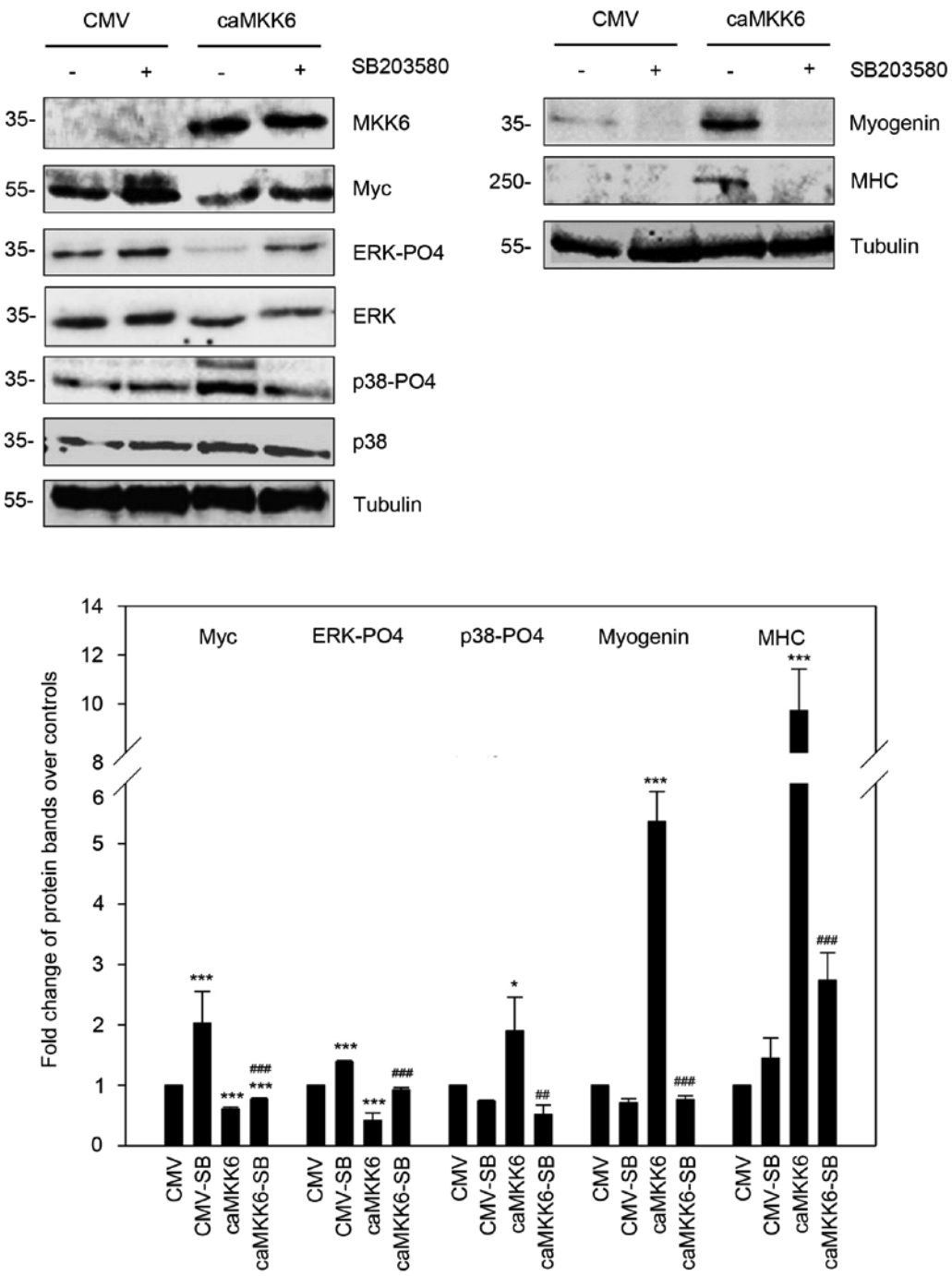

Figure 6. TE cell differentiation is dependent on MKK6/p38 pathway activation. TE cells transfected with CMV or caMKK6 were treated with 5 μM SB203580 or left untreated; western blots of MKK6, Myc, ERK-PO4, p38-PO4, myogenin and MHC were normalised to tubulin. Phospho-kinases were also normalised for unphosphorylated isoforms. The numbers on the left of the blots indicate the protein size (kDa). Lower panel represent histograms of the quantitative evaluations of the western blots expressed as the mean ± SD. Statistical analyses were performed using two-way ANOVA with Tukey's post hoc test: ***P<0.001; *P<0.05 vs. CMV; $^{###}$P<0.001; $^{##}$P<0.01 vs. caMKK6. Experiments were performed three times. TE cells, TE671 cells; MHC, myosin heavy chain.

(Fig. 9C). These results indicate that AKT is activated when the myogenic program is induced either by pharmacological treatment or by the enforced expression of the MKK6/p38 pathway.

Discussion

To the best of our knowledge, the present study is the first to demonstrate the differential role of the MKK class of MAP kinases, namely MKK6 and MKK3, in the ERMS cell system, specifically in the RD and TE cell lines. Both MKK3 and MKK6 are upstream kinases of p38, the difference between these kinases being that MKK3 markedly stimulates JNK, which is known to counteract myogenesis (32). Though both kinases are upstream pathways of p38 (33). The present study demonstrated, by using constitutively active isoforms (ca) of both, that caMKK6 markedly enhanced phospho-active p38, growth arrest and myogenic differentiation. By contrast, caMKK3 failed to induce active p38 and maintained the RD cells in a proliferative state. This finding

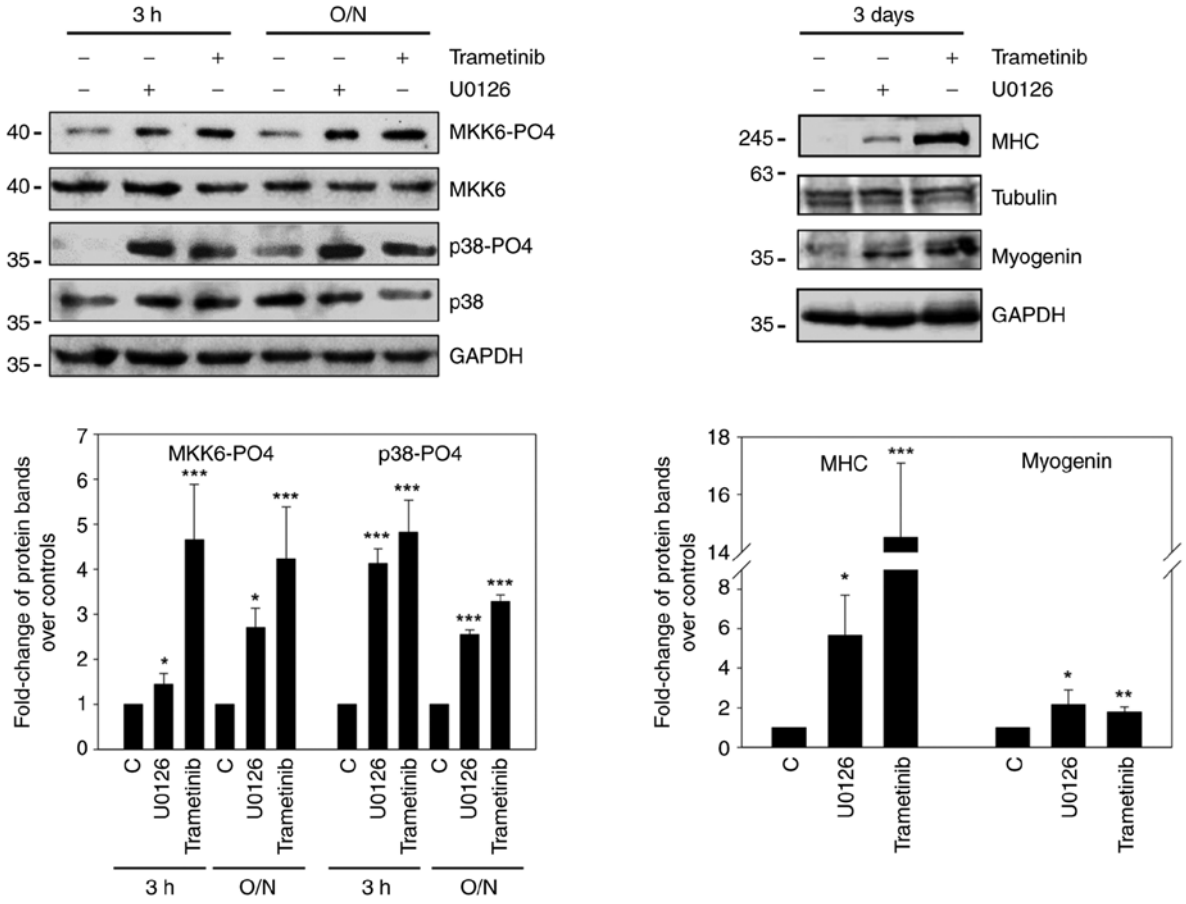

Figure 7. MKK6 is induced by MEK/ERK inhibitors in RD cells. RD cells were treated with 10 μM U0126 or 10 nM trametinib and the expression levels of MKK6-PO4, p38-PO4, MHC and myogenin were examined using western blot analysis. GAPDH and unphosphorylated kinases were used to normalise MKK6 and p38 (3 h and O/N panel); tubulin and GAPDH were used to normalise MHC and myogenin, respectively (3 days panel). The numbers on the left of the blots indicate the protein size (kDa). Lower panels represent histograms of the quantitative evaluations of the western blots, expressed as the mean ± SD. Statistical analyses were performed by using one-way ANOVA with Dunnett's post hoc test: ***P<0.001; **P<0.01; *P<0.05 vs. negative control. Experiments were performed three times. MHC, myosin heavy chain; O/N, overnight; C, negative control.

was demonstrated by the induction of proliferative markers in caMKK3-transfected cells and the downregulation of these markers in MKK6-overexpressing cells. When examining the effect of dnMKK3, which cannot be activated due to the replacement of Ser-189 and Thr-193 with Ala, it was found that it was unable to control Myc upregulation and ERK-PO4 kinase activation, whilst it still functioned in the control of cyclin D1 specific expression. This is intriguing data and future research is required to provide further insight into the related molecular mechanisms. Since the present study did not extend the investigation to other aspects aimed at deeply analysing the proliferative role of MKK3 in ERMS-derived cells, this point remains an open question, thus representing a limitation of the present study. The analysis of the growth potential of RD cells confirmed that caMKK3 sustains the proliferative phenotype, whereas caMKK6 abrogates it, as is shown by the biochemical data. The overexpression of Myc, phospho-active ERK and cyclin D1, under the enforced expression of caMKK3 which failed to activate p38, is indicative of a permanent proliferative state. This result supports the reported role of p38 in the cell cycle exit of myoblasts (34). Indeed, Perdiguero *et al* (34) reported that the genetic deficiency of p38α in myoblasts confers enhanced growth and results in defects in the withdrawal from the cell cycle, as well as in the formation of multinucleated myotubes. In agreement with the key role played by p38 in controlling the proliferative potential of RMS cells, the authors of the present study recently demonstrated that the inhibition of p38 activity, by using SB203580, increases the clonogenic ability of RD cells (35). In line with the role of p38 in controlling the growth and differentiation of RMS tumours, it was found that the anti-growth and promyogenic responses to U0126 were attenuated in the p38-silenced cells compared to SCR-transfected cells, thus corroborating p38 to actively participate in the antioncogenic responses induced by MEK/ERK inhibition.

Myc and myosin in RD cells transfected with caMKK3, caMKK6 or an empty vector (CMV) displayed a morphological pattern of cells that undergo myogenic differentiation with MKK6 overexpression and an increased transformed phenotype expression was observed in MKK3-overexpressing cells.

Finally, the meaning of the data of the two kinases in ERMS cell lines is coherent with those obtained by interrogating public transcriptomics datasets on RMS tumours. Indeed, bioinformatics analysis demonstrated that the expression levels of MKK3 and MKK6 were lower in patients with ERMS compared to normal muscle. In addition, in ERMS, MKK3

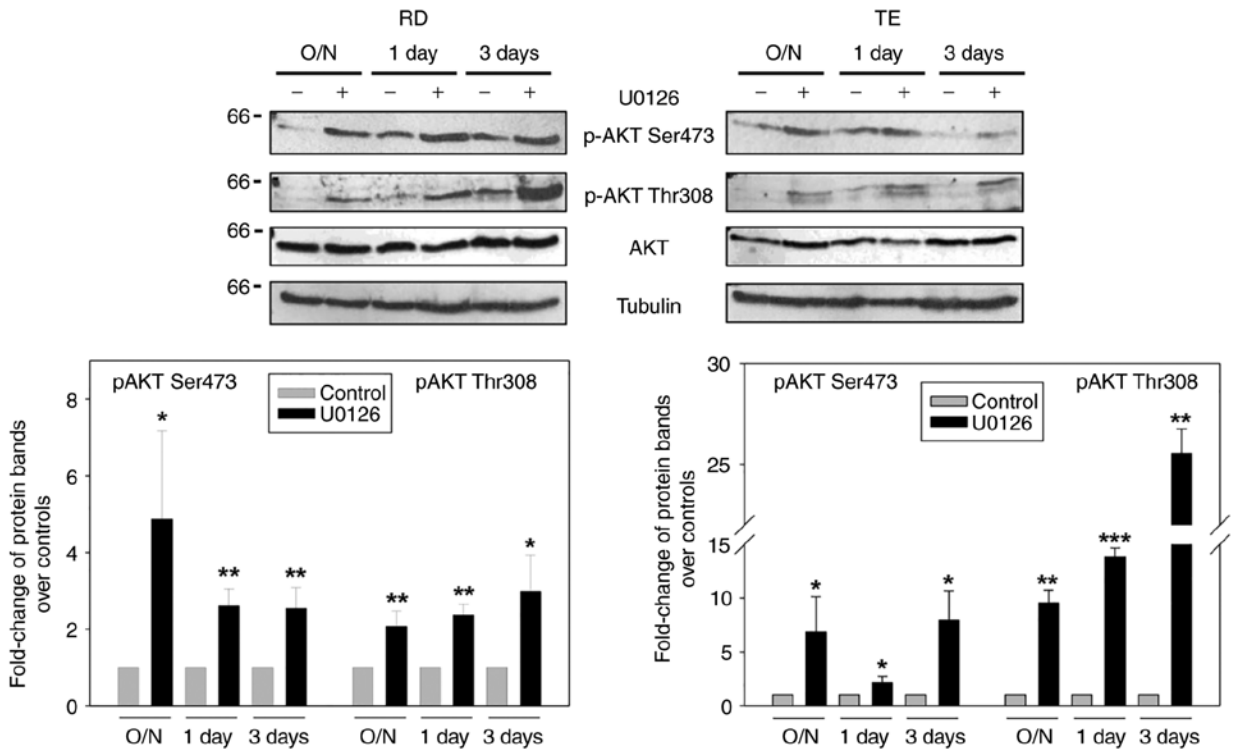

Figure 8. AKT is induced by MEK/ERK inhibition. RD and TE cells were treated with 10 μM U0126 or left untreated and lysates were analysed for AKT-PO4 Ser473 and AKT-PO4 Thr308 expression. Both phosphorylation levels were increased after O/N, 1 day and 3 days of treatments. The numbers on the left of the blots indicate the protein size (kDa). In the lower panels, quantitative evaluations of the western blots are shown as the mean ± SD. Statistical analyses were performed using a Student's t-test: ***P<0.001; **P<0.01; *P<0.05 vs. negative control (control). Experiments were performed twice. TE cells, TE671 cells; O/N, overnight.

expression was higher than that of MKK6, this suggesting that for the reversal of the ERMS oncogenic phenotype, the induction of MKK6 is strictly required.

In both RD and TE cell lines, MKK6/p38 pathway activation induced the downregulation of Myc and ERK-PO4 accompanied by increased MHC and myogenin expression. SB203580 treatment specifically abolished myogenic marker expression in both ERMS cell lines and counteracted the decrease in cell proliferation induced by caMKK6 ectopic expression.

Exploring the hypothesis that MEK inhibitors can mimic the effect of the enforced expression of caMKK6, it was demonstrated that one of the early responses to the MEK inhibitors U0126 or trametinib in both RD and TE cells was the activation of MKK6.

It has previously been reported that the enforced induction of MKK6/p38 pathways restores the myogenic differentiation of ERMS cell lines (10). The present study demonstrates for the first time, to the best of our knowledge, that extracellular signals, such as those induced by the MEK/ERK inhibitor within the pathological myogenesis of ERMS, restore the activation of endogenous MKK6/p38.

N-Ras and mutant p53, which both sustain the ERMS phenotype, are known to be related to p38 MAPK (36). The present study found a functional connection between MKK6, ERK, p38 and AKT that is orchestrated by the MEK/ERK inhibition or by the overexpression of caMKK6 (MKK6-EE) though not by caMKK3 (MKK3-Glu) expression. The functional connection between MKK6, ERK, p38 and AKT reflects that reported in a study on normal myoblasts, in which myogenesis was induced in differentiating medium (37). Serra *et al* (37) demonstrated the role played by responses at the chromatin level and the specific role of kinases in mediating the formation of transcription complexes that are active in muscle during the specific regeneration program. When they investigated myogenic precursors by focusing on p38 and ERK in a normal myogenic program, they found that p38 was required for the expression of MyoD-responsive genes and that ERK plays a biphasic role, peaking in undifferentiated and post-mitotic myoblasts.

Importantly, the effects of the MEK/ERK inhibitors on RMS is further supported by the similarity between the data of the present study and those of the aforementioned authors (37), as is shown by the ability of these inhibitors to induce a pathway in tumour cells, just as occurs in the normal myogenic program.

The fact that p38 activation is responsible for the concomitant decreases in ERK-PO4 and Myc levels demonstrates that the oncogenic functional partnering of active ERK and Myc (19) can be disrupted by activated p38 kinase. The concomitant ERK-PO4 and Myc downregulation also demonstrates that the increase in p38 activity and the attenuation of ERK activity promote the transition from the proliferation to the differentiation of ERMS cells.

While the crosstalk between ERK and p38 has previously been reported by the authors of the present study (13), as well as by other authors (38,39), the dependence of Myc levels on active p38 has not. The present study demonstrated that the

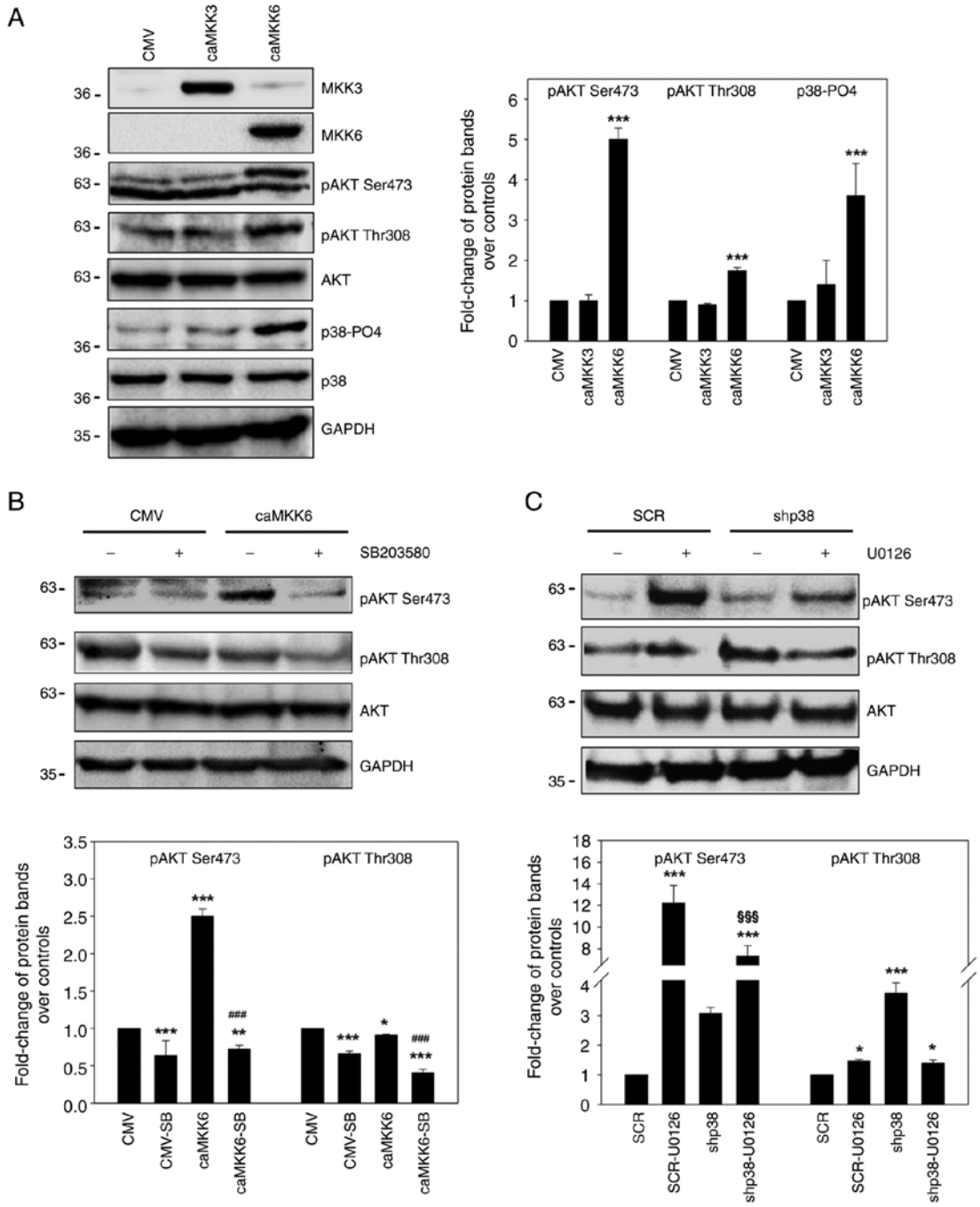

Figure 9. AKT activation is part of myogenic differentiation in RD cells. (A) Cells transfected with empty vector (CMV), caMKK3 or caMKK6 were analysed for AKT-PO4 Ser473, AKT-PO4 Thr308 and p38-PO4 expression. Both AKT phosphorylation sites and p38 were activated by caMKK6 transfection, whereas they were absent in CMV and caMKK3 transfected cells; MKK3 and MKK6 overexpression is shown as a transfection control. GAPDH was used for protein quantification. Phospho-kinases were also normalised for unphosphorylated isoforms. The numbers on the left of the blots indicate the protein size (kDa). Right panel represents histograms of the quantitative evaluations of the western blots, expressed as the mean ± SD. Statistical analyses were performed using one-way ANOVA with Dunnett's post hoc test: ***P<0.001 vs. CMV. (B) Western blots showing the reduced AKT phosphorylation in caMKK6-transfected RD cells treated with 5 μM SB203580. (C) Both AKT phosphorylation levels were not activated by U0126 in p38-silenced RD cells, whilst they were present in scramble control-transfected (SCR) cells treated with U0126. GAPDH was used as loading control. (B and C) The numbers on the left of the blots indicate the protein size (kDa). Lower panels represent histograms of the quantitative evaluations of the western blots, expressed as the mean ± SD. Statistical analyses were performed using two-way ANOVA with Tukey's post hoc test: ***P<0.001; **P<0.01; *P<0.05 vs. CMV or SCR; $^{\#\#\#}$P<0.001 vs. caMKK6; §§§P<0.001 vs. SCR-U0126. Experiments were performed twice.

activation of MKK6 by either MEK inhibitors or by caMKK6 overexpression altered the p38/ERK ratio towards a differentiation state, thereby suggesting that ERK and p38 kinases are regulated by a critical ratio during pathological myogenic differentiation.

In agreement with this, the balance of p38 and ERK-PO4 regulate cell differentiation in osteosarcoma and the use of MEK inhibitor has been suggested as a candidate for reverting malignant phenotype in this system (40). In keeping with this hypothesis, it is not surprising that SB203580 p38 inhibitor not

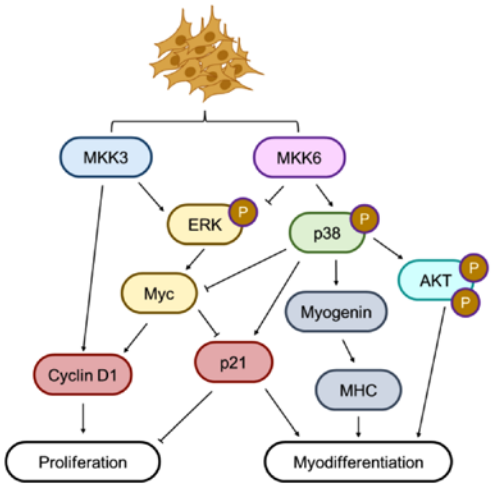

Figure 10. MKK3 and MKK6 kinases in ERMS cells. Summary diagram describing the differential role played by MKK3 and MKK6 in controlling growth arrest and myogenic differentiation in ERMS cells. MHC, myosin heavy chain.

only inhibits p38, but enhances ERK-PO4 and Myc expression, thereby corroborating the inverse association between ERK and p38 proteins in ERMS. Since Myc inactivation alone leads to growth arrest and myogenic differentiation of cultured ERMS cells (14), the MEK/ERK inhibitor can recruit the kinases capable of mediating Myc degradation, which in turn release myodifferentiation signals. Indeed, Myc accumulation is one of the oncogenic and anti-myogenic responses to Ras/MEK/ERK overactivation. Likewise, following MEK/ERK inhibition by either MEK/ERK inhibitor or caMKK6 enforced expression, the induction of AKT signalling is rapid and p38-dependent. The activation of AKT may be consequent to a variation in Myc expression (31). Indeed, Myc is known to impair PI3K/AKT activation levels (31), which suggests that PI3K/AKT may be released when Myc levels are reduced. AKT activation may play an important role in the restored myogenic program by MEK/ERK inhibition, which leads to the re-establishment of the MKK6/p38/AKT cascades. It is noteworthy that the activation of p38 by the inhibition of ERK has been reported to be linked to the apoptotic action of p38, which is modulated by the concomitant PI3K/AKT module (41). In targeted knockdown and in genetic knockout experiments, AKT proteins have been implicated in myogenic differentiation and myofiber maturation (24). The controlled activation of AKT in proliferating ERMS cells has the potential to be an additional promising option in differentiation therapy for the myogenic rescue of RMS tumours.

The present study defined the differential role of MKK kinases in controlling growth arrest and myogenic differentiation and in maintaining the proliferative phenotype in ERMS cells. In detail, MKK6 activation (by MEK inhibitor treatment or ectopic expression) triggers both p38 and AKT activation, which mediate the expression of myogenic markers, such as MHC and myogenin. Conversely, Myc, which is known to counteract myogenic programs is downregulated together with ERK-PO4 contributing to the growth arrest (Fig. 10). The finding that MEK/ERK inhibitors recover the MKK6/p38 axis inducing myogenic differentiation in RMS may lead to the use of them in combined and more advanced therapies for this aggressive solid tumour and prevent its dissemination. From a therapeutic point of view, the MEK/ERK inhibitor-based therapies had limited success (42); nevertheless, since ERK and Myc functional partnering in ERMS cells are disrupted by MKK6/p38 activation from MEK inhibitors, their concomitant inhibition lends itself to exploitation at the therapeutic level.

Acknowledgements

The authors would like to thank Professor Lewis Baker (Sapienza Language Centre, Sapienza University of Rome) for reviewing the English language in the manuscript. The authors are grateful to Professor Pier Lorenzo Puri (Development, Aging and Regeneration Program, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, USA) who provided the MKK6EE vector. The authors would also like to thank Dr Giulia Vitali (Department of Experimental Medicine, Sapienza University of Rome) for providing supporting with the western blot analyses.

Funding

The present study was partially funded by 'Progetti di Ricerca di Ateneo 2018' n. RM1181642707D875, Sapienza University of Rome Italy. The research leading to these results received funding from AIRC under IG 2020-ID.24696 project. The present study was also supported by 'Io, domani…' Associazione per la lotta contro i tumori infantili Onlus (Rome, Italy) with a postdoctoral fellowship.

Availability of data and materials

The datasets used and/or analysed during the present study are available from the corresponding author on reasonable request or from R2-Genomics Analysis and Visualization Platform website (http://r2.amc.nl).

Authors' contributions

BMZ, ADR, FMa, CC, SC, GB and FMe conceptualised and designed the study. ADR, BMZ, SC, AB, LS, CC, BLO and FMe performed the experiments and/or analysed and interpreted the data. SC carried out the bioinformatics and statistical analyses. BMZ, MB and CM were responsible for data curation. BMZ wrote the original draft of the manuscript. BMZ, SC and FMe critically reviewed and edited the manuscript. BMZ and SC prepared the figures. FMa, FMe and CM provided funds. authors have read and approved the final manuscript. ADR and BMZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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