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Differentiation of human rhabdomyosarcoma RD cells is regulated by reciprocal, functional interactions between myostatin, p38 and extracellular regulated kinase signalling pathways

Stefania Rossi [a], *Elena Stoppani* [a], *Pier Lorenzo Puri* [b,c], *Alessandro Fanzani* [a,*]

[a] *Department of Biomedical Sciences and Biotechnologies and Interuniversity Institute of Myology (IIM), University of Brescia, viale Europa 11, 25123 Brescia, Italy*

[b] *Dulbecco Telethon Institute (DTI), IRCCS Fondazione Santa Lucia and European Brain Research Institute, Roma, Italy*

[c] *Sanford-Burnham Medical Research Institute, La Jolla, USA*

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ABSTRACT

Rhabdomyosarcoma (RMS) includes heterogeneous tumours of mesenchymal derivation which are genetically committed to the myogenic lineage, but fail to complete terminal differentiation. Previous works have reported on deregulated myostatin, p38 and extracellular regulated kinase (ERK) signalling in RMS cell lines; however, the functional link between these pathways and their relative contribution to RMS pathogenesis and/or maintenance of the transformed phenotype *in vitro* are unclear. Herein we show that the constitutive expression of a dominant-negative form of activin receptor type IIb (dnACTRIIb), which inhibits myostatin signalling, decreased proliferation and promoted differentiation of the human RMS RD cell line. DnACTRIIb-dependent differentiation of RD cells correlated with a reduced SMAD2/3 (small mother against decapentaplegic) and ERK signalling and the activation of p38 pathway. Conversely, the expression of a constitutively activated ALK5 (activin receptor-like kinase) (caALK5) form, activating SMAD3 and ERK pathways, led to further impairment of RD differentiation. Pharmacological blockade of ERK pathway in RD cells was sufficient to replicate the biological phenotype observed in dnACTRIIb-expressing RD cells, and also recovered the differentiation of caALK5-expressing RD cells. Conversely, deliberate activation of p38 signalling mimics the effect of dnActRIIb and overcame the differentiation block in RD cells. These data indicate the existence of a network formed by myostatin/SMAD2/3, ERK and p38 pathways that, when deregulated, might contribute to the pathogenesis of RMS. The components of this network might, therefore, be a valuable target for interventions towards correcting the malignant phenotype of RMS.

1. Introduction

Rhabdomyosarcoma (RMS) is the most common paediatric soft tissue tumour, which is thought to arise from mesenchymal precursors committed to myogenic lineage.[1] As many other human malignancies, RMS derives from genetic alterations; the two predominant histological RMS variants, termed alveolar and embryonal, are, respectively, associated

* *Corresponding author*: Address: Department of Biomedical Sciences and Biotechnologies, University of Brescia, Viale Europa 11, 25123 Brescia, Italy. Tel.: +39 030 3717567; fax: +39 030 3701157.
E-mail address: fanzani@med.unibs.it (A. Fanzani).

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to translocations of chromosomes 2 and 13 and deregulation of imprinted genes in chromosome region 11p15.5.[2–5] The RMS embryonal RD cell line is a widespread tool to investigate the molecular pathways that contribute to the generation and maintenance of the malignant phenotype of cultured RMS cells. Previous studies showed that elevated myostatin expression,[6–8] deregulated extracellular regulated kinase (ERK) signalling[9–11] and deficient activation of the p38 pathway[12,13] contribute to the differentiation block in RD cells. Myostatin is a muscle-specific transforming growth factors-β (TGF-β) family member[14], which regulates muscle growth during development and post-natal life through the engagement of activin receptors type II (ACTRIIa and ACTRIIb).[15] Myostatin/ACTRII and trans-phosphorylation of type I ALK4 or 5 receptors (activin receptor-like kinase) activate a variety of downstream effectors,[16,17] including SMAD2/3 (small mother against decapentaplegic)[18,19] and the mitogen-activated protein kinases (MAPK) cascade.[20,21] However, the relative contribution of the myostatin signalling and MAPK cascades to rhabdomyosarcomagenesis, and their functional interactions in RMS cells, have not been established. Herein, we have expressed a dominant-negative ACTRIIb receptor (dnACTRIIb) or a constitutively activated ALK5 (caALK5) form in the RD cells, to antagonise or mimic the myostatin signalling,[22] respectively. The analysis of these cells led to the identification of a complex intracellular network, composed by the myostatin-SMAD2/3, ERK and p38 cascades, that control RD proliferation and differentiation. An exaggerated activation of myostatin-SMAD2/3 and ERK pathways blocks differentiation in RD cells. Conversely, activation of p38 signalling promotes RD differentiation, by countering the ERK and myostatin pathways.

2. Materials and methods

All reagents were from Sigma–Aldrich, if not otherwise indicated.

2.1. Cell cultures and pharmacological treatments

Human embryonal rhabdomyosarcoma RD cells were purchased from European Collection of Cell Cultures (ECACC). Cells were routinely maintained at 37 °C in 5% CO_2 humidified incubator and cultured at a sub-confluent density in high-glucose Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (Invitrogen) and 100 μg/ml penicillin–streptomycin antibiotic, defined as a growth medium (GM). To induce differentiation, confluent cells were daily supplemented with a differentiating medium (DM), consisting of DMEM supplemented with 2% horse serum. 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment (0.1 μM) was performed when the cells reached 80% confluence in GM, without replacing the medium over the entire time-course. The pharmacological compound SB203580 (5 μM) was used to inhibit the p38 activity, whereas PD98059 (5 μM) or U0126 (5 μM) compounds were used to inhibit the ERK activity. Recombinant human myostatin was purchased from BioVendor.

2.2. Cell transfections

Cell transfections were carried out with Lipofectamine 2000 reagent (Invitrogen), according to manufacturer's recommendations. To obtain stably transfected antibiotic-resistant clones, RD cells were co-transfected together with a pBabe plasmid harbouring a puromycin gene resistance, and transfectants were cultured in the presence of 1 μg/ml puromycin antibiotic.

2.3. Plasmids

MDAF2/dnACTRIIb vector harbours a membrane-bound form of murine activin receptor type IIb lacking the kinase domain (aminoacids 1–174),[22] whose expression is under the control of a myosin light chain promoter and 1/3 enhancer and simian virus 40 processing sites.[23] The vectors pCDNA-MKK6EE or -MKK6AA allow the expression of a constitutively activated or a kinase-dead MAP kinase kinase 6 (MKK6) form, respectively.[12] The vector pCDNA-caALK5 harbours a hemagglutinin (HA)-tagged point-mutated ALK5 receptor form (T204D) that results constitutively activated. The pGL3-$(CAGA)_{12}$-luc plasmid serves as a sensor for the detection of SMAD3 activity.[24]

2.4. Semi-quantitative RT-PCR analysis

Total RNA was obtained by tri-reagent extraction, digested with DNase (DNA-free, Ambion), and reverse-transcribed (2 μg) in the presence of 400 Units of Moloney Murine Leukaemia Virus Reverse Transcriptase enzyme (Promega). The following forward and reverse primers (250 nM each) were used: 5′-CTCGGGAGTGCATCTACTACAACG-3′ and 5′-TTCCGATGACGATACATCCAGAAG-3′ amplify a 415 bp (base pairs) long fragment of the murine ACTRIIb receptor; 5′-CACGGAAGGCATGCGCACGATTCT-3′ and 5′-TCGATGGGGTATATCAAGGTACGCT-3′ amplify a 591 bp long fragment of the human cyclin A1. Gene expression levels were normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression.

2.5. Antibodies

The following antibodies were used: mouse monoclonal anti-Cav-3 from BD Transduction Laboratories (clone 26), mouse monoclonal anti-myogenin from Santa Cruz Biotechnology (clone F5D), mouse monoclonal anti-MyHC from Hybridoma Bank (University of Iowa), rabbit polyclonal anti-HA from Sigma–Aldrich and a mouse monoclonal anti-alpha-tubulin from Sigma–Aldrich (clone B-5-1-2). Rabbit polyclonal antibodies from Cell Signaling were used to detect phosphorylated p38 (Thr^{180}/Tyr^{182}), SMAD2 (Ser^{465}/Ser^{467}) and SMAD3 (Ser^{423}/Ser^{425}) forms versus total forms. Mouse monoclonal antibodies from Santa Cruz Biotechnology were used to detect phosphorylated ERK (Tyr^{204}) forms versus total forms.

2.6. Immunoblotting

Equal amounts of protein samples, assayed by bicinchoninic acid method (Pierce), were resolved by SDS–PAGE under reducing conditions and transferred to PVDF membranes.

Incubation with specific primary antibodies was followed by horseradish peroxidase-conjugated secondary antibodies (Chemicon), and the immunocomplexes were visualised using enhanced chemiluminescence reagent (Chemicon). To detect myogenin, caveolin 3 (Cav-3), myosin heavy chain (MyHC) and alpha-tubulin, homogenates were prepared by harvesting the cells in RIPA lysis buffer, consisting of 20 mM Tris–HCl pH 7.6, 50 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS and proteases inhibitors (Roche Molecular Biochemicals). For immunodetection of the phosphorylated p38, ERK, SMAD2 and 3 versus total forms, cell homogenates were prepared by using a lysis buffer composed of 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.2% Triton X-100 and a mix of proteases and phosphatases inhibitors (0.5 mM NaF and Na_3VO_4).

2.7. *Immunofluorescence*

RD cells were seeded onto sterile coverslips (12 mm) coated with 20 μg/ml laminin (Roche); the cells were then fixed with ice cold methanol for 10 min at −20 °C, washed with phosphate buffered solution (PBS), treated with 3% bovine serum albumin (BSA)/PBS for 15 min, and incubated for 2 h in a humid atmosphere in the presence of a 1:200 diluted mouse monoclonal MyHC antibody (Hybridoma Bank, University of Iowa), followed by incubation with a 1:500 diluted anti-mouse Cy3 conjugated secondary antibody (Li StarFish), in the presence of Hoechst reagent to stain nuclei. Fluorescent staining was observed under an Axiovert S100 microscope (Zeiss). Pictures were taken with a digital camera (SensiCam) using the Image-Pro Plus software version 6.2.

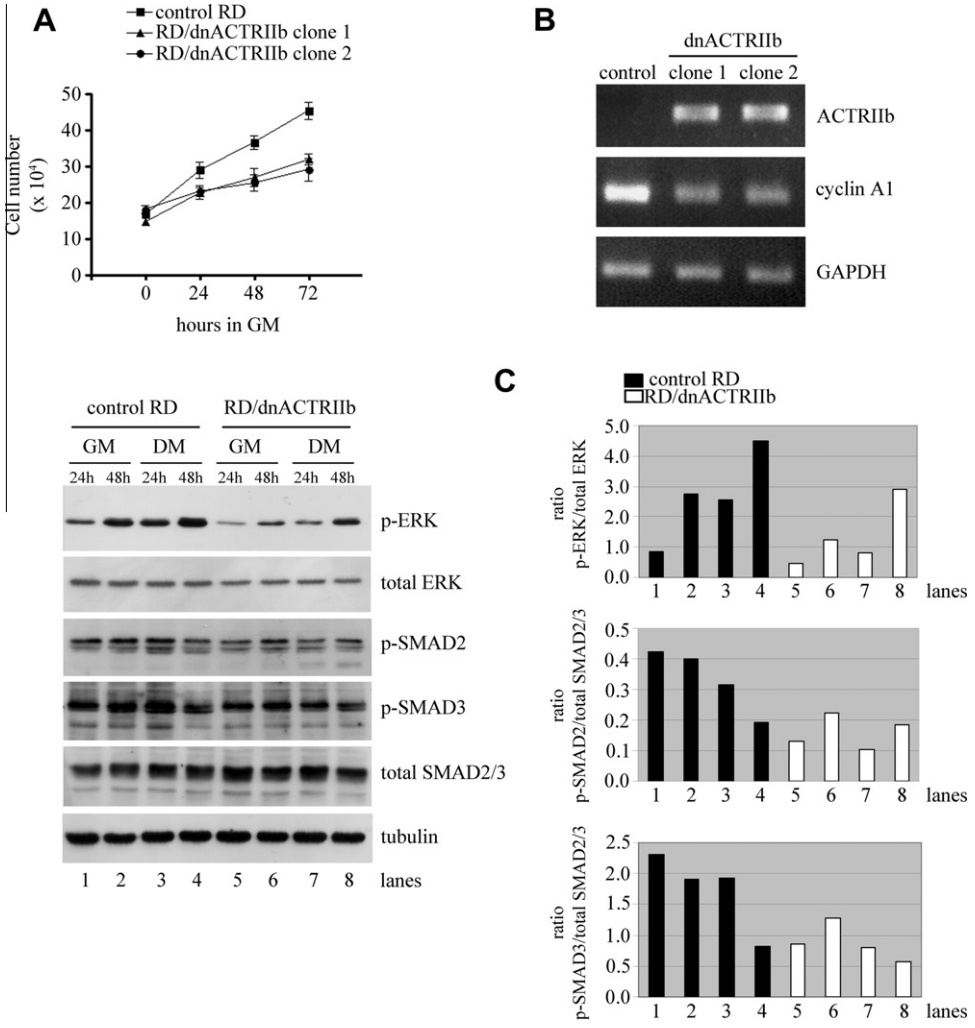

Fig. 1 – Delivery of a dominant-negative activin receptor type IIb form (dnACTRIIb) delays RD cell proliferation and reduces ERK and SMAD2 and 3 phosphorylation. (A) Graphic representation of the proliferative capacity of control RD and RD/dnACTRIIb clones, as evaluated by performing a cell counting over a time-course in GM for up to 72 h. At the beginning of the kinetic, the number of the cells seeded was 15,000 cells/cm^2. Results are the mean values of three independent experiments. (B) Semi-quantitative RT-PCR analysis was performed to compare the transcript expression of ACTRIIb and cyclin A1 between control RD and RD/dnACTRIIb clones cultured in GM. GAPDH amplification was used to normalise the transcript expression levels. (C) Immunoblotting and relative densitometric quantification showing the phosphorylation levels of ERK, SMAD2 and SMAD3 versus total forms in control and RD/dnACTRIIb cells cultured in GM or DM for up to 48 h. Tubulin was used as loading control.

3. Results

3.1. *A dominant-negative form of activin receptor type IIb delays RD cell proliferation and reduces the phosphorylation levels of ERK, SMAD2 and SMAD3*

We stably transfected the human embryonal RD cells with an expression vector harbouring a dominant-negative activin receptor type IIb (dnACTRIIb) mutant, which was reported to induce doubling of muscle mass in transgenic mice by antagonising the myostatin signalling.[22] After antibiotic selection, few resistant clones were selected; in particular, our analysis focused on two different transfectants, namely RD/dnACTRIIb clone 1 and 2, exhibiting a decrease in the proliferation rate by about 40% compared to control cells, as graphically reported after a cell counting assay (Fig. 1A). To measure the amount of transgene expression, we performed a semi-quantitative RT-PCR analysis to compare the abundance of ACTRIIb transcript between RD cells transfected with an empty vector (control) or with dnACTRIIb vector (Fig. 1B). Accordingly, dnACTRIIb expression coincided with the down-regulation of cyclin A1 (Fig. 1B), a gene typically deregulated in RD cells, which contributes to their uncontrolled proliferation.[25] These data indicate that the withdrawal from the cell cycle might be anticipated in RD/dnACTRIIb clones, as compared to control cells. ACTRIIb is a cell surface type II TGF-β receptor, which is bound by myostatin,[15] activins[26] and GDF11.[27] Upon ligand binding, TGF-β receptors initiate a SMAD-dependent and -

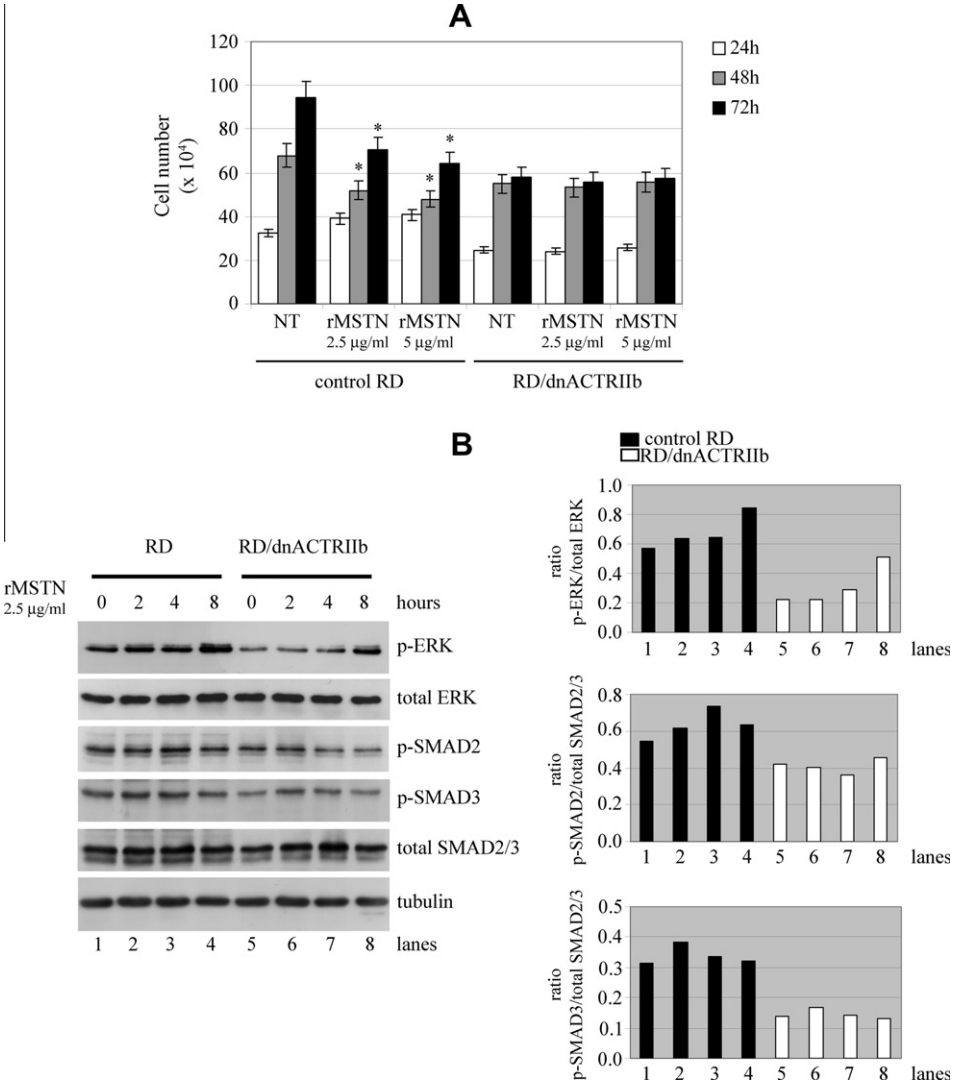

Fig. 2 – Exogenous administration of human recombinant myostatin decreases the proliferation and enhances the phosphorylation of ERK, SMAD2 and 3 pathways in RD cells, but not in RD/dnACTRIIb cells. (A) RD and RD/dnACTRIIb cells, seeded with a density of 20,000 cells/cm^2, were counted after 24, 48 and 72 h of growth in GM in the absence (NT, not treated) or presence of daily administration of 2.5 or 5 μg/ml recombinant myostatin (rMSTN). Results are the mean values of two independent experiments. Significant at $^*p < 0.05$ versus NT. (B) Immunoblotting and relative densitometric quantification showing the phosphorylation levels of ERK, SMAD2 and SMAD3 versus total forms in control and RD/dnACTRIIb cells cultured in GM, in the absence or presence of 2.5 μg/ml rMSTN for the indicated time-points. Tubulin was used as loading control.

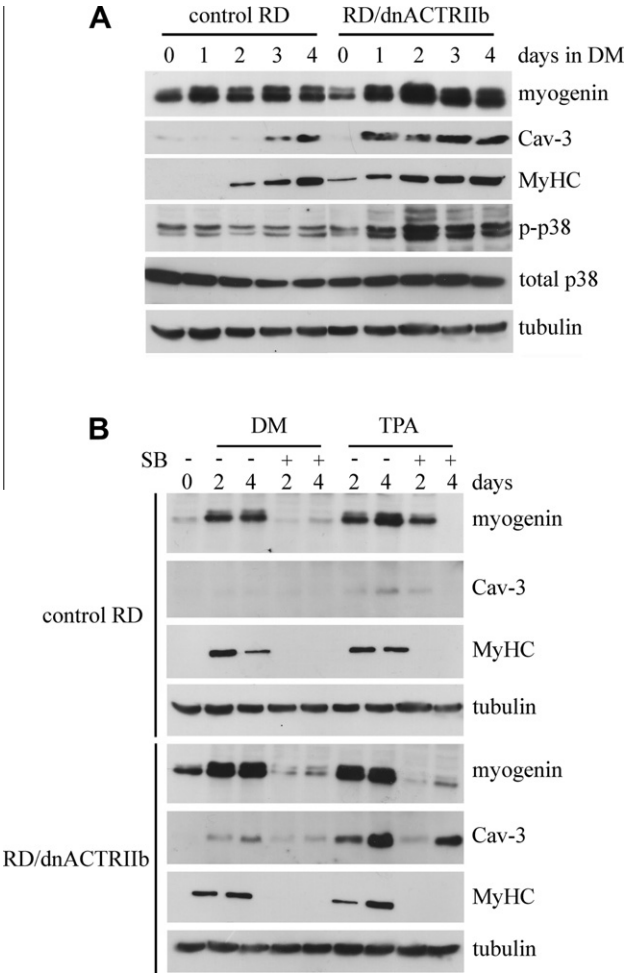

Fig. 3 – Forced dnACTRIIb expression enhances the expression of several myogenic markers via p38 activation. (A) Immunoblotting showing the levels of myogenin, Cav-3, MyHC and phosphorylated p38 versus total forms, as detected in control and RD/dnACTRIIb cells over a time-course differentiation. Tubulin was used as loading control. (B) Immunoblotting showing the levels of myogenin, Cav-3 and MyHC in control and RD/dnACTRIIb cells, as detected after DM or TPA exposure over a time-course differentiation, in the absence or presence of 5 μM SB compound. Tubulin was used as loading control.

independent signalling.[16,17] In particular, most of the effects exerted by myostatin rely on the activation of SMAD2/3[18,19] and ERK pathways,[21] and, therefore, we investigated their activation status upon ectopic dnACTRIIb expression. Since the biological behaviour of both dnACTRIIb clones was undistinguishable, hereafter we employed the clone 1 (generally termed dnACTRIIb) for the subsequent experiments. Control and dnACTRIIb cells were cultured in GM for up to 24–48 h and, once the confluence was reached, were switched to DM for an additional 24–48 h. A decreased phosphorylation of ERK, SMAD2 and 3 was observed in dnACTRIIb cells compared to control (Fig. 1C), regardless of the culture conditions. Thus, the introduction of a kinase-deficient ACTRIIb mutant reduces the trans-phosphorylation of SMAD proteins and interferes with the ERK pathway. To further prove that dnACTRIIb form is effectively able to counteract the action of myostatin, recombinant myostatin (rMSTN) was administered to both control and dnACTRIIb cells cultured in GM for up to 72 h. As shown in Fig. 2A, in the presence of 2.5 or 5 μg/ml rMSTN the proliferation rate of control RD cells was significantly reduced within 72 h, whereas the growth of RD/dnACTRIIb cells was unaffected. As detected by immunoblotting and densitometric band analysis, the phosphorylation levels of ERK, SMAD2 and 3 were increased in RD cells, but were unaffected in RD/dnACTRIIb cells upon treatment with rMSTN (Fig. 2B).

Taken together, these data indicate an inverse relationship between ACTRIIb-activated signalling and the ability of RD cells to arrest the cell cycle.

3.2. *Ectopic expression of the dnACTRIIb form enhances the differentiation of RD cells in a p38-dependent manner*

Down-modulation of the ERK pathway is often predictive of growth arrest and differentiation in RD cells.[28–30] Since dnACTRIIb cells exhibited reduced ERK and SMAD2 and 3 pathways, we cultured control and RD/dnACTRIIb cells in DM to measure the expression of different myogenic markers. Increased levels of myogenin, Cav-3 and MyHC were detected in RD/dnACTRIIb cells, in comparison to control cells (Fig. 3A), further indicating that the delivery of dnACTRIIb first attenuated the proliferation, and then stimulated the differentiation. The simultaneous block of the cell cycle followed by the activation of a differentiation programme is reminiscent of the effects observed in response to reactivation of the p38 pathway in a subset of RMS cell lines showing deficient p38 activity.[12] Accordingly, the enhanced differentiation of RD/dnACTRIIb cells was accompanied by the increased levels of phosphorylated p38 form, as compared to control cells (Fig. 3A). To establish the functional impact of p38 pathway on the dnACTRIIb-induced RD differentiation, the activity of p38 kinases alpha and beta was either blocked by SB203580 inhibitor (SB, 5 μM) or stimulated by the presence of 0.1 μM TPA, a pharmacological trigger.[13,31] Inhibition of the p38 pathway abrogated the differentiation in RD/dnACTRIIb cells (Fig. 3B). Conversely, activation of p38 pathway by either DM or TPA treatment increased the protein levels of myogenin, Cav-3 and MyHC in RD/dnACTRIIb cells, in comparison control RD cells (Fig. 3B). Morphologically, RD/dnACTRIIb cells cultured in DM showed the typical morphology of differentiated muscle cells (multinucleation) and displayed increased MyHC staining, as compared to control RD (Fig. 4A). Remarkably, the administration of TPA induced near totality of RD/dnACTRIIb cells to differentiate and form MyHC positive myotubes, in comparison with TPA-treated control cells (Fig. 4A). In particular, in the presence of DM or TPA, the percentage of MyHC positive myotubes in dnACTRIIb cells was about two and threefold increased compared to control, respectively (Fig. 4B). Remarkably, differentiation of both RD/dnACTRIIb and control cells induced by DM or TPA was prevented by the continuous blockade of p38 pathway through SB administration, as demonstrated by the round-shape cell morphology and the absence of MyHC positive myotubes (Fig. 4A and B). To definitely correlate the effects of dnACTRIIb on the p38 activation, we transiently transfected a constitutive active form of MKK6, termed MKK6EE, which activates p38 kinases,

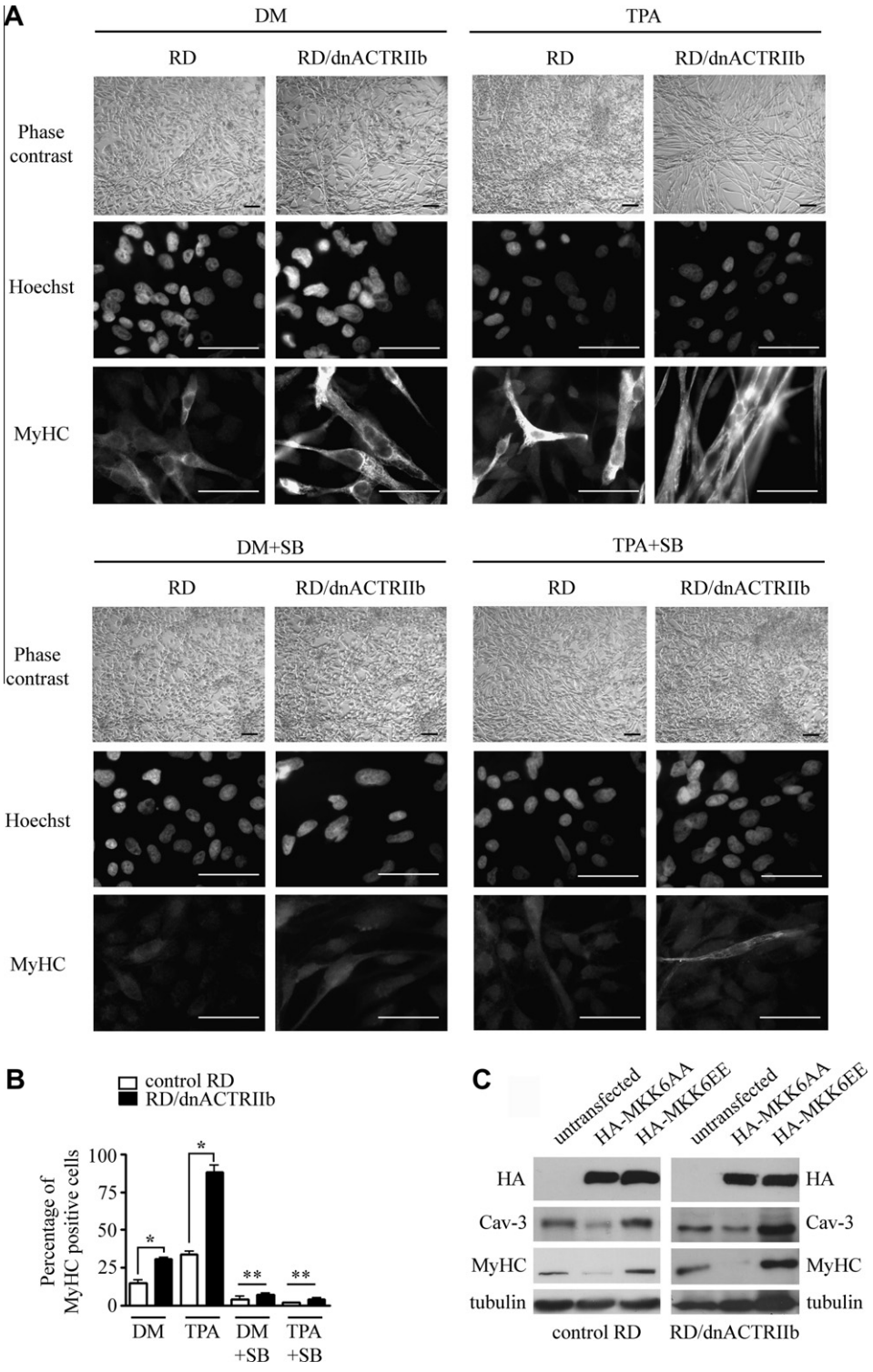

Fig. 4 – The morphological differentiation of RD/dnACTRIIb cells is abolished or further improved by the pharmacological inhibition or stimulation of p38 activity, respectively. (A) Morphological visualisation of control and RD/dnACTRIIb cells treated with DM or TPA for up to day 4, in the absence or presence of the p38 inhibitor SB (DMSO was used as vehicle), as obtained by phase contrast pictures or by immunofluorescent MyHC staining. Hoechst staining was performed to visualise the total number of nuclei present in each microscopic field. Bars = 100 μm. (B) The graph reports the percentage of MyHC positive myotubes observed under the conditions seen above, as calculated by the ratio between the number of MyHC positive cells and the total number of Hoechst-positive nuclei counted in 10 microscopic field. Significant at $^{*}p < 0.05$ versus control and at $^{**}p < 0.05$ versus DM or TPA. (C) After 72 h of transient transfection of HA-MKK6AA and -EE forms, immunoblotting was performed to verify the amount of HA-tagged proteins, Cav-3 and MyHC in control and RD/dnACTRIIb cells. Tubulin was used as loading control.

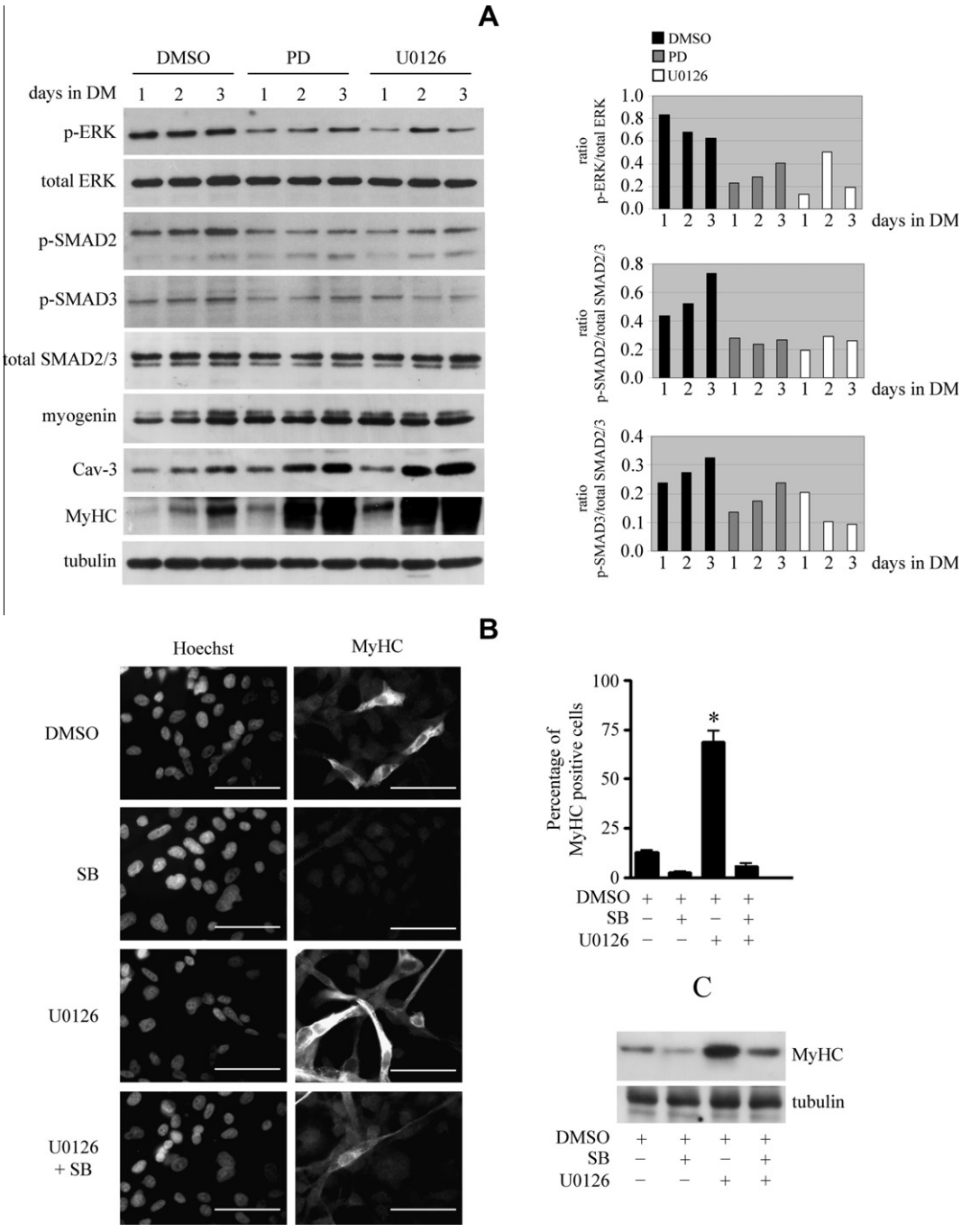

Fig. 5 – Chemical prevention of ERK signalling leads to a down-regulation of SMAD2 and 3 pathways and enhances the myogenic conversion of RD cells via p38 activation. (A) RD cells were daily treated with the vehicle (DMSO) or with either 5 μM PD or U0126 inhibitors for up to day 3. Under these conditions, immunoblotting and relative densitometric quantification show the phosphorylation levels of ERK, SMAD2 and 3 versus total forms, and the amounts of myogenin, Cav-3 and MyHC. Tubulin was used as loading control. (B) Immunofluorescent staining of MyHC was employed to label differentiating RD cells exposed to DM for up to day 3, in the presence of daily administration of SB, U0126 or simultaneous U0126 and SB treatment, in comparison with DMSO. Bars = 100 μm. Quantification of MyHC positive cells is graphically reported as the ratio between the number of MyHC positive cells and the total number of Hoechst-positive nuclei counted in each microscopic field. Significant at $^{*}p < 0.05$ versus DMSO. (C) Under the conditions seen above, immunoblotting was performed to evaluate the levels of MyHC protein. Tubulin was used as loading control.

or its phosphorylation-resistant inactive form, termed MKK6AA, which functions with a dominant-negative fashion.[12] After 72 h of transfection, HA-tagged MKK6EE and -AA forms were detected in control and RD/dnACTRIIb cells (Fig. 4C). Compared to untransfected cells, Cav-3 and MyHC were increased after MKK6EE expression in both control and

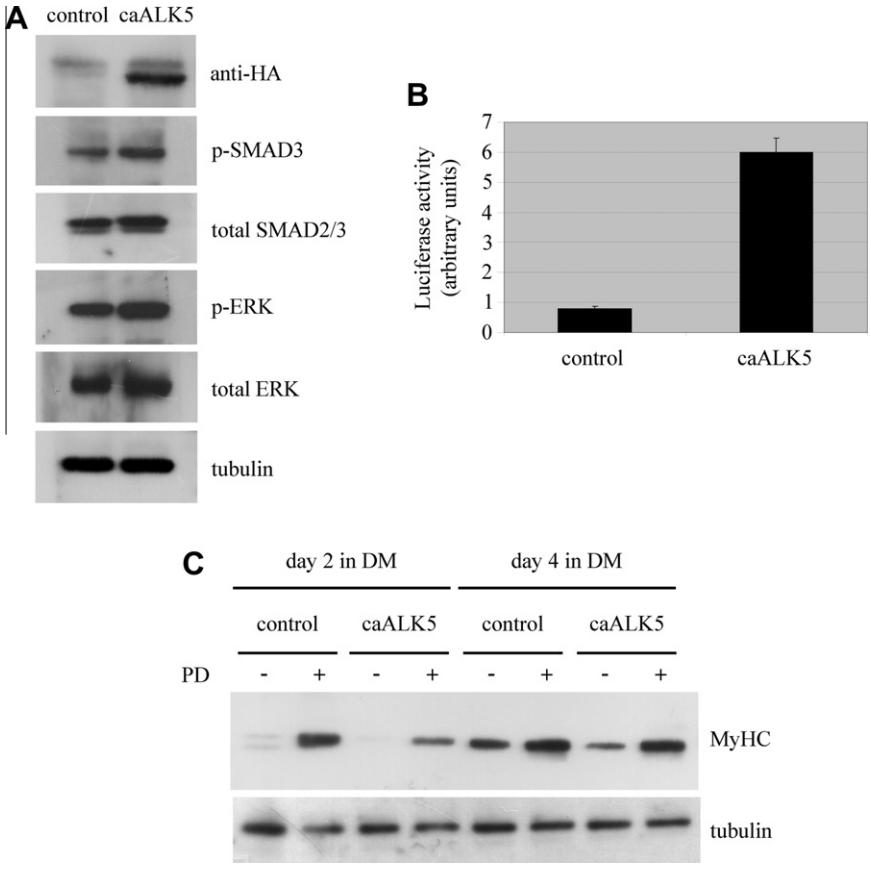

Fig. 6 – The impairment of RD differentiation, triggered by the expression of a constitutively activated ALK5 receptor, is countered by the pharmacological ERK inhibition. (A) Immunoblotting showing the phosphorylation levels of SMAD3 and ERK versus total forms in control and RD-caALK5 cells. Tubulin was used as loading control. (B) A luciferase reporter assay was performed on control and RD-caALK5 cells, after the transient transfection of a SMAD-sensor for up to 24 h. The reporter activity is expressed as the ratio between Firefly and Renilla luciferases. (C) Immunoblotting showing the expression of MyHC in control and RD-caALK5 cells, left untreated or treated with the ERK inhibitor PD in the presence of DM for up to day 2 or 4. Tubulin was used as loading control.

RD/dnACTRIIb cells, whereas the expression of MKK6AA reduced Cav-3 and MyHC levels (Fig. 4C). Thus, the interruption of myostatin signalling, as obtained through the expression of dnACTRIIb form or the constitutive activation of the p38 pathway, shows equivalent effects on RD cells, including the inhibition of proliferation and the induction of differentiation. Importantly, the differentiation is invariably prevented by the blockade of p38 pathway.

3.3. *Pharmacological ERK inhibition in RD cells is per se sufficient to trigger a p38-dependent differentiation which causes attenuation of SMAD2 and 3 phosphorylation*

A previous work has shown that inhibition of the ERK pathway restores the differentiation in RD cells, an effect coincident with p38 activation.[13] We wished to establish a functional relationship between ERK, p38 and SMAD2/3 pathways in RD cells; to this purpose, we performed the pharmacological ERK inhibition in RD cells with two different compounds, namely PD98059 (PD) and UO126. As shown by immunoblotting and densitometric band analysis (Fig. 5A), daily administration of either PD or U0126 compounds prevented the ERK phosphorylation, as detected over a 3 days time-course. Under these conditions, the phosphorylation levels of SMAD2 and 3 were reduced, an effect accompanied by an increased expression of myogenin, Cav-3 and MyHC. RD differentiation induced by ERK inhibition was univocally linked to the p38 activation, as the expression of MyHC, monitored through either immunofluorescence (Fig. 5B) or immunoblotting (Fig. 5C), increased in U0126-treated cells compared to dimethylsulphoxide (DMSO)-treated cells, but was almost completely abolished upon simultaneous administration of U0126 with the p38 inhibitor SB. Taken together, these data confirm previous findings showing that RD differentiation induced by pharmacological prevention of the ERK pathway requires the simultaneous activation of p38 pathway. They also indicate that the down-modulation of SMAD2/3 pathway plays a permissive role in the differentiation of RD cells in which the ERK pathway has been inhibited.

3.4. *Expression of a constitutive active ALK5 receptor determines increased SMAD3 and ERK phosphorylation levels in RD cells*

Upon binding with myostatin, ACTRIIb receptor commonly employs ALK4 or ALK5 receptors to signal via SMAD-depen-

dent or -independent pathways.[18–21] To mimic the action of myostatin, we generated a stably transfected RD cell line harbouring a constitutively activated HA-tagged ALK5 (caALK5) form, whose expression was confirmed by immunoblotting (Fig. 6A). RD-caALK5 cells showed an increased phosphorylation of SMAD3, in comparison to control cells (Fig. 6A). To prove that, in the presence of caALK5 receptor, the observed increase in SMAD3 phosphorylation led to activation of the downstream effectors, we employed a SMAD-sensor, consisting of a tandem repetition of $(CAGA)_{12}$ elements that allow SMAD recruitment.[24] As shown in Fig. 6B, the luciferase activity of SMAD-sensor was greatly increased in RD-caALK5 cells, as compared to control cells, confirming that the SMAD3 pathway is activated in these cells. Interestingly, RD-caALK5 cells also exhibited increased levels of the phosphorylated ERK forms (Fig. 6A). To verify the effects produced by caALK5 receptor on the differentiation of RD cells, we cultured control and RD-caALK5 cells in DM, in the absence or presence of the pharmacological ERK inhibitor PD. After 2 and 4 days of treatments, morphological differentiation was monitored by immunoblotting analysis of MyHC. As shown in Fig. 6C, the expression of MyHC was reduced in RD-caALK5 cells compared to control cells, indicating that the over-activation of SMAD3 and ERK pathways diminishes the ability of RD cells to differentiate. Interestingly, ERK inhibition recovered the differentiation of RD-caALK5 cells after 4 days of treatment, as observed for control cells (Fig. 6C), once again suggesting that the inhibition of the ERK pathway plays a dominant role over the SMAD pathway in RD cells.

4. Discussion

The data reported above showed for the first time that a kinase-deficient ACTRIIb form promotes differentiation in the RMS embryonal RD cells. ACTRII (ACTRIIa and ACTRIIb) are TGF-β type II receptors that bind potent negative regulators of skeletal muscle mass, such as myostatin, activins and GDF11.[15,26,27] Previous works have shown that elevated levels of myostatin or increased TGF-β signalling inhibit proliferation and differentiation of RMS cells, including RD cell line.[6,7,32] These effects resemble those observed in normal myoblasts.[33] Accordingly, myostatin blockade promotes terminal differentiation of embryonal RD cells.[6,8] Loss of myostatin elicits an abnormal development of muscle mass in mammals,[22] and the muscular 'phenocopy' is observed in transgenic mice carrying a dnACTRIIb form,[15] suggesting that most of the effects of myostatin rely on the pathways triggered through this receptor. Upon ligand binding, two ACTRIIb dimers bind with two TβRI receptors (ALK receptors), enabling TβRII to phosphorylate the short GS domain of TβRI on serines and threonines, which initiate the SMAD-mediated signalling. Additionally, TβRI is also tyrosine phosphorylated in response to TGF-β ligands,[34] allowing recruitment of ShcA/Grb2/Sos complex that translocates to plasma membrane and activates Ras, which in turn stimulates ERK phosphorylation. Although activation of ERK MAP kinase is usually much weaker than in response to receptor tyrosine kinase signalling, its activation is thought to be important in regulating SMAD function and various TGF-β-induced responses.[16] Indeed, our study shows that the increased differentiation of RD cells, expressing the kinase-deficient dnACTRIIb receptor, coincides with a down-regulation of ERK and SMAD2 and 3 pathways, and the activation of p38 pathway, which is typically deficient in RD cells.[12] In addition, RD cells treated with an ERK inhibitor resemble dnActRIIb cells, being characterised by a p38-dependent differentiation and a down-modulation of SMAD2 and 3 pathways. Thus, this confirms that a functional antagonism between ERK and p38 pathways contributes to determine proliferation versus differentiation in the embryonal RD cells,[12,28–30] as already described in normal myoblasts[35] and other cell types.[36] ERK pathway is frequently over-activated in RMS cells from embryonal derivation, such as RD cells, because of the presence of activating RAS mutations,[37] leading to a reduced differentiation featured by the block of p38 pathway.[12] Accordingly, MEK/ERK inhibition is sufficient to promote differentiation and reduce RMS tumour growth as a consequence of the p38 activity restoration,[28–30] whereas the forced activation of p38, as obtained via expression of the upstream MKK6 kinase,[12] increases differentiation and reduces the ERK pathway activation. Thus, it is reasonable that these pathways negatively influence each other, as previously suggested.[36] In support of the functional relationship between ERK and p38 pathways, our data further suggest that the activation of SMAD2 and 3 pathways may cooperate together with the ERK pathway to impair differentiation, while a p38-dependent differentiation requires the block of ERK and the attenuation of SMAD2 and 3 pathways. This evidence suggests a previously unappreciated functional relationship between ERK, SMAD and p38 pathways in the regulation of proliferation and differentiation of muscle progenitors. In RD cells, an exaggerated myostatin signalling appears to promote SMAD and ERK activation, which leads to the inhibition of p38 pathway, thereby inhibiting differentiation. This network is relevant, when considering the impact of deregulated p38 signalling in RMS and other cancer cells.[38,39] In muscle cells p38 directs the assembly of the MyoD-associated complex in the regulatory regions of muscle-specific genes,[40] as observed in the case of MyoD-E47 heterodimers,[41] and allows the recruitment of SWI/SNF,[42,43] and Ash2L-containing mixed-lineage leukaemia (MLL) methyltransferase complex.[44] Interestingly, RD cells fail to form MyoD-E protein heterodimers[45] and have an impaired recruitment of SWI/SNF chromatin complex at the muscle loci.[46] Moreover, down-regulation or mutations of SWI/SNF components have been described in RMS.[47,48] It is likely that sustained ERK signalling in RMS, due to RAS mutations or activated myostatin pathway, inhibits the formation of the MyoD-associated complex on the chromatin of muscle genes indirectly, by preventing activation of the p38 signalling. In this context, the potential contribution of SMAD pathway remains to be established. In addition, independent control of MyoD activity by the ERK upstream activator – MEK1 – was suggested by previous studies[49] and could contribute to regulate muscle gene expression in RD cells. Thus, it is not surprising that the block of myostatin pathway leads to differentiation of RD cells by a sustained p38 activation. In apparent contrast to our findings, it has been shown that myostatin promotes activation of p38 in RMS A204 cells through the TGF-β-activated kinase 1

(TAK1),[50] suggesting that blunting myostatin pathway should down-regulate p38 rather than activate it. However, beyond the different origin of RD and A204 RMS lines, it is well established that the biological effects exerted by TGF-β ligands, such as myostatin, rely on a transient activation of SMAD-dependent pathways[14,15,19] and MAPK pathways, including JNK,[20] ERK[21] and p38.[50] Instead, RD differentiation requires a long-lasting activation of p38, and this should presumably occur through a TAK1-independent pathway. Overall, our findings indicate that interventions that target the myostatin/ERK/p38 network can be effective in promoting differentiation of RMS cells.

Statement of author contributions

S.R. and E.S. conceived and carried out experiments. A.F. and P.L.P. conceived the experiments, analysed data and wrote the paper. All authors had final approval of the submitted and published versions.

Author's statement

The authors declare that the material includes an original research, which has not been previously published and has not been submitted for publication elsewhere while under consideration.

Conflict of interest statement

None declared.

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