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ORIGINAL ARTICLE

Failure to downregulate the BAF53a subunit of the SWI/SNF chromatin remodeling complex contributes to the differentiation block in rhabdomyosarcoma

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Rhabdomyosarcoma (RMS), the most common soft tissue sarcoma in children and young adults, is characterized by a partially differentiated myogenic phenotype. We have previously shown that the blocking of tumor growth and resumption of differentiation can be achieved by re-expression of miR-206, a muscle-enriched microRNA missing in RMS. In this work, we focused on BAF53a, one of the genes downregulated in miR-206-expressing RMS cells, which codes for a subunit of the SWI/SNF chromatin remodeling complex. Here we show that the BAF53a transcript is significantly higher in primary RMS tumors than in normal muscle, and is a direct target of miR-206. Sustained expression of BAF53a interferes with differentiation in myogenic cells, whereas its silencing in RMS cells increases expression of myogenic markers and inhibits proliferation and anchorage-independent growth. Accordingly, BAF53a silencing also impairs embryonal RMS and alveolar RMS tumor growth, inducing their morphological and biochemical differentiation. These results indicate that failure to downregulate the BAF53a subunit may contribute to the pathogenesis of RMS, and suggest that BAF53a may represent a novel therapeutic target for this tumor.

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INTRODUCTION

ATP-dependent remodeling complexes regulate transcription by promoting nucleosome release or repositioning along the chromatin. The SWI-SNF complex consists of an ATPase (BRG1 or BRM, mutually exclusive in mammals) responsible for chromatin unwinding, and of up to 12 additional components, the BRG1-associated factors (BAFs). BRG1 or BRM are associated with a core of highly conserved subunits (BAF47, BAF155 and BAF170) and with a fixed number of additional subunits, which may vary because of tissue- or stage-specific expression of distinct family members.[1] During differentiation and development, highly regulated permutations between these members lead to the assembly of lineage- and stage-specific SWI-SNF complexes. For example, in the transition from neural progenitors (np) to differentiated neurons (n), several subunits undergo isoforms exchange. In particular, the BAF45a and BAF53a subunits of npBAF complexes are replaced by the homologous BAF45b and BAF53b. Preventing this switch impairs neuronal differentiation. The brain-specific BAF53b subunit (which cannot be substituted by BAF53a) is responsible for the recruitment of nBAF complexes to the promoters of genes for dendritic outgrowth.[2] In myogenesis, the BAF60c subunit (which is muscle-enriched) is already expressed in proliferating myoblasts, where it marks myogenic loci for transcription by forming a pre-complex with MyoD at their promoters.[3] At the onset of differentiation, p38MAPK-induced BAF60c phosphorylation results in recruitment of the other subunits to form a transcriptionally active 'myogenic' SWI-SNF complex.[3] Contrary to the specific expression of some subunits in differentiated tissues, the BAF53a isoform is expressed in ES cells and in rapidly proliferating progenitors of all tissues. For example, in the hematopoietic system, the mRNA for BAF53a is high in stem cells and multipotent progenitors, but rapidly decreases in more mature bone marrow populations. Accordingly, BAF53a is necessary for the maintenance of hematopoietic stem and progenitor cells.[4] Similarly, in the skin BAF53a is necessary to maintain the undifferentiated state of epidermal progenitors and its conditional loss leads to cell cycle exit and terminal differentiation.[5] It remains to be seen whether an exchange of BAF53 subunits, similar to that described in neurogenesis, also occurs during differentiation of other lineages, and whether failure of downregulating BAF53a could be involved in cancer. So far, components of the SWI-SNF complex have been shown to behave mainly as tumor suppressors. Loss of heterozygosity at the Brg1 and BRM loci occurs in a variety of human cancers, and transgenic mouse models indicate that heterozygous loss of either ATPase can potentiate tumor development.[6] A highly tumorigenic mouse model also results from the conditional knock-out of the gene encoding the core BAF47 subunit,[7] which has been found mutated in rhabdoid tumors and in some primary rhabdomyosarcoma (RMS).[8]

RMS (reviewed in Merlino and Helman[9]) is the most common soft tissue sarcoma of childhood and is characterized by coexpression of markers of proliferation and myogenic differentiation. Although RMS cells express the myogenic 'master gene' MyoD, the latter is not transcriptionally active and this blocks myogenic differentiation.[10] The two major subtypes of

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RMS, alveolar (ARMS) and embryonal (ERMS), are clinically and genetically distinct. ERMS harbor diverse lesions, such as loss of the p16 or p53 tumor suppressors and loss of imprinting at the IGF2 locus, whereas ARMS harbor either the PAX-3/FOXO1 (70%) or the PAX-7/FOXO1 fusion genes (10%).[11] Nowadays, the 5-year survival rate for patients with non-metastatic ERMS is >70%. This figure results from a combination of radiotherapy, multi-agent chemotherapy and surgery, and reflects the importance of an accurate and timely diagnosis in specialized centers.[12,13] However, both ERMS and ARMS are still associated with poor overall survival (<30%) when metastatic at diagnosis.[14] Moreover traditional therapies are often not effective in children with metastatic and recurrent disease and can cause long-term side effects including secondary tumors. Therefore, novel more efficient and not-toxic therapeutic strategies are needed to cure high-risk patients and to improve the quality of their life.

We previously reported that re-introduction of physiological levels of miR-206, a MyoD-dependent muscle-specific microRNA missing in RMS, is sufficient to block ERMS and ARMS growth and to promote their differentiation.[15] This differentiation-inducing effect was confirmed by Missiaglia *et al.*[16] in several RMS cell lines. They also showed, by gene expression profiling of 84 RMS samples, that low miR-206 significantly correlated with poor overall survival and with the presence of metastasis at diagnosis.[16] Along with others, we have shown that miR-206 downregulates the Met receptor, which is often overexpressed in RMS and is essential for RMS cells survival.[17,18] A more recent study has revealed that in myogenic cells miR-206 acts as a switch for MyoD activity by changing the availability of E-protein partners and by increasing its own level of transcription, thereby locking the cells into a committed differentiation program.[19] In the present work, we link the arrested myogenesis observed in RMS to the failure of downregulating the BAF53a subunit of the SWI-SNF chromatin remodeling complex. We show that the BAF53a subunit is overexpressed in ERMS and ARMS primary tumors and cell lines, compared with normal muscle and proliferating myoblasts, and that BAF53a downregulation occurs in normal myoblasts and in RMS cells that are forced to differentiate. We show that BAF53a is post-transcriptionally downregulated by miR-206 and that its overexpression interferes with miR-206-mediated differentiation. Furthermore, BAF53a silencing is sufficient to revert the malignant phenotype and to reactivate the differentiation program of RMS cells. Finally, we show that conditional silencing of BAF53a in a xenograft mouse model of RMS blocks tumor growth and induces expression of late myogenic markers. To sum it up, this work shows that persistence of the BAF53a subunit of the SWI-SNF complex may contribute to RMS pathogenesis, and thus that BAF53a may represent a novel therapeutic target for this tumor. Finally, this work adds chromatin remodeling to the multiple controls that miR-206 exercises in the transition from growth to differentiation in myogenic cells.

RESULTS

BAF53a is overexpressed in RMS primary tumors and cell lines and is downregulated on 12-O-tetradecanoylphorbol-1-acetate-induced differentiation

The mRNA for the BAF53a subunit of the SWI-SNF complex was one of the transcripts downregulated in RMS cells by forced expression of miR-206.[15] As BAF53a is downregulated during neural, hematopoietic and epidermal differentiation,[2,4,5] we asked whether persistence of high levels of BAF53a had a role in RMS pathogenesis. Thereby we compared the level of expression of BAF53a mRNA in tumor biopsies of 101 individuals with a confirmed diagnosis of RMS with that of 30 normal muscle biopsies.[20] BAF53a was overexpressed about fivefold with respect to human skeletal muscle in all RMS subtypes ($P<0.001$, Figure 1a). Similarly, in a panel of ERMS and ARMS cell lines the level of BAF53a protein was found to be higher than in proliferating normal human myoblasts (Figure 1b). On the other hand, the level of BAF53a protein decreased significantly in concomitance with the appearance of the myosin heavy chain (MHC) late muscle marker, both in human myoblasts switched to low serum (Figure 1b, left panel) and in RD (ERMS) and RH4 (ARMS) cells forced to differentiate in low serum by 12-O-tetradecanoylphorbol-1-acetate addition (Figure 1b, right panel).

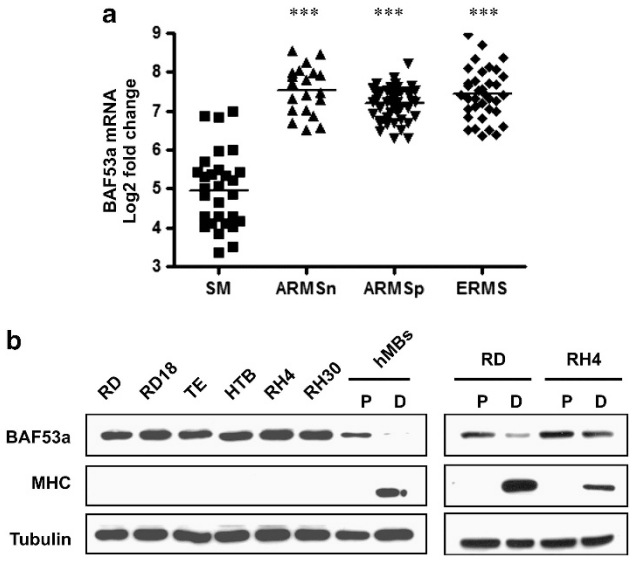

Figure 1. BAF53a is overexpressed in primary RMS tumors and cell lines and is downregulated on myogenic differentiation. (**a**) Differential expression of BAF53a mRNA in human skeletal muscle (SM), alveolar RMS negative for the PAX3/7-FOXO1 fusion gene (ARMSn), alveolar RMS positive for the PAX3/7- FOXO1 fusion gene (ARMSp) and embryonal RMS (ERMS); ***$P<0.001$. (The publicly available data set used are specified in Material and methods section). (**b**) Left panel: western blot analysis for the indicated proteins in proliferating ERMS (RD, RD18, TE, HTB) and ARMS (RH4, RH30) cell lines and in human skeletal myoblasts (hMBs) in proliferating conditions (P) and after differentiation in low serum (D). Right panel: western blot analysis for BAF53a expression in RD (ERMS) and RH4 (ARMS) cell lines in proliferating conditions (P) and after differentiation (D) induced by 12-O-tetradecanoylphorbol-1-acetate addiction in low serum.

The BAF53a protein is downregulated in miR-206-expressing RMS cells, as well as in differentiating satellite cells and MyoD-converted 10T1/2 fibroblasts

Western blot analysis of total extracts of RD18 ERMS and RH4 ARMS cells, induced to differentiate by conditional miR-206 expression, showed a strong downmodulation of BAF53a in concomitance with the upregulation of myogenic markers such as myogenin, muscle creatine kinase, desmin and MHC (Figures 2a and b). To verify whether BAF53a downmodulation occurs physiologically at the onset of myogenesis, we extended this analysis to satellite cells and to MyoD-converted 10T1/2 fibroblasts. Also in these systems, BAF53a downmodulation occurred in concomitance with the upregulation of myogenic genes (Figures 2c and d). We obtained further demonstration of the inverse correlation between nuclear BAF53a and myogenesis by performing double immunofluorescence with BAF53a and MHC antibodies on NIH10T1/2 fibroblasts after 3 days of MyoD induction. Figure 2e shows that nuclear BAF53a staining is strongly downregulated in cells positive for MHC, whereas the signal is high in the nuclei of MHC-negative cells, confirming that BAF53a downregulation occurs concomitantly with terminal myogenic differentiation.

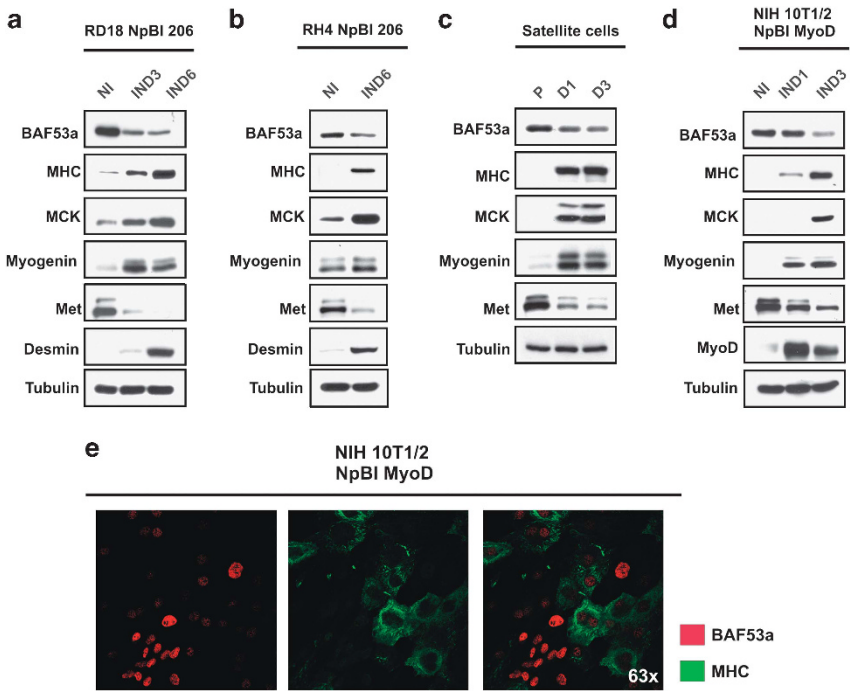

Figure 2. The BAF53a protein is downregulated in miR-206-expressing RMS cells, as well as in differentiating satellite cells and MyoD-converted 10T1/2 fibroblasts. Western blot analysis for the indicated proteins in (**a**, **b**) RD18 and RH4 RMS cells infected with a conditional miR-206-expressing lentiviral vector (NpBI-206), treated or not with doxycycline for the indicated days (miR-206 induced, IND; miR-206 not induced, NI). Cells were always kept in high serum, (**c**) satellite cells in proliferating conditions (P) and 1 and 3 days after switching to low serum, (**d**) NIH10T1/2 fibroblasts infected with a conditional MyoD-expressing lentiviral vector (NpBI-MyoD), treated or not with doxycycline for the indicated days. In cells described in (**a**–**d**) downregulation of the Met receptor, a direct target of miR-206, is shown as positive control for miR-206 induction. (**e**) Representative BAF53a (left, red), MHC (center, green) and merged (right) immunostainings of NIH10T1/2 NpBI-MyoD cells after 3 days of MyoD induction by doxycycline treatment.

BAF53a is mainly nuclear and it is found in a complex with Brg1

A cytoplasmic isoform of BAF53a has been described.[21] To further verify the subcellular localization of BAF53a in myogenic cells, we separated the nuclear and cytoplasmic fractions of miR-206-expressing RD18 cells, and of differentiating NIH10T1/2 MyoD fibroblasts and mouse satellite cells. Western blot confirmed that BAF53a is mainly nuclear, and that this fraction is downmodulated on differentiation (Supplementary Figure S1A). Nuclear extracts of RD18 cells were also used to verify the association of BAF53a to the SWI-SNF complex. BAF53a was co-immunoprecipitated by Brg1-specific antibodies and vice versa, confirming the association (Supplementary Figure S1B).

BAF53a is a direct target of miR-206

To establish whether miR-206 was responsible for the decrease in BAF53a, we transduced two non-myogenic cell lines (human HEK293 and murine NIH10T1/2 fibroblasts) with an inducible bidirectional lentiviral vector expressing miR-206 and green fluorescent protein (GFP) as a reporter. After 3 days of doxycycline treatment, the percentage of GFP-expressing HEK293 and NIH10T1/2 cells was monitored by fluorescence-activated cell sorting analysis. In both cases, GFP-positive cells were around 90% of the total, indicating a very high efficiency of transduction. Furthermore, the GFP-positive populations appeared to be highly homogeneous (Supplementary Figure S2). In these cells, BAF53a decreased significantly on miR-206 induction (Figure 3a), whereas the level of miR-206 expression remained within 'physiological' limits, below the level observed in differentiating mouse satellite cells on growth factor withdrawal (Figure 3b). Thus, miR-206 and BAF53a are inversely correlated. As the introduction of miR-206 in these cell lines is not sufficient to convert them to a myogenic fate, this confirms that downmodulation of BAF53a is a result of miR-206 expression rather than a consequence of its differentiation-promoting effect.

To determine whether miR-206 interacts directly with the BAF53a transcript, we first searched for miR-206 recognition elements (MRE) in both the human and murine 3′-untranslated regions (UTRs) using five different microRNA target prediction softwares (Targetscan, MiRWalk, miRanda, MicroCosm and RNAhybrid). We found a perfect MRE (seven base pairing) in the human BAF53a 3′-UTR (MRE1, Figure 3c, upper panel), which, however, is not conserved in rat and mouse. An additional MRE is present in both the human and mouse transcripts (five out of seven base pairing; MRE2, Figure 3c, upper panel). We then generated luciferase sensors with the wild-type and mutated human and murine 3′-UTRs. The constructs were transfected in HEK293 cells, where miR-206 could be induced by doxycycline. MiR-206 induction caused a $\approx$50% decrease in luciferase activity using the human 3′-UTR sensor (WT) and a $\approx$30% decrease using the mouse 3′-UTR (WT), respectively. No decrease in luciferase activity was seen using the mutated 3′-UTRs (Figure 3c, lower panel).

BAF53a interferes with myogenic differentiation

To investigate the effect of preventing miR-206-mediated BAF53a downmodulation during myogenesis, we generated a lentiviral vector that produces a BAF53a transcript lacking the 3′-UTR. Sustained BAF53a levels in miR-206-expressing RD18 and RH4 cells impaired myogenic differentiation, as shown by the reduced level of expression of muscle-specific genes such as desmin and MHC (Figures 4a and b). It should be noted that in these cells miR-206 induction was not affected by the increase in BAF53a (Supplementary Figures S3A and B). In fact, the endogenous

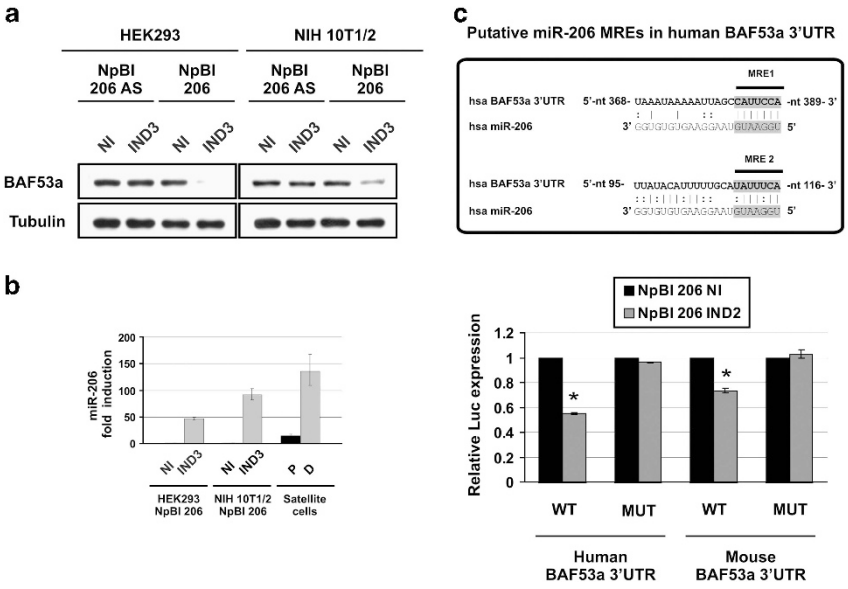

Figure 3. BAF53a is a direct target of miR-206. (**a**) Western blot analysis for the indicated proteins in HEK293 and NIH10T1/2 cells infected with either the control (NpBI-206 AS) or the conditionally expressing miR-206 (NpBI-206) lentiviral vector, treated (IND) or not (NI) with doxycycline for 3 days. (**b**) Real-time PCR for miR-206 in HEK293 NpBI-206, NIH10T1/2 NpBI-206 and in satellite cells in proliferating and differentiating conditions. (**c**) Upper panel: putative miR-206 MREs in the human (hsa) BAF53a 3′-UTR. Lower panel: relative luciferase expression in HEK293 NpBI-206 cells transfected with sensor vectors carrying the human and mouse BAF53a 3′-UTRs either wild-type (WT) or mutated (MUT), after 2 days of miR-206 induction with doxycycline. Mean values (± s.d.) are from three independent experiments, $*P < 0.05$.

BAF53a (which runs slightly ahead of its flagged counterpart) was downregulated on miR-206 induction (Figures 4a and b). The BAF53a gain-of-function experiment was done also in satellite cells, and in NIH10T1/2 fibroblasts, which rapidly undergo myogenic differentiation on MyoD induction. Western blot analysis confirmed that in both cases increased BAF53a levels impaired expression of muscle markers on differentiation (Figures 4c and d). The above results altogether indicate that BAF53a downregulation is necessary for optimal myogenic differentiation.

Conditional silencing of BAF53a interferes with the transformed phenotype of ERMS and ARMS cells and promotes myogenic differentiation

To verify whether BAF53a silencing in RMS cells could affect their transformed phenotype and ultimately promote differentiation, we infected RD18 and RH4 cells with an inducible system that allows doxycycline-dependent expression of a BAF53a-directed short hairpin RNA (shRNA). In both cell lines, doxycycline addition to the culture medium caused a strong reduction of the proliferation rate (Figures 5a and b), in concomitance with BAF53a silencing (Figures 5e and f). We next examined if BAF53a silencing could affect anchorage-independent growth of ERMS and ARMS cells. BAF53a silencing markedly decreased soft agar colony formation (Figures 5c and d). It should be noted that silencing of BAF53a was sufficient to induce expression of early (myogenin) and late (desmin, MHC) myogenic markers especially in RD18 (ERMS) but also in RH4 (ARMS) cells in spite of the high serum (10% fetal bovine serum) present in the culture medium (Figures 5e and f). In RH4 cells, which have a higher basal level of myogenin and muscle creatine kinase with respect to RD18 cells, the increase in differentiation markers was evident only for desmin (Figure 5f). Furthermore, while after 6 days of induction of the shRNA the MHC-positive cells were close to 30% in RD18, in RH4 they were only 4% (Supplementary Figures S3A and B). On the other hand, a slightly higher percentage of RH4 cells underwent apoptosis (Supplementary Figure S4C). In both cell lines, miR-206 expression increased following BAF53a silencing (Supplementary Figures S4D and E), indicating resumption of MyoD-dependent transcriptional activity.[22] Supplementary Figure S5 shows that results essentially overlapping with those described above were obtained with a second BAF53a-directed shRNA.

Conditional silencing of BAF53a impairs ERMS and ARMS tumor growth

To assess whether BAF53a silencing could have therapeutic potential for RMS treatment, we injected nude mice with RD18 and RH4 cells where a BAF53a-directed or a control shRNA could be induced by doxycycline administration. After 2 weeks of doxycycline treatment, while tumors where a control shRNA was induced continued to grow, tumors where BAF53a had been silenced were significantly smaller (Figures 6a and b). Histological analysis of the tumors showed a reduction of the Ki67 proliferation marker and upregulation of MHC expression with respect to controls, more evident in RD18 than in RH4 xenografts (Figures 6c and d, upper panels). Western blots of tumor samples confirmed MHC positivity on silencing of BAF53a (Figures 6e and f, lower panels). Altogether these and the above results indicate that BAF53a silencing interferes with the transformed phenotype of RMS cells *in vitro* and impairs RMS tumor growth *in vivo*, essentially by inducing myogenic differentiation.

DISCUSSION

In previous work, we provided proof of concept that miR-206, a skeletal muscle-specific microRNA, could be used to block tumor growth by forcing RMS cells to resume differentiation.[15] Its anti-proliferative effect could be explained by downregulation of a number of validated targets such as Pola I[23] and cyclin D2[24,25] (components of the DNA replication and cell cycle machinery), Pax-3 and Pax-7[26,27] (transcription factors of early muscle progenitors), and the Met receptor[15] (growth factor signaling). However, miR-206 was able not only to stop RMS cells growth but also to switch their transcriptome toward that of mature muscle, suggesting an 'instructive' function in reprogramming the

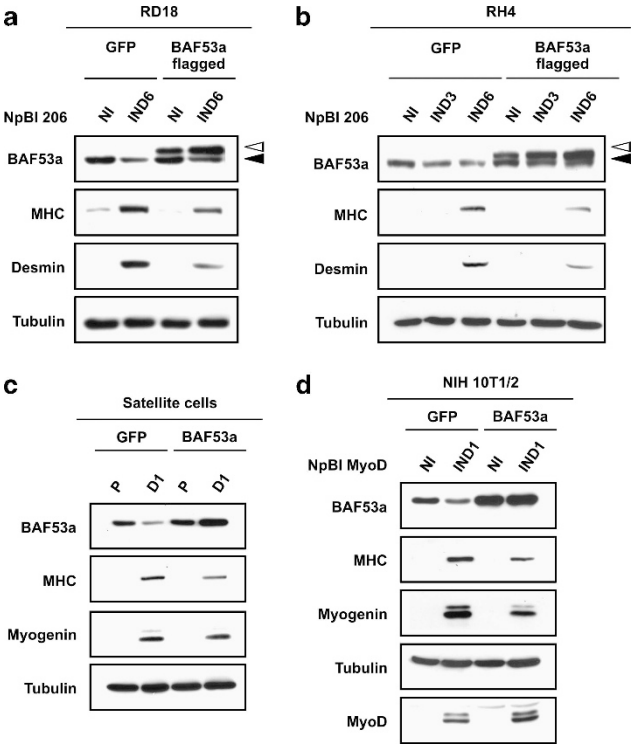

Figure 4. BAF53a overexpression impairs miR-206- and MyoD-induced myogenic differentiation. Western blot analysis for the indicated proteins in (**a**, **b**) RD18 and RH4 NpBI-206 cells over-expressing GFP as a control, or a flagged-BAF53a (open arrow head), in the absence of doxycycline (miR-206 not induced, NI) and after 6 days of doxycycline treatment (miR-206 induced, IND), (**c**) satellite cells overexpressing GFP as a control, or BAF53a (not flagged), in proliferating conditions (P) and after 1 day of growth in low serum (D1), (**d**) NIH10T1/2 NpBI-MyoD fibroblasts overexpressing GFP as a control or BAF53a (not flagged) in the absence of doxycycline (MyoD not induced, NI) and after 1 day of doxycycline treatment (MyoD induced, IND).

genome. Thus, we chose to further pursue BAF53a, among other potential miR-206 targets, because of the importance of chromatin remodeling in the transcriptional changes occurring during myogenic differentiation. In fact it has been shown that SWI/SNF chromatin remodeling enzymes are required for MyoD and myogenin-mediated induction of muscle genes, as well as for the expression of myogenic microRNAs.[28–31] Previous studies have revealed that the transition from neuroblast to post-mitotic neuron occurs via the assembly of a 'neurogenic' SWI/SNF complex where BAF53b replaces BAF53a, following microRNA-mediated downregulation of the latter.[32] This suggested to us that a similar BAF53a downregulation could also be required in myogenesis. Furthermore, our interest in BAF53a grew when we found in a large cohort of primary RMS tumors that its level of expression was significantly higher compared with normal muscle.

SWI-SNF components had been previously shown to be involved in a large spectrum of cancer types (reviewed in Wu[33]). Although BAFs were described mainly as oncosuppressors, the expression level of some subunits has been found increased in several malignancies. For example, BRG1 is increased in prostate, gastric, colorectal tumors and in melanomas. In cells derived from the last two cancers the knock-down of BRG1 significantly reduced cell proliferation. Furthermore, BRG1 is required for tumor formation after BAF47 deletion, indicating that in BAF47-negative BAF complexes BRG1 acts as an oncogene, rather than a tumor suppressor. In this work, we showed that BAF53a also seems to behave functionally as an oncogene. In fact RMS cells expressing a BAF53a mRNA devoid of 3′-UTR, were resistant to differentiation induced by the muscle-specific miR-206. Conversely, BAF53a silencing partially reverted the phenotype of RMS cells, impairing tumor growth. The differentiation-inducing effect of BAF53a silencing was higher in RD 18 ERMS cells than in RH4 ARMS cells. This could be related to the fact that the alveolar subtype is already more differentiated down the myogenic lineage. In both cell lines, however, failure to downregulate BAF53a seems to contribute to the tumor cells' inability to complete differentiation.

Having established that downregulation of BAF53a is a necessary step in RMS differentiation, we showed that is also required in myogenesis. This suggests an inhibitory role of the BAF53a-containing SWI-SNF complex on the transcription of myogenic genes. This conclusion is strengthened by recent work in hematopoietic and epidermis, showing that BAF53a is essential for the maintenance of progenitors, prevents activation of the differentiation program and must be downregulated for differentiation to proceed.[4,5] Its replacement with BAF53b, however, remains unique to the neurons, in keeping with the tissue specificity of expression of this subunit.[34] Although the two previous papers and our own work provide definitive evidence in three distinct differentiating systems for the need to downregulate BAF53a during differentiation, we are the first ones to suggest that persistence of BAF53a may be causal in tumorigenesis.

During myogenesis there is an increase first in the level of miR-206, which seems to be involved in the early stages of differentiation, and then in the level of its homolog miR-1, which appears to control the maintenance of the differentiated state.[15] The two microRNAs share the same seed sequence and are the most abundant in muscle. Although we were completing this work, another group identified BAF60b and BAF47 as additional miR-1/206 targets, and showed that their overexpression has a negative effect on the differentiation of C2C12 myoblasts.[35] However, when we re-examined the public databanks comparing expression profiles of RMS primary tumors with normal muscle for BAF60b and BAF47, their increase in the tumors was very modest (1.5-fold for BAF60B and 2-fold for BAF 47, data not shown). The fact that BAF53a seems to be significantly more elevated (fivefold), suggests that miR-206 may have a different importance in the regulation of these subunits. Furthermore, considering that in our hands BAF53a silencing was sufficient to trigger differentiation in RMS cells, it is likely that the control of BAF53a levels has a predominant role in the transition from growth to differentiation both in normal and deranged myogenesis. Thus, the picture emerging in myogenesis seems to involve miR-1/206-mediated downregulation of BAF53a, BAF60b and BAF47, accompanied by an increase in BAF 60C.[3] Finally, the BRG1 ATPase itself contains a MRE for miR-1/206 and was found to be downregulated in RD cells 48 h after transfection of a miR-1 mimic.[36] It has been suggested that during myogenesis this subunit may be replaced by the alternative catalytic subunit BRM. Although BRG1 is required for the activation of early muscle genes BRM seems to be essential for completion of terminal differentiation.[37] Indeed knock-out mice for BRM have increased muscle mass, suggesting altered control of cellular proliferation.[38]

In recent years, 'differentiation therapy' has been proposed by us and others[39–46] for RMS, a pediatric cancer for which a non-toxic approach would be highly desirable, given the young age of most patients. This strategy is based on the observation of the phenotype rather than the genotype of RMS cells. In fact, regardless of the genetic lesions and in spite of the conflicting opinions on the cell of origin of RMS (a satellite cell, a myoblast or a more primitive mesenchymal precursor), RMS cells appear to be somehow 'frozen' in a state of incomplete myogenic differentiation. Thus, removing this block could have therapeutic value. MiR-206 is potentially attractive as an anticancer drug because of its pleiotropic action, which allows ERMS and ARMS genetic heterogeneity to be overcome, and may bypass the risk of eliciting resistance.[47]

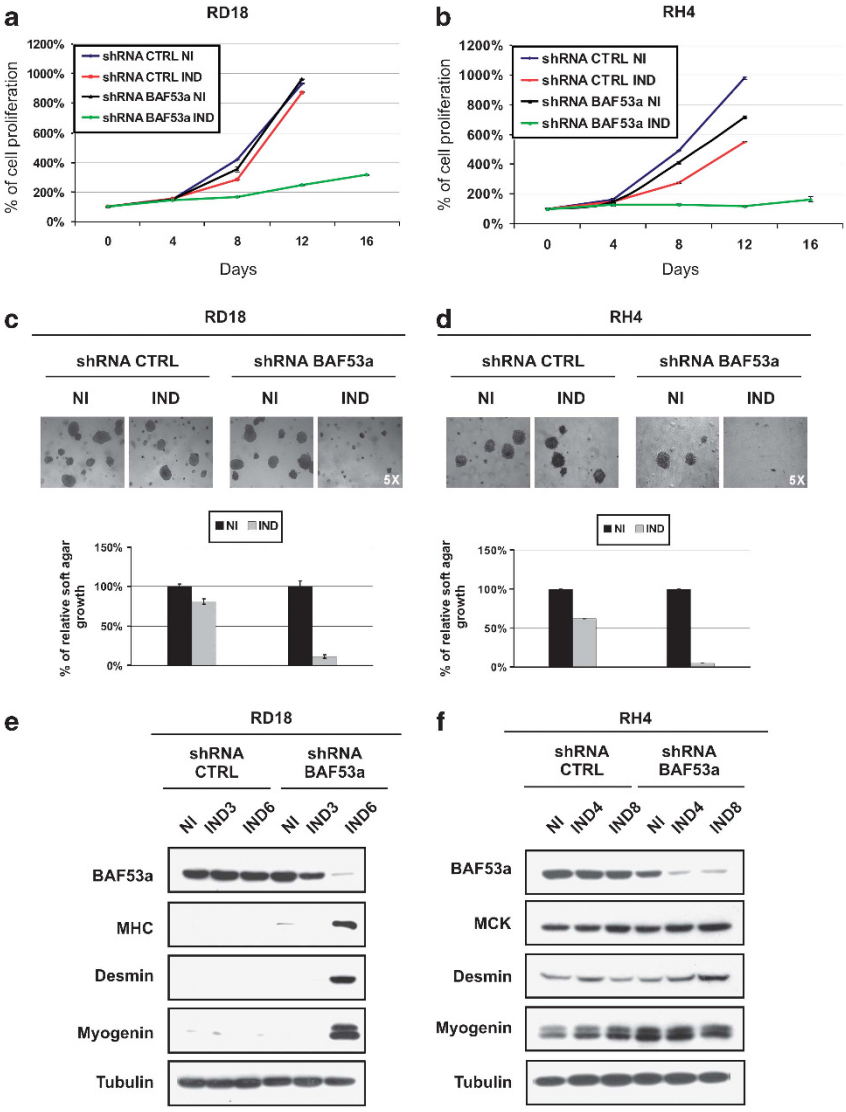

Figure 5. Conditional silencing of BAF53a impairs RMS cells proliferation and anchorage-independent growth by inducing myogenic differentiation. (**a**, **b**) MTT analysis of RD18 and RH4 cells with inducible expression of a control and an anti-BAF53a shRNA. Cells were kept for the indicated days in the absence (shRNA not induced, NI) or in the presence (shRNA induced, IND) of doxycycline. The number of cells at day 0 was set at 100%. Mean values (± s.d.) are from three independent experiments. (**c**, **d**) Upper panels: representative images of soft agar growth assays of the same cells. Lower panels: quantification of the soft agar growth assays. The number of colonies obtained from the cells in NI conditions was set at 100%. (**e**, **f**) Western blot analysis of the indicated proteins in RD18 and RH4 cells kept for the indicated days in the absence (shRNA not induced, NI) or in the presence (shRNA induced, IND) of doxycycline. For results obtained with a second shRNA see Supplementary Figure S5.

However, converting it into a drug implies solving the problem of delivery, which is still a major bottleneck for the use of microRNAs as therapeutics. In this work, we showed that downmodulation of BAF53a is a critical event downstream of miR-206 for forcing RMS cells to resume differentiation. Although BAF53a may not be directly druggable, gaining further insights on the molecular mechanisms underlying the role of the SWI-SNF remodeling complex in physiological and miR-206-induced myogenesis may shed new light on the possible use of epigenetic drugs for the differentiation therapy of RMS.

MATERIALS AND METHODS

All reagents, unless otherwise specified, were from Sigma-Aldrich (St Louis, MO, USA). All transfections were performed with Lipofectamine 2000 (Life Technologies, Paisley, UK) according to the manufacturer's instructions.

Gene expression data analysis

We assessed the mRNA levels of BAF53a in a panel of 101 RMS patient samples relative to 30 skeletal muscle samples using a previously published patient ITCC/CIT data set (Innovative Therapies for Children with Cancer/Carte d'Identité des Tumeurs[48] available at http://www.ebi.ac.uk/arrayexpress/experiments/E-TABM-1202/) and public Affymetrix expression profiling data for skeletal muscle, available at GEO (http://www.ncbi.nlm.nih.gov/geo/).[20] All samples, assayed on the Affymetrix Human Genome U133 Plus 2.0 Array platform (Santa Clara, CA, USA) have been normalized by the RMA algorithm as implemented in R free software environment, and annoted with Affymetrix annotation (release na32). Differential expression has been evaluated by limma package.

Cell cultures

ERMS (RD, RD18) and ARMS (RH4, RH30) cells, NIH 10T1/2 fibroblasts and HEK293 were grown in Dulbecco's modified Eagle's medium (Euroclone,

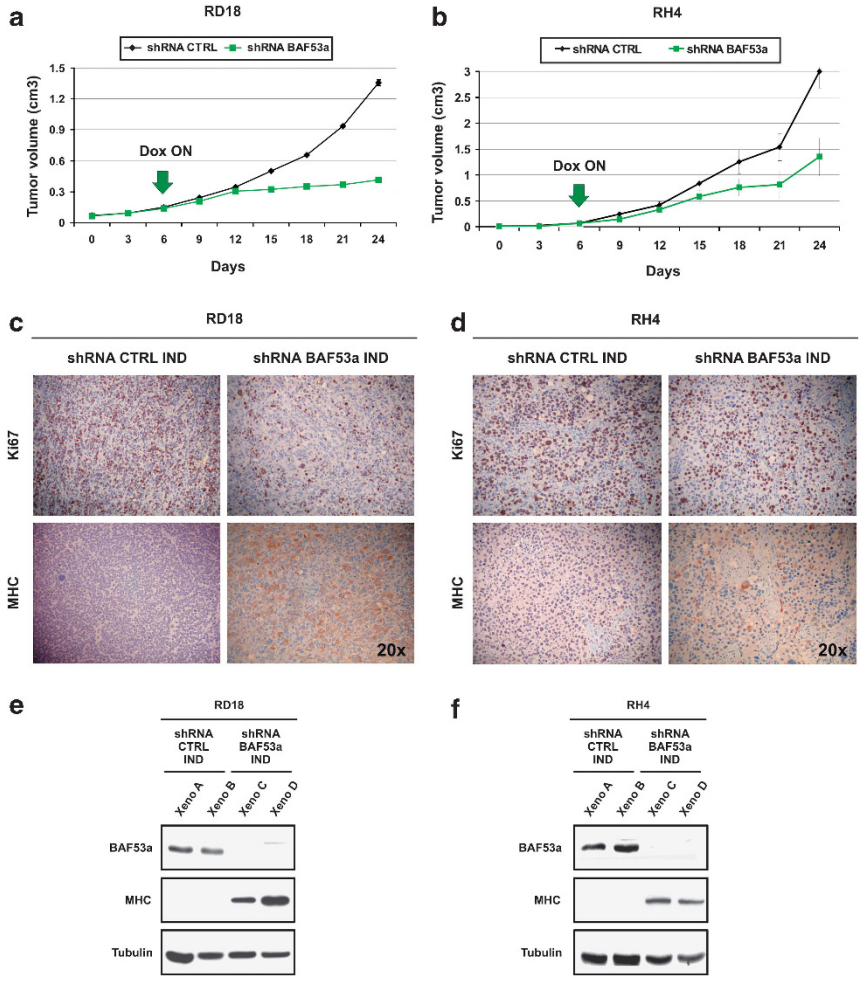

Figure 6. *In vivo* BAF53a silencing blocks ERMS and ARMS tumor growth and induces myogenic differentiation. (**a**, **b**) RD18 and RH4 cells with inducible expression of a control shRNA or of an anti-BAF53a shRNA were subcutaneously injected in nude mice. A total of 5 of 10 mice bearing RMS tumors were given drinking water containing 1 mg/ml of doxycycline, starting on the day indicated by the arrow, to induce the shRNA expression (green line). Tumor volume was measured every 3 days, starting when the tumors became palpable. (**c**, **d**) Immunohistochemical analysis of sections of tumors harvested from doxycycline-treated animals (shRNA CTRL or shRNA BAF53a induced, IND). Ki67 was used as a marker for proliferating cells; MHC was used as a marker for differentiated cells. (**e**, **f**) Western blots analysis of RD18 and RH4 tumors harvested from mice treated with doxycycline for 18 days (shRNA CTRL induced, IND: Xeno A and B; shRNA BAF53a induced, IND: Xeno C and D).

Pero, Italy) supplemented with 10% fetal bovine serum (Euroclone). Murine satellite cells were isolated as from hind-limb muscles of 18-day-old *Ink4a–/–* mice and cultured as described before.[15] All cells were incubated at 37 °C in a 5% CO_2-water-saturated atmosphere, and media were supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Human myoblasts (C-12530 PromoCell GmbH, Heidelberg, Germany), were maintained in proliferating conditions in PromoCell Cell Growth Medium supplemented with growth factors (C-23060 and C23160, PromoCell GmbH). Differentiation (D) was obtained switching human myoblasts for 15 days to differentiating medium with appropriate supplements (C-23161 and C-39366, PromoCell GmbH). ERMS (RD) and ARMS (RH4) cell lines were differentiated in low serum by 12-O-tetradecanoylphorbol-1-acetate addiction as described.[20]

Lentiviral vector construction

All primers/oligonucleotides used for vectors construction are listed in Supplementary Table 1. NpBI-206 sense and NpBI-206 antisense bidirectional lentiviral vectors (NpBI-206S, AS) were generated as described before.[15] To generate the NpBI-MyoD vector, the insert was recovered from a MyoD pcDNA vector provided by Pier Lorenzo Puri and subcloned into the blunted-*Nhe*I NpBI-206 AS vector. Human BAF53a complementary DNA was PCR amplified from a commercial pBluescript vector (code 17879, Addgene, Cambridge, MA, USA) with primers A and B. The PCR product was cut with *Eco*RV and *Sal*I restriction enzymes and cloned into the *Bam*HI-blunted-*Sal*I sites of the pCCL.sin.PPT.hPGK.GFPWpre vector, originally provided by Luigi Naldini (San Raffaele-Telethon Institute for Gene Therapy, Milano, Italy).[49] The same procedure was used for the generation of a lentiviral vector carrying a flagged human BAF53a complementary DNA, which was PCR amplified with primers C and D. PLKO.1 lentiviral vectors (BAF53a sh1: code TRCN0000072277- BAF53a sh2: code TRCN0000072274) expressing shRNAs against BAF53a and the control shRNA (code: SHC002) were purchased from Sigma-Aldrich. The shRNAs were PCR amplified from the PLKO vectors with primers E and F. PCR products were then subcloned into the *Eco*RI/*Cla*I restriction sites of the pPLVTH-inducible lentiviral vector as described before.[17] All vectors were sequenced before using them for lentivirus production.

Lentiviral vectors production and cellular transduction

High titer lentiviral vector stocks were produced in HEK 293T cells by transfection of the modified transfer vector pCCL.sin.PPT.hPGK.GFPWpre and packaging vectors pMDLg/pRRE, pRSVRev and pMD2.VSVG. Regulatable expression using NpBI vectors was obtained as described before.[15] pPLVTH and LV-tTR-KRAB were co-transfected with pCMV-dR8.74, pRSV-Rev and pMD2.VSVG packaging vectors. Inducible expression of pLVTH vectors was obtained as described before.[17] To induce the expression of inducible constructs, cells were treated with 1 ug/ml of doxycycline.

Western blots

Total cell extracts were made with RIPA buffer (50 mM TrisHCl pH 8, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% NP40) supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM Na_3VO_4 and protease inhibitor cocktail. Nuclear-cytoplasmic extracts were prepared by using a Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol. Protein lysates were quantified with the Bio-Rad (Hercules, CA, USA) protein assay, resolved in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred to Hybond ECL Nitrocellulose Membranes (Amersham Biosciences, Piscataway, NJ, USA). Proteins were visualized with horseradish peroxidase-conjugated secondary antibodies and SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA). Original western blots before brightness and contrast adjustments are shown in Supplementary Figure S5.

Antibodies

Anti-Met was from Zymed Paisley, UK; anti-MHC, anti-BRG1, anti-BAF53A (E-3) and anti-myogenin were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); anti-α-tubulin (B-5-1- 1) was from Sigma-Aldrich; anti-desmin and anti-MyoD were from DAKO (Glostrup, Denmark); anti-muscle creatine kinase and anti-MHC used in immunofluorescence and immunohistochemistry were from Developmental Studies Hybridoma Bank (Iowa City, IA, USA).

Co-immunoprecipitation assay

Nuclear lysates were prepared by using a hypotonic buffer (Tris HCl 10 mM pH 7.9; KCl 10 mM; MgCl2 1.5 mM) plus 0.05% NP40. Nuclei were resuspended into high salt buffer (Tris HCl 20 mM pH7.9; 25% glycerol; 420 mM NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA). Nuclear extracts were then diluted in IP buffer (50 mM Tris HCl pH 7.9; 150 mM NaCl; 1 mM EDTA; 5 mM $MgCl_2$; 0.1% NP-40; 20% glycerol) and pre-cleared with protein G dynabeads (Life Technologies) for 2 h on a rotating platform. Pre-cleared lysates were then incubated O/N with either BRG1 or BAF53a antibodies and subsequently incubated with protein G dynabeads for 2 h at on a rotating platform. All these steps were performed at 4 °C. Dynabeads were finally eluted by boiling in Laemmli Buffer.

Sensor vectors production and luciferase assay

Human and mouse BAF53a 3′-UTRs were PCR amplified from genomic DNA using, respectively, primers G-H and K-L (Supplementary Table 1). The mutated human and mouse 3′-UTRs (sequences listed in Supplementary Table 1) were both synthesized by GeneArt (Life Technologies). All 3′-UTRs were cloned into psiCHECKTM-2 Vector (Promega, Madison, WI, USA) downstream of Renilla luciferase using the *Xho*I/*Not*I restriction sites. The vectors were sequenced before using them for luciferase assays. The psiCHECKTM-2 vectors were transfected into the indicated cell lines and the luciferase assay was performed with the Dual-Luciferase Reporter Assay System (Promega). Luminescence was measured with the 'Dual Glow' protocol of the Glowmax MULTI + Detection System (Promega). The luciferase signal obtained from the *Renilla* (Renilla *reniformis)* was normalized against the *firefly* (Photinus *pyralis)* luciferase intraplasmid control. The values obtained for the 'not induced' cells were set as 1.

Immunofluorescence

For MHC and BAF53a detection, cells seeded on 24-wells were fixed with 4% paraformaldehyde, saturated in blocking solution (3% bovine serum albumin in phosphate-buffered saline) for 1 h. Once permeabilized with 0.3% Triton X-100, cells were incubated with primary antibody (MHC from Hybridoma Bank or BAF53a from Abcam, Cambridge, UK) for 1 h and then with secondary antibody (either Alexa-555 or Alexa-633 from Invitrogen, Paisley, UK) for 30 min. Nuclei were stained with 4,6-diamidino-2-phenylindole. Images were acquired for NIH NpBI-MyoD through a Leica confocal microscope (Leica TCS SP5 software) or through a Leica DMIRE2 fluorescence microscope (Leica application Suite software version 2.8.1., Leica, Wetzlar, Germany).

Cells proliferation, anchorage-independent growth, *in vivo* tumorigenesis assays and immunohistochemistry were performed as described before.[19]

Statistics

Student's *t*-tests was used to calculate the statistical significance. *P*-values of <0.05 were considered statistically significant. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. Bars indicate s.e.m.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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