Validation of Met as a Therapeutic Target in Alveolar and Embryonal Rhabdomyosarcoma

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Abstract

Rhabdomyosarcoma (RMS) is a highly malignant soft-tissue tumor of childhood deriving from skeletal muscle cells. RMS can be classified in two major histologic subtypes: embryonal (ERMS) and alveolar (ARMS), the latter being characterized by the PAX3/7-FKHR translocation. Here we first investigated whether the Met receptor, a transcriptional target of PAX3 and PAX7, has a role in PAX3-FKHR-mediated transformation. Following PAX3-FKHR transduction, Met was up-regulated in mouse embryonal fibroblasts (MEF), NIH 3T3 and C2C12 cells, and they all acquired anchorage independence. This property was lost in low serum but addition of hepatocyte growth factor/scatter factor (HGF/SF) rescued soft-agar growth. Genetic proof that Met is necessary for this PAX3-FKHR-mediated effect was obtained by transducing with PAX3-FKHR MEFS derived from Met mutant (Met<sup>+/−</sup>) and wild-type (Met<sup>+/+</sup>) embryos. Only Met<sup>+/+</sup> MEFS acquired anchorage-independent growth whereas PAX3-FKHR-transduced Met<sup>+/−</sup> cells were unable to form colonies in soft agar. To verify if Met had a role in RMS maintenance, we silenced the receptor by transducing ERMS and ARMS cell lines with an inducible lentivirus expressing an anti-Met short hairpin RNA (shRNA). Met down-regulation significantly affected RMS cells proliferation, survival, invasiveness, and anchorage-independent growth. Finally, induction of the Met-directed shRNA promoted a dramatic reduction of tumor mass in a xenograft model of RMS. Our data show that both ARMS- and ERMS-derived cell lines, in spite of the genetic drift which may have occurred in years of culture, seem to have retained an “addiction” to the Met oncogene and suggest that Met may represent a target of choice to develop novel therapeutic strategies for ARMS. (Cancer Res 2006; 66(9): 4742-9)

Introduction

Among the broad category of small round blue cell tumors, rhabdomyosarcoma (RMS) stands out as the most common soft-tissue sarcoma in childhood and as the third soft-tissue sarcoma in the adult (1). Although the precise cell type from which the tumor originates is still a matter of debate, the evidence points towards the myogenic lineage. RMS expresses a variety of markers typical of both embryonic and mature skeletal muscle. In addition, cytoplasmic striations, a skeletal muscle-specific feature, can also be detected in these tumors (2).

A widely employed histologic classification of RMS defines three subtypes, differing from each other for body location, occurrence, mean patient age, and prognosis. Embryonal rhabdomyosarcoma (ERMS) is composed of spindle-shaped cells with a stromal rich appearance and it occurs mainly in the head and neck region, including the orbita. The alveolar subtype (ARMS) typically consists of small round densely packed cells, resembling pulmonary alveoli, and it occurs more often in the trunk and extremities. The pleomorphic variant has minor incidence and does not occur in pediatric patients (2).

Alveolar histology is an independent predictor of worse outcome: a 5-year survival rate of <30% has been reported in children with metastatic ARMS. The most relevant feature of this subtype is the presence of one of two possible chromosomal translocations, t(2;13)(q35;q14) and t(1;13)(p36;q14), which result in expression of the chimeric PAX3-FKHR and PAX7-FKHR transcription factors, respectively (3–6). In contrast to ARMS, no diagnostic genetic lesions have been identified in ERMS. However, ERMS are characterized by frequent loss of heterozygosity at the 11p15 locus, a region harboring the genes for insulin-like growth factor 2 (IGF2), H19, p57<sup>kip2</sup>, and associated with alterations of imprinting (2). Expression profiling of ARMS and ERMS has revealed that the two signatures differ widely, to the point that in the rare cases lacking the classic chromosomal translocation, it is possible to diagnose the ARMS subtype on the basis of the transcriptional profile alone (7).

The PAX3/PAX7-FKHR translocations yield fusion proteins which are thought to have enhanced transcriptional activity. They consist of the DNA binding domain of PAX3 or PAX7 linked to the transactivation domain of the FKHR transcription factor. Both PAX3 and PAX7 are involved in skeletal muscle development. PAX3 is a key regulator of myogenesis (8) whereas PAX7 is the master gene of satellite cells specification (9). In myogenic precursors, PAX3 activates transcription of several target genes, among which myod, lady-bird, and c-met (10–12). The latter codes for a cell-surface receptor which, at limb level, mediates delamination of myoblast precursors from the epithelial dermomyotome and their migration in the limb bud. This occurs in response to the hepatocyte growth factor/scatter factor (HGF/SF) ligand. Both pax3 and c-met loss of function mutant embryos lack limb muscles (13, 14). Conversely, in embryos where the pax3 gene is replaced by the PAX3-FKHRCDNAs, myogenic precursors overexpress Met and undergo ectopic delamination. This phenotype can be rescued by bringing the PAX3-FKHCRI in a Met-null background (12), thus indicating that Met is responsible for the aberrant PAX3-FKHR-induced delamination.
Met is highly expressed in ARMS cell lines established from human tumors (15) and HGF/SF promotes their motility and resistance to chemotherapy (16). Consistently, Met was found to be up-regulated in a murine model of ARMS, obtained by bringing a conditional knock-in PAX3-FKHR allele in an Ink-4a/Arf−/− background (17, 18). Interestingly, Met seems to be expressed also in human ERMS, sometimes at levels comparable with those seen in ARMS (19, 20). ERMS lack the t(2;13)(q35;q14) translocation, but in this case, MET transcription is likely to be driven by the wild-type form of either PAX3 or PAX7, one of which seems to be always up-regulated in this tumor type (21). Furthermore, by bringing in an Ink-4a/Arf−/− background transgenic mice ectopically expressing HGF/SF, a highly penetrant model of ERMS has been obtained (22).

The evidence described above suggests that, in spite of their distinct molecular profiles, histology, and clinical outcome, Met may be effective in the treatment of RMS. Necessary for RMS maintenance and suggest that Met-directed therapies may be effective in the treatment of RMS.

In this work, we first established that Met is an essential mediator of the oncogenic properties of PAX3-FKHR by showing that HGF/SF rescued the ability of PAX3-FKHR–expressing cells to grow in soft agar in low serum, and that fibroblasts from mutant mouse embryos expressing a signaling–dead Met receptor (MetD/D; ref. 14) were unable to form colonies following PAX3-FKHR transduction. We then used lentiviral-mediated inducible RNA interference to find out whether Met is essential for the survival of RMS cell lines, both alveolar and embryonal. Doxycycline-induced down-regulation of the Met receptor significantly affected proliferation, survival, invasiveness, and anchorage-independent growth of both ARMS and ERMS cells. Finally, we proved that Met down-modulation can arrest tumor growth also in vivo by treating with doxycycline nude mice bearing xenotransplants of RMS transduced with the inducible lentivirus. Histologic analysis of the regressing tumors showed that Met silencing was accompanied by an increase in apoptotic and a reduction in proliferating cells. These data indicate that Met may be necessary for RMS maintenance and suggest that Met-directed therapies may be effective in the treatment of RMS.

Materials and Methods

Reagents. All reagents, unless specified, were from Sigma-Aldrich (St. Louis, MO).

Cells. Human RMS cells of embryonal (RD, RD18, and CCA) and alveolar (SJ-RH30, SJ-RH4, and RMZ-RC2) histotype and HER-2/neu-driven murine RMS cells were provided by Dr. Pier-Luigi Lollini (Amedeo Cancer Research Laboratories, Rome, Italy). GTL-16 cells are a gastric carcinoma cell line which was kindly provided by Dr. Beat W. Schafer (Department of Pediatrics, Division of Oncology, University Children’s Hospital, Zurich, Switzerland). P8a-puro/PAX3-FKHR was kindly provided from Open Biosystems (Huntsville, AL). P8a-puro/PAX3-FKHR was kindly provided from Open Biosystems (Huntsville, AL).

Vector construction. The pSUPER vector containing the Met-specific short hairpin RNA (shRNA) M3 (23) was kindly provided by Dr. Silvia Giordano (IRCC, University of Turin). The pSUPER vector containing the TM2 control sequence was previously described (24). Constitutive Lenti-M3 was generated by subcloning the H1-RNA-promoter-M3 shRNA cassette from pSUPER in EcoRI-Xhol sites of the vector pCCL.sin.PPT.hPGK. GFPWpre (27). Conditional Lenti-M3 was generated by subcloning the H1-RNA-promoter-M3 shRNA cassette from pSUPER in EcoRI-Cclal sites of the vector pLVTH. pLVTH and LV-TRK-RAB were kindly provided by Dr. Didier Trono (Department of Microbiology and Molecular Medicine, Faculty of Medicine, University of Geneva, Geneva, Switzerland). PLKO.1 lentiviral vectors expressing the shRNAs against PAX3 (NM_000438) were purchased from Open Biosystems (Huntsville, AL). P8a-puro/PAX3-FKHR was kindly provided from Dr. Beat W. Schafer (Department of Pediatrics, Division of Oncology, University Children’s Hospital, Zurich, Switzerland). P8a-puro/ eGFP construct was generated by subcloning the eGFP from pB-I-Egfp (Clontech, Mountain View, CA) in BsmHI-SacI sites of the vector pLVTH. pLVTH and LV-TRK-RAB were transfected with lipofectamine 2000 (Invitrogen, Carlsbad, CA) and using polybrene. Cells were grown until subconfluence, then trypsinized and centrifuged at 300 g for 10 minutes, and seeded at a concentration of 2 × 10⁵ cells per well in 96-well plates. Stock cultures were grown in DMEM supplemented with 10% FBS for two population doublings and then frozen.

Antibodies. Antibody against Met (C-12), antimouse Met (B-2), anti–extracellular signal-regulated kinase (ERK) 2 (C-14), anti-p53 (FL-393), anti–NF-κB p65 (C-20), and anti–eukaryotic translation initiation factor 2α (eIF2α; FL-315) were from Santa Cruz Biotechnology (Santa Cruz, CA); anti–α-tubulin (B-5-1-1) and anti–diphospho-ERK-1/2 (M-8139) were from Sigma-Aldrich (St. Louis, MO); anti–phospho-Akt (Ser473), anti–Akt, anti–cleaved caspase 3 (Asp175), anti–cleaved caspase 7 (Asp394), and anti–phospho-Met (Tyr1234/1235) were from Cell Signaling Technology (Danvers, MA); anti–green fluorescent protein (GFP) was from Molecular Probes (Eugene, OR); anti–phospho-eIF2α (Ab18) was from Oncogene Research (San Diego, CA); and anti–Ki67 was from Novocastra (Newcastle, United Kingdom).

Western blot. Cells were seeded in 96-well plates at a density of 2 × 10⁴ per well in the presence or absence of doxycycline (20 ng/mL). Cell proliferation was evaluated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide labeling reagent (Roche, Indianapolis, IN) by counting cells...

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in a Burker's hemocytometer chamber. The number of cells at day 1 was set at 100%.

Semiquantitative reverse transcription-PCR. PCR was done according to standard procedures. RNA was extracted from RMs cells by Trizol (Invitrogen, Carlsbad, CA). Total RNA, 0.4 μg, was used for reverse transcription by SuperScript III Reverse Transcriptase (Invitrogen) according to the instructions of the manufacturer. cDNA was then amplified using the following primers: actin-s 5'-TGGCCAGCGAGCCAGGAG-3' and actin-as 5'-AGATCGCTGCTGGCCACCA-3' for actin, PAX3-s 5'-AGAGGAAAACGCGGAAAGGCG-3' and PAX3-as 5'-TGCTCTTTCCATCTTGGCACG-3' for PAX3; HGF-s 5'-CAGCATGTCCTCCTGCATCTC-3' and HGF-as 5'-TGGTGTGGTATCATGGAACC-3' for HGF. PCR reaction was done using the following variables: 94°C 1 minute, 61°C 30 seconds for 35 cycles. Relative intensity between actin fragments was used to verify if equal amounts of cDNA template were used.

Anchorage-independent cell growth assay. Cells were suspended in 0.35% type VII low melting agarose in DMEM (10% or 2% FBS) at 2 × 10^5 per well and plated on a layer of 0.7% agarose in DMEM (10% or 2% FBS) in six-well culture plates and cultured at 37°C with 5% CO2 with or without human recombinant HGF (4 ng/mL). HGF-containing medium was replaced every 5 days. After 2 weeks, colonies >100 μm in diameter were counted.

In vitro invasion assay. Invasiveness was examined by using the membrane invasion culture system (Transwell polycarbonate membranes 6.5 mm diameter, 8 μm pore size; Costar, Cambridge, MA) according to the standard protocol. Briefly, RMS cells were grown in the presence or absence of doxycycline (20 ng/mL) for 48 hours and 2 × 10^5 cells were seeded onto the upper well of transwells previously coated with 50 μL of Matrigel Basement Membrane Matrix (Becton Dickinson, Franklin Lakes, NJ). After 48 hours, the noninvasive cells on the upper surface of the membrane were removed with a cotton swab. Cells that migrated through the Matrigel matrix and attached to the lower surface of membrane were fixed with 11% glutaraldehyde, stained with cresyl violet, solubilized in 10% acetic acid and quantified by spectrophotometric analysis (595 nm).

Immunohistochemistry. Tumor samples were collected at the indicated times, fixed, and embedded in paraffin. Sections were rehydrated and processed with four antigen retrieval cycles of 5 minutes each in microwave oven (800 W) in citrate buffer (pH 6). After blocking for 1 hour at room temperature with 0.1% Triton X-100 and 10% normal goat serum in PBS, the sections were incubated with primary antibodies in the same solution at room temperature overnight. Primary antibodies were added at a dilution of 1:300. Sections were further processed with biotinylated secondary antibodies (Vector). Avidin-biotin-peroxidase complex (ABC, Vector, Burlingame, CA) and finally visualized with 3,3′-diaminobenzidine (Roche).

Apoptosis analysis. Apoptosis was measured by flow cytometry after staining with Annexin V. Cells (1 × 10^5) were trypsinized, washed in PBS, and incubated for 15 minutes at 37°C in HEPES buffer solution [10 mmol/L HEPES (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L CaCl2] with 0.5 μL biotin-conjugated Annexin V (BD Pharmingen Biosciences, San Diego, CA). Annexin V binding was revealed by additional incubation with 0.5 μL streptavidin-alkaline phosphocyanin (BD Pharmingen Biosciences). Cells were analyzed by FACScan using CellQuest Software (BD Pharmingen Biosciences).

In vivo tumorigenesis assay. Cells were trypsinized and resuspended at 1 × 10^6/mL in sterile PBS. One hundred microliters were injected s.c. into the flank of female nu/nu mice (Charles River, Wilmington, MA). Tumor dimensions were measured with vernier calipers every 3 days and tumor volumes were calculated as the volume of a sphere. Conditional anti-Met shRNAs were induced in mice using 1 mg/mL doxycycline in drinking water.

Results
c-met is up-regulated by PAX3-FKHR and PAX3-FKHR oncogenicity is dependent on the HGF/SF-Met axis. Up-regulation of c-met at the transcriptional level by PAX3 protein has previously been reported (15, 20). However, it has also been reported that no variation in Met expression was detected following PAX3-FKHR transduction (29). To verify Met up-regulation by PAX3-FKHR and to establish its role in mediating the oncopgenic properties of PAX3-FKHR, we established stable cell lines (NIH 3T3 fibroblasts, C2C12 myoblasts, and a newly established 3T3-like fibroblast line here termed “MEF”) by infecting them with an eukaryotic retroviral vector carrying the PAX3-FKHR or the EGFP cDNA as control. Met was indeed up-regulated at the protein level in all three PAX3-FKHR–expressing cell lines (Fig. 1A). PAX3-FKHR is known to be a “weak” oncogene and, in fact, it was unable to induce foci in the NIH 3T3 cells and in the 3T3-like MEFs. However, it proved capable of conferring anchorage-independent growth to all cell lines. To verify if this property was dependent on stimulation of HGF-Met axis, PAX3-FKHR– and EGFP-expressing cells were seeded in soft agar in different serum conditions. In 10% FBS, PAX3-FKHR did induce soft-agar colony formation in all cell types, but in low serum (2% FBS), this response was strongly impaired. When HGF (4 ng/mL) was added to 2% FBS, the ability of PAX3-FKHR–expressing cells to grow without anchorage was rescued to the 10% FBS level (Fig. 1B). The increase in colony formation observed in the controls can be ascribed to the basal level of Met expression.

To obtain genetic proof that the oncogenic potential of PAX3-FKHR depends on Met signaling, we used primary MEFs from mutant embryos expressing a signaling-dead Met receptor (Met(D735A)). Primary wild-type MEFs and their mutant counterpart were infected with either PAX3-FKHR or EGFP retroviruses and tested for anchorage-independent growth. Only PAX3-FKHR–transduced MEFs that were wild-type for Met did grow in soft agar whereas Met(D735A) MEFs failed to form any colony (Fig. 1C). On the basis of all the above data, we conclude that Met is an essential mediator of PAX3-FKHR–induced transformation.

Constitutive and inducible lentiviral expression of a shRNA against Met (M3) down-regulates the Met receptor in RD18 ERMS cells. To study Met function in RMS, we first assessed the level of expression of the receptor and its ligand in different RMS cells. Both ERMS (RD, RD18, and CCA) and ARMS cells (SJ-RH4, SJ-RH30, and RMZ-RC2) expressed Met (Fig. 2A). In these cells, the Met receptor was phosphorylated at confluence when the medium became conditioned by the low amounts of released HGF/SF (Fig. 2A; ref. 16). To verify whether Met silencing could affect their viability, we infected the RMS cells with a lentiviral vector that leads to constitutive expression of an anti-Met shRNA (M3) and EGFP as a marker (26, 27, 30). Biochemical analysis of RD18-transduced cells showed that Met was specifically down-regulated in a dose-dependent manner whereas expression of other endogenous proteins was unaffected (Fig. 2B). Furthermore, we did not observe any up-regulation of the phosphorylated form of eIF2α, a signal of activation of the IFN-mediated response that is occasionally elicited by double-stranded RNA. Surprisingly, in RD18 cells, constitutive Met down-regulation induced a proliferative block followed by apoptosis (data not shown). To rule out the possibility that this effect could be due to the lentiviral vector, we decided to use an inducible system (requiring co-infection with two lentiviruses, LV-TTR-KRAB and LV-TThshRNA-M3) in which the expression of the anti-Met shRNA was doxycycline dependent (31). In a first series of experiments, we tested the ability of this system to down-regulate the expression of Met receptor in RD18 ERMS cells. The cells, co-transduced with LV-TTR-KRAB and LV-TThshRNA-M3, in the absence of doxycycline, expressed Met at levels comparable to those of wild-type cells because the tTR-KRAB protein repressed the production of the anti-Met shRNA (Fig. 2C). In contrast, doxycycline addition to the culture medium of the double-transduced cells resulted in a Met down-modulation as robust as that observed in RD18 cells constitutively expressing the M3 shRNA sequence. After
1 day of drug administration, Met was already strongly downregulated (Fig. 2C). Concomitantly, the downstream response to HGF, exemplified by phosphorylation of Akt and ERK, was reduced (Fig. 2C).

Conditional Met silencing induces inhibition of cell proliferation and promotes apoptosis in both ERMS and ARMS cell lines. Conditional silencing of Met receptor led to a substantial inhibition of the proliferation rate in both ERMS and ARMS (Fig. 3A). In contrast, RMS cells infected with a lentivirus expressing a control shRNA directed against Tpr-Met (TM2) were not affected (Fig. 3A).

A critical feature of transformed cells is their increased resistance to apoptosis. We wondered whether in RMS cells reduction of Met protein would be sufficient to overcome this mechanism of escape. RMS cells transduced with the inducible M3 lentivirus were scored for apoptosis at different time points after doxycycline administration. The number of apoptotic cells increased progressively, reaching >70% of the population by day 5 (Fig. 3B). In contrast, no increase of apoptosis was observed in uninduced cells or in cells expressing the control TM2 shRNA (Fig. 3B).

Finally, biochemical analysis showed a strong correlation between Met down-modulation and activation of the apoptotic pathway in RMS cells. After 3 days of doxycycline administration, when Met was almost completely silenced, activation of caspase 3 and 7 became detectable (Fig. 3C). To further confirm the specificity of these results, we transduced positive and negative control cell lines with the constitutive M3 lentivirus. As a positive control, we used the U87 glioblastoma cell line, known to be sensitive to Met-directed shRNA (32). As negative controls, we used RMS cells derived from a HER-2/neu transgenic mouse line (33), C2C12 myoblasts (which express Met), and Ewing’s sarcoma cells (which do not express Met). Whereas Met down-regulation caused massive cell death in U87 cells (data not shown), it did not induce either alteration of the proliferation rate or apoptosis in all other control cells (Supplementary Fig. S1).

Conditional Met silencing induces inhibition of invasiveness in vitro and block of anchorage-independent growth. We next investigated if conditional Met silencing could cause reversion of the oncogenic properties of ERMS and ARMS cells (invasiveness and anchorage-independent growth). To analyze the invasive ability of RMS cells, we did Transwell assays. After 2 days of doxycycline administration, cells were plated in the upper well of transwells, previously coated with Matrigel, and cell migration to the other side of the filter was evaluated after 48 hours. Met silencing rapidly reduced the rate of migration across the extracellular matrix (Fig. 4A). We next examined how our conditional Met silencing could affect the ability of RMS cells to grow in soft agar. As expected, the number of anchorage-independent colonies was markedly decreased in ERMS and ARMS cells in which Met had been conditionally silenced whereas no reduction was measured in uninduced M3 cells and in cells transduced with the control TM2 shRNA (Fig. 4B).

Conditional silencing of the Met receptor in a xenograft model of ERMS blocks neoplastic growth and efficiently reduces the tumor mass without detectable side effects. It was previously reported that inhibition of PAX3-FKHR and PAX3 by antisense oligonucleotides induced apoptosis in both ERMS and ARMS cells (34). We have now shown that ARMS and, more surprisingly, ERMS cells are sensitive to Met silencing. To test the hypothesis that Met down-regulation could have a therapeutic value in RMS treatment, we injected nude mice with RD18 cells expressing the inducible anti-Met M3 shRNA and RD18 TM2 cells. When tumors originated from RD18 M3 and RD18 TM2 cells reached a volume of 1.5 cm³ (day 24), doxycycline was administered to the mice in the

Figure 1. PAX3-FKHR up-regulates Met and confers Met-mediated anchorage independence. A, Western blots with anti-Met antibodies of anti-Met immunoprecipitates from extracts of NIH 3T3, C2C12, and MEF cell lines stably expressing PAX3-FKHR (P-F) or GFP. Tubulin was used as a loading control. B, relative soft-agar colony number of NIH 3T3, C2C12, and MEF cell lines expressing either PAX3-FKHR or GFP, grown in high serum (10% FBS), low serum (2% FBS) and low serum supplemented with 4 ng/mL of HGF (2% FBS + HGF). C, relative soft-agar colony number of PAX3-FKHR-transduced Met⁺⁺ and Met⁺⁻ primary fibroblasts grown in high serum (10% FBS). The number of colonies obtained from control cells was set at 100% in (B) and at 35% in (C). Columns, mean from three independent experiments done in triplicate; bars, SD. *, P < 0.05 (Student's t test).
drinking water. After 2 weeks, there was a substantial reduction of the mass of the tumors where Met had been silenced (Fig. 5A). Histologic analysis done at different time points showed that Met was almost completely down-regulated and that the apoptotic pathway was already activated after only 3 days of doxycycline induction whereas a strong reduction in proliferation was evident after 8 days of treatment (Fig. 5B). In vivo induction of the anti-Met shRNA in the tumors did not affect the feeding behavior and did not perturb the weight of the treated animals.

**Discussion**

The development of new therapies for RMS has been hampered by the lack of knowledge about the basic molecular mechanisms involved in its ethiology and maintenance. In the case of ARMS, since the product of the PAX3-FKHR translocation is a more potent activator than PAX3 but retains its binding specificity, it has been proposed that tumorigenesis may depend on aberrant up-regulation of PAX3 target genes. In this perspective, c-met is an intriguing nodal point to look for because, on one hand, it is included among the effectors of PAX3 in the execution of the myogenic program (10) and, on the other hand, it has been shown to be involved in many types of cancer (35). Furthermore, Met has been found to be highly expressed in ARMS specimens (15, 19, 20) and is present also in ERMS (20), albeit at more variable levels.

In this work, we first showed that Met expression is essential for PAX3-FKHR-mediated transformation. Met was up-regulated in PAX3-FKHR–transduced MEFs, NIH 3T3, and C2C12 cells, all of which acquired anchorage independence. However, PAX3-FKHR–transduced cells gave rise to colonies only in agar supplemented with 10% FBS, but not with 2% FBS. Soft-agar growth in low serum was rescued by chronic stimulation with HGF, proving that the oncogenic effect of PAX3-FKHR could be reconstituted by merely activating Met signaling. Furthermore, PAX3-FKHR did not induce formation of any colonies in fibroblasts derived from mutant mice expressing a signaling-dead Met receptor (MetD/D), yielding genetic proof that PAX3-FKHR indeed acts by activating the HGF-Met axis.

Up-regulation of Met in ARMS, which are characterized by the PAX3-FKHR translocation, is easily explained by the enhanced transcriptional activity of the chimeric protein. However, the receptor is also present in ERMS where its expression could depend on the sustained levels of PAX3 or PAX7 (21). We confirmed this hypothesis by showing that down-modulation of PAX3 in RD cells via RNA interference resulted in a concomitant reduction of Met protein (Supplementary Fig. S2).

Validation of the biological effect of a gene in cultured cells can be sought by gene silencing. We silenced Met expression in ERMS and ARMS cell lines by transducing them with a lentiviral system that allowed to control the expression of an anti-Met shRNA (M3) by doxycycline administration. Met silencing significantly impaired cell replication, survival, invasiveness, and anchorage-independent growth of RMS cells. The anti-Met shRNA also induced massive cell death in U87 glioblastoma cells, a line that was chosen as a positive control.

**Figure 2.** Lentiviral delivered M3 anti-Met shRNA down-regulates efficiently and specifically the Met receptor and inhibits its downstream effectors. A. **top,** HGF expression analysis (reverse transcription-PCR) in the indicated RMS cell lines; middle and bottom, Western blot analysis of Met and p-Met in RMS (50 μg/lane) and control GTL-16 cells (2 μg/lane). B. Western blot of Met, GFP, Akt, p65, p53, ERK, p-eIF2α, eIF2α, and tubulin in extracts of RD18 cells infected at the indicated viral titers with a lentivirus constitutively expressing the M3 anti-Met shRNA. Lentiviral-delivered TM2 shRNA was used as a control. C. Western blot of Met, p-Akt, Akt, p-ERK, ERK, p-eIF2α, and tubulin in extracts of RD18 cells transduced with inducible M3 lentivirus (or with control constitutive TM2 lentivirus). Cells were either treated with 20 ng/mL of doxycycline (DOX) for 24 hours or were left untreated (NO DOX). After an overnight starving, HGF/SF (8 ng/mL) was added to the medium for 10 minutes before lysing the cells. Equivalent amounts of lysate (10 μg/lane) were loaded in each lane.
control because its viability is known to be sustained by Met expression (32). Conversely, transduction with the M3 lentivirus of C2C12 myoblasts, murine HER-2/neu-dependent RMS cells, and Ewing’s sarcoma cells did not induce any alteration of growth or apoptosis, indicating that Met acted as a dominant oncogene only in the cells where its silencing was crucial to viability. More precisely, silencing of the Met receptor impaired cell replication and increased apoptotic cell death. The ability of RMS cells to migrate through a Matrigel barrier and to form colonies in agar was also significantly reduced after Met knock-down.

When the effect of Met silencing was analyzed in vivo in a xenograft mouse model of RMS, turning on the M3 shRNA by doxycycline administration in the drinking water was sufficient to block tumor growth and to induce an impressive reduction of the tumor mass. Histologic analysis revealed that Met silencing was accompanied by an increase of apoptotic and by a reduction of Ki67-positive cells.

In conclusion, we have provided evidence for a role of Met in the maintenance and survival of both ARMS and ERMS. The finding of a common genetic element driving the two tumor types is somehow surprising because these tumors, besides being distinct for clinical presentation, histology, prognosis, and response to therapy, differ widely also in molecular terms, as shown by the presence of a specific genetic lesion only in ARMS.

Figure 3. Conditional down-regulation of the Met receptor by the M3 shRNA in RD18 and SJ-RH30 RMS cells decreases proliferation, increases apoptosis, and activates the caspase cascade. A, proliferation of RMS cells was evaluated by seeding 2 × 10⁶ cells per well (on 96-well plates) ± doxycycline (20 ng/mL) and counting them for 5 days. B, apoptosis of RMS cells was analyzed by seeding 1 × 10⁵ cells per well (on six-well plates), adding doxycycline in the culture medium for 1 to 5 days, and revealing apoptotic cells through Annexin V-allophycocyanin staining and FACS analysis. Points and columns, mean from three independent experiments done in triplicate; bars, SD. C, Western blot analysis of Met, GFP, cleaved caspase 3, cleaved caspase 7, and tubulin in lysates (50 μg/lane) of RD18 and SJ-RH30, where the M3 shRNA was induced for the indicated times. Similar results were obtained in two additional embryonal (RD and CCA) and one additional alveolar (SJ-RH4) cell lines.
and by their nonoverlapping gene expression profiles. A role for Met in ARMS, given the genetic evidence for a PAX3-FKHR-Met transcriptional axis, was perhaps predictable. It is intriguing to hypothesize that, due to its ability to confer invasive properties, Met could be functional to the worse aspect of this malignancy, its metastatic spread. It was more surprising to find that Met is also necessary for ERMS maintenance, given the milder nature of this malignancy and the absence of an obvious genetic lesion responsible for Met up-regulation. In this respect, it should be noted that others considered the possibility of finding activating point mutation in Met in ERMS, but the result of sequence analysis done on RMS cell lines was negative (16). On the other hand, a strong hint for Met involvement in ERMS was provided by the histology of the RMS found by Dr. G. Merlino’s group in the murine model generated by bringing the HGF/SF transgene on an Ink4a/Arf−/− background (22).

Met signaling is known to keep myoblasts cycling and to delay their differentiation (36). This feature is likely to represent the link between the two tumors and may explain why the PAX3/FKHR genomic translocation is positively selected specifically in the myogenic lineage (37, 38). However, it is likely that the interactions and cross-talk of Met with other molecules, such as the IGF-I receptor (39), as well as its role in the establishment of the malignancy, may differ in the two tumor types.

Met has been implicated in a wide number of cancers (35) and a variety of Met-directed approaches of potential therapeutic value have been described (40). However, the experience of the epidermal growth factor receptor inhibitor gefitinib, which failed to show any benefit in two large randomized placebo-controlled trials including patients selected only on the basis of receptor expression, has taught us the importance, for the validation of a new drug, of identifying a cohort of patients where the targeted molecule is causal (41, 42). According to our data, both ARMS and ERMS are “addicted” to the Met oncogene and thus are likely to respond to Met-directed therapies. These may be particularly significant for the more aggressive forms of ARMS, which are among the most incurable childhood malignancies.

Figure 4. Conditional down-regulation of the Met receptor by the M3 shRNA reduces invasiveness and inhibits anchorage-independent growth of embryonal and alveolar cell lines. A, invasiveness of the indicated RMS cells was evaluated by seeding 1×10⁵ cells after 2 days of doxycycline administration (20 ng/mL) on the upper side of an 8 μm pore size transwell chamber, coated with Matrigel. After 48 hours, cells that migrated to the lower side of the membrane were fixed, stained in cresyl violet solution, solubilized in acetic acid, and colorimetrically quantified. B, soft-agar growth was evaluated by resuspending RMS cells (2×10⁴ per well in six-well plates) in 0.35% agar. After 2 weeks of culture at 37° C, colonies with a diameter >100 μm were counted. The number of colonies obtained from uninduced M3 cells (M3-DOX) was set at 100%. Columns, mean from three independent experiments done in triplicate; bars, SD.

Figure 5. Conditional down-regulation of the Met receptor by the M3 shRNA inhibits growth and promotes regression of tumors derived from RD18 cells in a xenograft mouse model of RMS. A, tumor growth was evaluated by injecting s.c. six nude mice with RD18 cells transduced by inducible Lent-M3 and three nude mice with Lent-TM2-transduced RD18 cells (1×10⁶ per injection). On day 24, doxycycline (1 mg/mL) was added to the drinking water of three M3 and three TM2 tumor-bearing mice. Tumor growth was measured every 3 days. Bars, SD. B, top, anti-Met immunohistochemistry of M3- or TM2-expressing tumors after 3 days of doxycycline administration. Middle, anti–cleaved caspase 3 immunohistochemistry of M3- or TM2-expressing tumors after 3 days of doxycycline administration. Bottom, Ki67 staining of M3- or TM2-expressing tumors 8 days after doxycycline administration.

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