SHORT COMMUNICATION

Hsa-mir-145 is the top EWS-FLI1-repressed microRNA involved in a positive feedback loop in Ewing's sarcoma

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EWS-FLI1 is a chromosome translocation-derived chimeric transcription factor that has a central and rate-limiting role in the pathogenesis of Ewing's sarcoma. Although the EWS-FLI1 transcriptomic signature has been extensively characterized on the mRNA level, information on its impact on non-coding RNA expression is lacking. We have performed a genome-wide analysis of microRNAs affected by RNAi-mediated silencing of EWS-FLI1 in Ewing's sarcoma cell lines, and differentially expressed between primary Ewing's sarcoma and mesenchymal progenitor cells. Here, we report on the identification of hsa-mir-145 as the top EWS-FLI1-repressed microRNA. Upon knockdown of EWS-FLI1, hsa-mir-145 expression dramatically increases in all Ewing's sarcoma cell lines tested. Vice versa, ectopic expression of the microRNA in Ewing's sarcoma cell lines strongly reduced EWS-FLI1 protein, whereas transfection of an anti-mir to hsa-mir-145 increased the EWS-FLI1 levels. Reporter gene assays revealed that this modulation of EWS-FLI1 protein was mediated by the microRNA targeting the FLI1 3′-untranslated region. Mutual regulations of EWS-FLI1 and hsa-mir-145 were mirrored by an inverse correlation between their expression levels in four of the Ewing's sarcoma cell lines tested. Consistent with the role of EWS-FLI1 in Ewing's sarcoma growth regulation, forced hsa-mir-145 expression halted Ewing's sarcoma cell line growth. These results identify feedback regulation between EWS-FLI1 and hsa-mir-145 as an important component of the EWS-FLI1-mediated Ewing's sarcomagenesis that may open a new avenue to future microRNA-mediated therapy of this devastating malignant disease.

Introduction

Ewing's sarcoma is the second most frequent bone cancer affecting mostly children and young adults. Its largely undifferentiated small round cell appearance is reminiscent of a stem cell disease, and recent genomic studies identified mesenchymal progenitor cells (MPCs) as the most related normal tissue and likely candidate tissue of Ewing's sarcoma origin (Torchia *et al.*, 2003; Riggi *et al.*, 2005, 2008; Tirode *et al.*, 2007; Kauer *et al.*, 2009). At the genetic level, Ewing's sarcoma is characterized by gene fusions of the TLS, EWS and TAF15 (TET) family gene *EWS* with an ETS-transcription factor gene, most frequently *FLI1* (Kovar, 2010). Gene expression profiling of primary Ewing's sarcomas compared with MPCs, and of Ewing's sarcoma cell lines upon RNAi-mediated EWS-FLI1 silencing confirmed the rearranged ETS transcription factor as the major driver of aberrant gene expression in this disease (Kauer *et al.*, 2009). The EWS-FLI1 fusion protein binds to DNA by the C-terminal ETS DNA binding domain, whereas the N-terminal EWS domain mediates transcriptional activation of ETS cognate targets (Bailly *et al.*, 1994; Lessnick *et al.*, 1995). EWS-FLI1-activated genes in Ewing's sarcoma annotate preferentially to proliferation-associated functions (Kauer *et al.*, 2009). However, genomic studies identified a similar number of EWS-FLI1-repressed genes in Ewing's sarcoma, which appear to have an important role in aberrant signaling, and the disturbed developmental program and differentiation arrest of the tumor cells (Hancock and Lessnick, 2007; Tirode *et al.*, 2007; Kauer *et al.*, 2009). Consequently, factors that modulate EWS-FLI1 expression are predicted to affect tumor growth and differentiation by changing the expression of EWS-FLI1 signature genes. So far, this has been demonstrated for hypoxia (Aryee *et al.*, 2010) and for post-translational modifications of EWS-FLI1 (Bachmaier *et al.*, 2009). The mechanisms of EWS-FLI1-mediated gene suppression remains largely unknown, but an important role for microRNAs has recently been suggested (Kovar, 2010).

MicroRNAs are a class of evolutionary conserved small non-coding double-stranded RNA molecules that have a pivotal role in post-transcriptional gene silencing by either inhibiting translation, causing

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mRNA degradation, or by a combination of both mechanisms. They have recently attracted increasing attention because of their involvement in the pathogenesis of diverse diseases, including cardiovascular disease, stroke, neurodegenerative disease, diabetes, liver disease, kidney disease, infectious disease and cancer (Garzon et al., 2009; Taft et al., 2010). In humans, more than 850 microRNAs have been identified so far, each of them are predicted to target hundreds of genes so that at least 30% of mRNAs are likely under the control of microRNA (Taft et al., 2010). MicroRNAs are expressed in a tissue-specific manner and orchestrate cellular differentiation and organismal development, specifically by regulating stemness and stem cell differentiation (Sartipy et al., 2009).

To study the role of microRNAs in Ewing's sarcoma pathogenesis, we have performed a genome-wide screen for microRNAs that are affected by RNAi-mediated modulation of EWS-FLI1 in Ewing's sarcoma cell lines and differentially expressed between primary Ewing's sarcoma and MPC. Here, we report on the identification of hsa-mir-145, a central regulator of stemness and validated tumor suppressor that is suppressed in many cancers, among the top EWS-FLI1-repressed microRNAs in Ewing's sarcoma. A number of targets for hsa-mir-145 have already been reported, including FSCN1 in esophageal and bladder cancer (Chiyomaru et al., 2010; Kano et al., 2010), YES and STAT1 in colon cancer (Gregersen et al., 2010), and MUC1 and RTKN in breast cancer (Wang et al., 2009; Sachdeva and Mo, 2010). Importantly, hsa-mir-145 was found to repress the core pluripotency factors OCT4, SOX2 and KLF4, as well as MYC (Sachdeva et al., 2009; Xu et al., 2009) and, thus, is involved in the maturation of several tissues including intestine (Zeng et al., 2009) and smooth muscle (Cordes et al., 2009). As hsa-mir-145 was predicted to target the 3′-untranslated region of FLI1 mRNA (Lewis et al., 2003), which has only recently been verified experimentally in mouse microvascular endothelial cells (Larsson et al., 2009), and FLI1 and EWS-FLI1 share the same 3′-untranslated region, we tested for the sensitivity of the EWS-FLI1 chimeric gene product to hsa-mir-145 regulation in Ewing's sarcoma cells. Here, we report that EWS-FLI1 levels in Ewing's sarcoma are balanced by feedback regulation with hsa-mir-145.

Results and discussion

EWS-FLI1 was transiently silenced in five of the Ewing's sarcoma cell lines by transfection of a small-hairpin RNA expression vector as previously described (Ban et al., 2008). Transfected cells were selected for 3 days with puromycin. Changes in expression of 650 microRNAs were monitored for 4 days after transfection relative to control transfections with a non-targeting small-hairpin RNA using quantitative stem-loop reverse transcription PCR technology (Mestdagh et al., 2008). Using the same technology, we also compared miR expression levels between five Ewing's sarcoma samples and MPC preparations of six healthy individuals. Statistical testing for both comparisons, followed by filtering for concurrently regulated miRs, revealed that hsa-mir-145 was the top candidate for regulation by EWS-FLI1 (Table 1).

Repeated validation experiments were performed in four of the Ewing's sarcoma cell lines (SK-N-MC, STA-ET-1, TC252 and WE68). MicroRNA expression levels were calculated relative to invariably expressed RNUB6 RNA. As demonstrated in Figure 1a, mature hsa-mir-145 expression was found increased on average between threefold (decrease of 1.6 Cq values; TC252) and 110-fold (decrease of 6.8 Cq values; STA-ET-1) upon silencing of EWS-FLI1 in the cell lines, suggesting that EWS-FLI1 suppresses hsa-mir-145 expression in Ewing's sarcoma. Surprisingly, analysis of five primary tumors revealed higher hsa-mir-145 expression levels than in any of the studied cell lines. However, when compared with six MPC samples, hsa-mir-145 expression was still lower in the tumors than in the reference tissue (1.66-fold, 0.6 Cq values, $P = 0.012$) consistent with suppression of hsa-mir-145 by EWS-FLI1 in Ewing's sarcoma. Extending this analysis to a total of nine Ewing's sarcoma family of tumors (ESFTs) cell lines and 23 primary tumors confirmed about 130-fold mean hsa-mir-145 expression difference between cell lines and tumors (Figure 1b). This result may be largely because of contamination of tumor samples with microvascular endothelial cells previously demonstrated to highly express hsa-mir-145 (Larsson et al., 2009). In fact, separate analyses of tumoral and stromal components of one tumor upon microdissection revealed markedly increased hsa-mir-145 levels in the tumor stroma over the purified ESFT cells, which expressed lower amounts than the undissected tumor (Figure 1c).

We next tested whether ectopic cytomegalovirus promoter-driven expression of a cloned 342-nucleotide long primary hsa-mir-145 fragment (pri-mir-145) affects EWS-FLI1 expression in Ewing's sarcoma cell lines. Figure 2a demonstrates a strong reduction of EWS-FLI1 protein levels in the presence of pri-mir-145 in TC252 cells, which was also seen upon direct introduction of a commercially available mature mir-145 oligoribonucleotide (Ambion, Applied Biosystems, Foster City, CA, USA), but not with a non-targeting control microRNA. Conversely, transfection of an anti-mir-145 oligonucleotide (Ambion, Applied Biosystems) increased EWS-FLI1 protein levels in TC252 cells.

Consistent with these findings, ectopic pri-mir-145 expression and transfected mature mir-145-modulated luciferase activity by more than fivefold in a reporter gene assay using firefly luciferase fused to a proximal 610 bp fragment from the human FLI1 3′-untranslated region containing three predicted hsa-mir-145 target sequences (TargetScan; http://www.targetscan.org/) (Figure 2b). Co-transfection of the anti-mir-145 oligonucleotide with pri-mir-145 or with the mature mir-145 fully rescued luciferase activity, confirming the specificity of the suppressive pri-mir-145 effect on the FLI1 3′-untranslated region.

Table 1 EWS-FLI1 regulated microRNAs in Ewinǵs sarcoma

mir	EWS-FLI1 knockdown in five ESFT cell lines			Comparison of five primary ESFT with six MPC		
	Fold change	Q-value	P-value	Fold change	Q-value	P-value
EWS-FLI1 repressed						
hsa-mir-145	3.2222	0.0015	0	−1.4885	0.0704	0.0044
hsa-mir-424	1.982	0.0017	0	−1.7378	0.038	0.0017
hsa-mir-21	1.8667	0.0029	0	−1.455	0.0707	0.0051
hsa-mir-214.star	1.7531	0.0026	0	−1.5978	0.0402	0.0019
hsa-mir-214	1.5264	0.0112	0.0002	−1.061	0.1731	0.0147
hsa-mir-28-5p	1.3484	0.0249	0.0006	−1.0885	0.1422	0.0114
hsa-mir-424.star	1.1057	0.1339	0.0073	−3.6564	0.0002	0
hsa-mir-27a.star	1.1017	0.0316	0.0012	−2.0636	0.0134	0.0005
hsa-mir-22.star	1.078	0.0627	0.0031	−2.1958	0.008	0.0002
hsa-mir-409-3p	1.0266	0.1361	0.0083	−1.9741	0.0699	0.0046
hsa-mir-21.star	0.8191	0.188	0.0147	−1.9652	0.0138	0.0005
hsa-mir-125b	0.749	0.1506	0.0102	−1.7503	0.0432	0.0023
hsa-mir-708	0.72	0.1925	0.0155	−3.5188	0.0002	0
hsa-mir-135b	0.7047	0.1307	0.0074	−1.9925	0.0133	0.0004
EWS-FLI1 activated						
hsa-mir-500	−0.5089	0.1562	0.0126	3.2699	0.0045	0.0002
hsa-mir-126.star	−0.5516	0.1685	0.0139	5.3377	0	0
hsa-mir-93.star	−0.5911	0.1932	0.0176	1.9946	0.1318	0.016
hsa-mir-505	−0.5995	0.1035	0.005	2.5715	0.0326	0.0023
hsa-mir-128	−0.6893	0.119	0.0062	2.1889	0.0938	0.0098
hsa-mir-126	−0.7602	0.0724	0.003	5.7069	0	0
hsa-mir-9	−0.7769	0.1074	0.0049	4.2576	0.0006	0
hsa-mir-101	−0.7777	0.133	0.0084	3.1182	0.0077	0.0003
hsa-mir-425.star	−0.8362	0.0621	0.0022	2.0361	0.121	0.0134
hsa-mir-592	−0.8482	0.1422	0.0096	4.1559	0.0004	0
hsa-mir-340.star	−0.8841	0.0555	0.0013	2.354	0.0562	0.0049
hsa-mir-505.star	−1.0487	0.031	0.0005	1.9638	0.1314	0.0157
hsa-mir-652	−1.0637	0.0484	0.0011	2.7847	0.0179	0.001
hsa-mir-150	−1.3157	0.0067	0	7.8548	0	0
hsa-mir-20a.star	−1.6308	0.004	0	2.2371	0.0948	0.0097

Abbreviations: MPC, mesenchymal progenitor cell; ESFT, Ewing's sarcoma family of tumors.
Raw Cq values, as measured with the Applied Biosystems Megaplex system, (Applied Biosystems, Foster City, CA, USA) were read into *R* statistical environment (Gentleman *et al.*, 2004) in which all statistical analyses were performed. After the initial analysis of the raw Cq value data, the maximum Cq value was set to 30 to preclude high variances of fold changes of miRs with very low expression. After this step, the data set was normalized by quantile normalization (Mar *et al.*, 2009; Rao *et al.*, 2008). For the knockdown experiments, differentially expressed microRNAs were determined using the ratios in each of the five cell lines of knockdown versus control as input for a rank product non-parametric method ('RankProd', (Breitling *et al.*, 2004)). Fold changes are presented on a log2 scale. Differentially expressed microRNAs between five ESFT and six mesenchymal progenitor cell samples were identified using a two-class test in the 'RankProd' package. All *P*-values were corrected for multiple testing using the 'Benjamini–Hochberg' method (giving *Q*-values). microRNAs that showed evidence for regulation in both conditions, EWS-FLI1 knockdown and primary Ewing's tumors versus MPC, were identified by applying a non-stringent *Q*-value cutoff of $Q < 0.2$ (corresponding to a maximum 20% false positives) in either of the data set, intersecting the resulting microRNA lists and excluding microRNAs whose sign of expression change (presence versus absence of EWS-FLI1) was not consistent in both the data sets.

As basal hsa-mir-145 levels varied between individual Ewing's sarcoma cell lines (Figure 1), and EWS-FLI1 protein levels could be increased by antagonizing residual hsa-mir-145 expression and decreased by forcing hsa-mir-145 in Ewing's sarcoma cells (Figure 2a), we predicted that basal hsa-mir-145 and EWS-FLI1 protein levels should inversely correlate with each other. To test this assumption, microRNAs and total protein were extracted from the same samples of four Ewing's sarcoma cell lines grown in parallel. Figure 3 demonstrates that the highest hsa-mir-145 expression levels (lowest Cq values relative to RNUB6) were found in cell lines A673 and SK-N-MC, and the lowest levels (highest Cq values relative to RNUB6) were seen in WE68 cells, whereas TC252 cells were intermediate for hsa-mir-145. Consistent with the hypothesis of positive feedback regulation between the microRNA and EWS-FLI1 expression, high hsa-mir-145 was associated with low EWS-FLI1 protein in A673 and SK-N-MC, whereas low hsa-mir-145 was accompanied with the highest EWS-FLI1 protein expression in WE68 cells.

EWS-FLI1 has been identified as the major driver of Ewing's sarcoma proliferation, and antagonizing EWS-FLI1 expression with small-interfering RNA, short-hairpin RNA and antisense RNA, or dominant-negative constructs results in growth inhibition of Ewing's sarcoma cells *in vitro* and *in vivo* (for a recent review see Kovar, 2010). Likewise, suppression of EWS-FLI1 expression by transfection of pri-mir-145 stalled the growth of three ESFT cell lines (SK-N-MC, TC252 and WE68) under anchorage-dependent growth conditions (Figure 4a), and significantly decreased their ability to form colonies in soft agar, as demonstrated for SK-N-MC and TC252 cells in Figure 4b.
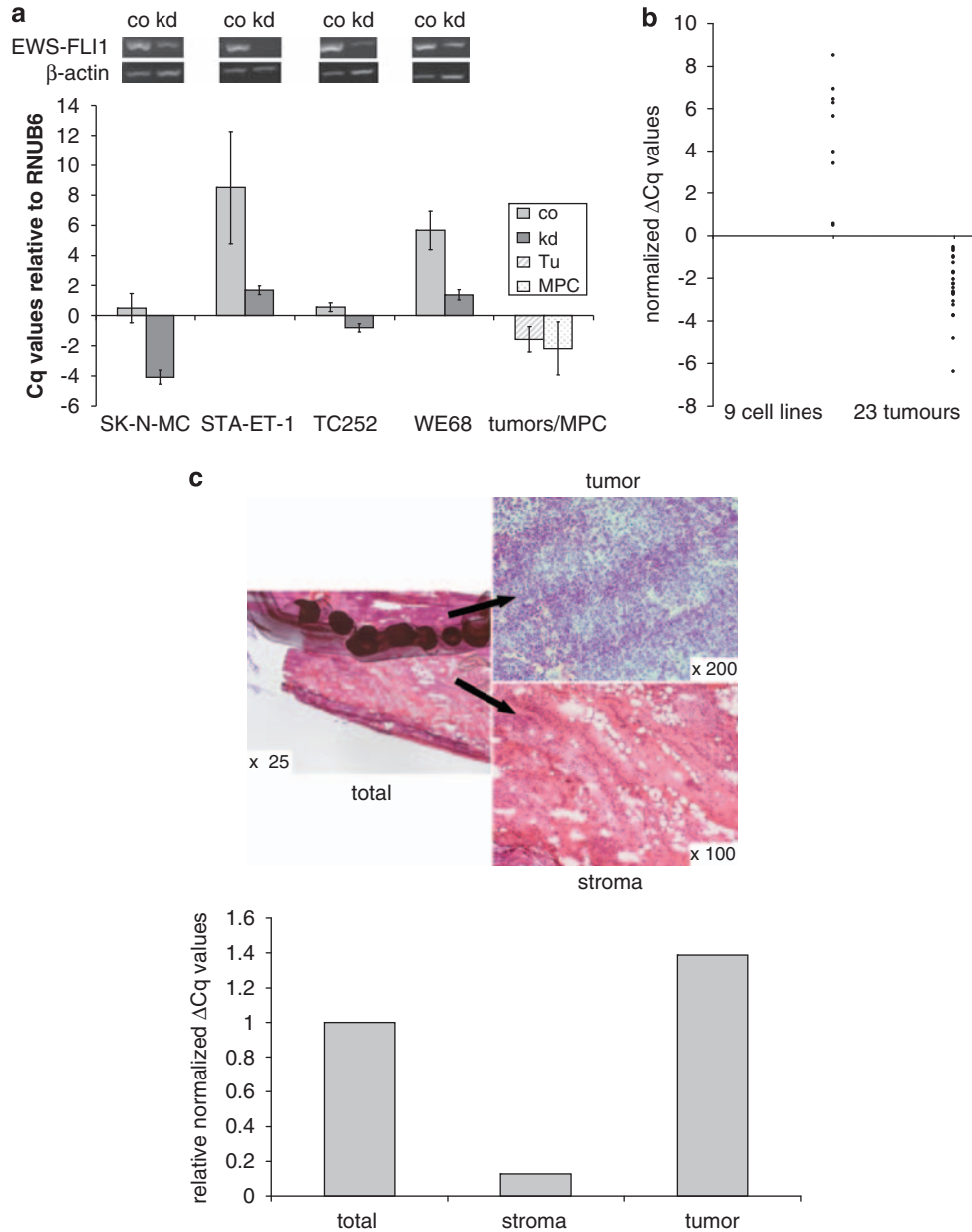
Figure 1 Expression of hsa-mir-145 in Ewing's sarcoma cell lines upon EWS-FLI1 silencing, in primary Ewing's sarcomas and in MPC. (**a**) RNAi-mediated silencing of EWS-FLI1 markedly increases hsa-mir-145 expression in ESFT cell lines. Ewing's sarcoma cell lines were transiently transfected with a non-targeting shRNA (co) or with an shRNA targeting the EWS-FLI1 fusion region (kd), and puromycin selected for 3 days as previously described (Ban et al., 2008). On day 4 after transfection, total RNA was extracted and subjected to EWS-FLI1 mRNA and hsa-mir-145 quantification. For the analysis of primary tumors (Tu) and MPC preparations, fresh frozen material from primary ESFT and bone marrow-derived MPC from healthy donors were used. (**b**) Hsa-mir-145 expression is generally higher in primary tumors than in ESFT cell lines. (**c**) Analysis of a microdissected primary extra-retrospinal ESFT of the soft tissue. Tumor cell-enriched areas were marked by a pathologist (black painted line) on a hematoxilin and eosin stained slide (upper left), and corresponding tumor cell-rich (tumor) and tumor cell-poor (stroma) areas (upper right) of unstained slides were used for tissue-specific RNA extraction and hsa-mir-145 expression (lower panel). Total RNA was extracted using the miRNeasy kit (Quiagen, Hilden, Germany) according to the manufacturer's recommendations and transcribed into cDNA. For quantification of mature hsa-mir-145 by stem-loop reverse transcription real-time PCR, a commercially available TaqMan Mature MicroRNA Assay was used (Applied Biosystems). Hsa-mir-145 expression is presented in Cq values relative to RNUB6 expression, which was determined in parallel, and which we found to be unaffected by EWS-FLI1 modulation (data not shown). EWS-FLI1-specific PCR to monitor EWS-FLI1 silencing was performed according to a standard protocol (Le Deley et al., 2010).

Conversely, transfection of the anti-mir-145, resulting in slightly elevated EWS-FLI1 protein levels, lead to a small increase in the colony-forming ability of ESFT cells, which was however not statistically significant (Figure 4c).

In this study, we identified hsa-mir-145 as a natural antagonist of EWS-FLI1, and therefore modulation of hsa-mir-145 appears to be an essential component of EWS-FLI1-mediated oncogenesis. Our results suggest that Ewing's sarcoma growth is controlled by a delicate
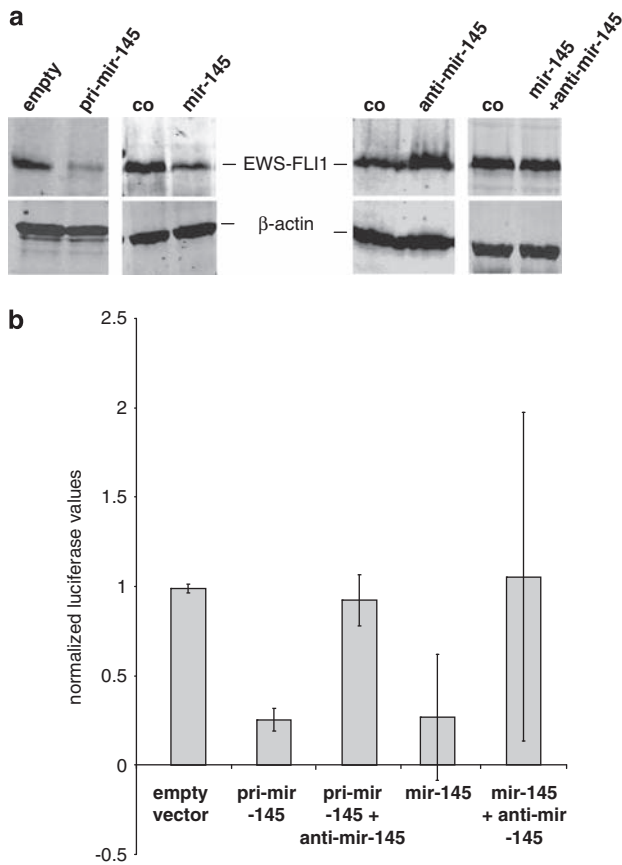
Figure 2 Hsa-mir-145 targets EWS-FLI1 in Ewing's sarcoma. (**a**) Effect of hsa-mir-145 on EWS-FLI1 expression. To generate a puromycin-selectable cytomegalovirus promoter-driven pri-mir-145 expression construct, a 346 bp genomic fragment containing hsa-mir-145 flanked on both sides by approximately 150 bp was amplified from genomic DNA using primers 5′-GGGGTACCGCT AGCAGAGCAATAAGCCACATCCG-3′ and 5′-CCGCTCGAG TTACCTCCAGGGACAGCCTTC-3′, which was inserted between Nhe1 and Xho1 sites of pIRES2-EGFP (Clontech, Saint-Germain-en-Laye, France), in which the IRES-driven EGFP cassette was replaced by a puromycin resistance cassette. Ewing's sarcoma cell line TC252 was transfected with either the empty vector or the pri-mir-145 expression construct using Lipofect AMINE Plus reagent (Invitrogen, Groningen, The Netherlands), subjected to 3 days of puromycin selection for 24 h after transfection, and EWS-FLI1 protein expression was monitored by immunoblotting using the FLI1-specific monoclonal antibody (mAb) 7.3 (kindly provided by O Delattre, Paris, France). For transfection of a mature hsa-mir-145 mimic, an Ambion chemically modified small double-stranded Pre-miR miRNA was used (Applied Biosystems). For control, Ambion's negative control#1 was transfected (co). (**b**) Reporter gene assays to monitor mir-145 effects on FLI1 3′-untranslated region were performed in TC252 cells. A pGL3 construct (Promega, Mannheim, Germany) containing about 610 bp from the FLI1 3′-untranslated region between the stop codon of the FLI1 reading frame and an Xba1 site fused to firefly luciferase was co-transfected with either empty pIRES-puro (empty vector) or pri-mir-145 construct, or with the commercial mature mir-145 in the absence or presence of a commercially available anti-mir-145 (anti-miR, Ambion, Applied Biosystems), along with thymidine kinase promoter-driven Renilla luciferase (pRL-TK, Promega) for transfection efficiency normalization purposes. Gene reporter assays were carried out at 48 h after transfection with the Dual Glo Luciferase assay kit (Promega). Reporter activity is presented as normalized luciferase activity relative to the empty vector control transfection.
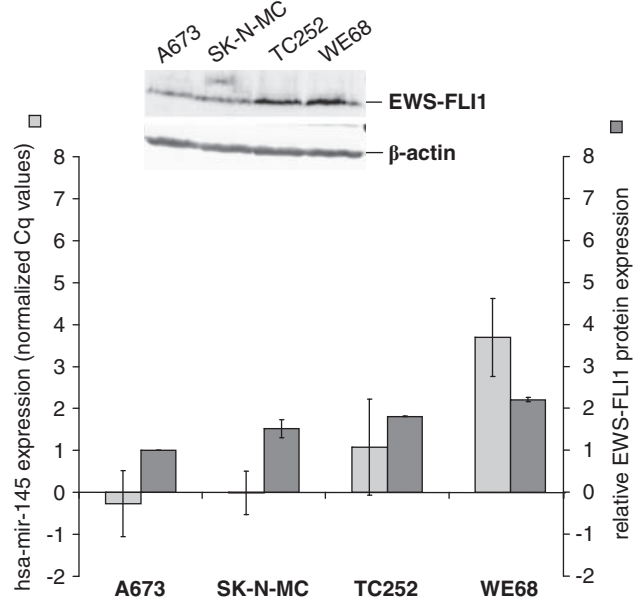

Figure 3 Inverse correlation between hsa-mir-145 and EWS-FLI1 protein expression. The indicated Ewing's sarcoma cell lines were grown under identical conditions until about 90% confluency, total RNA and protein were extracted from aliquots of the same cultures in parallel, and mature hsa-mir-145 expression was quantified as in Figure 1. EWS-FLI1 protein quantification on the immunoblot presented on top of the figure was achieved by fluorometry using a LI-COR Odyssey Imaging system (LI-COR Biosciences, Bad Homburg, Germany). Hsa-mir-145 expression (light gray columns) is presented in Cq values relative to RNUB6 expression (left y axis). Note that high Cq values reflect low microRNA expression. EWS-FLI1 protein expression (dark gray columns) after normalization to β-actin is presented relative to EWS-FLI1 protein levels in A673 cells, which was arbitrarily set 1 (right y axis).

balance between EWS-FLI1 and hsa-mir-145 that forms a positive feedback loop. The finding that hsa-mir-145 is among the top EWS-FLI1-repressed microRNAs is intriguing because of its documented role in the maturation and differentiation of stem cells (Xu et al., 2009). We have previously demonstrated that EWS-FLI1-repressed genes frequently annotate to differentiation-associated functions (Kauer et al., 2009). Hsa-mir-145 gives another such example. Although EWS-FLI1 has been shown to impose a partial neural differentiation program on the tumor cells (for review see Kovar, 2010), our results suggest that, simultaneously, it may maintain a certain degree of stemness by modulating hsa-mir-145, which is known to inhibit stem cell transcription factors Oct4, Sox2, Klf4 and Myc (Sachdeva et al., 2009; Xu et al., 2009). By modulating hsa-mir-145 expression, EWS-FLI1 not only blocks its own negative regulator in a feedback loop but it may also inhibit terminal differentiation of the presumed mesenchymal stem cell precursor of the tumor. Although this finding may at least partially explain the largely undifferentiated phenotype of Ewing's sarcoma, EWS-FLI1-mediated suppression of hsa-mir-145 does not confer multipotency to the tumor cells, which is only achieved upon long-term silencing of the fusion oncogene (Tirode et al., 2007).
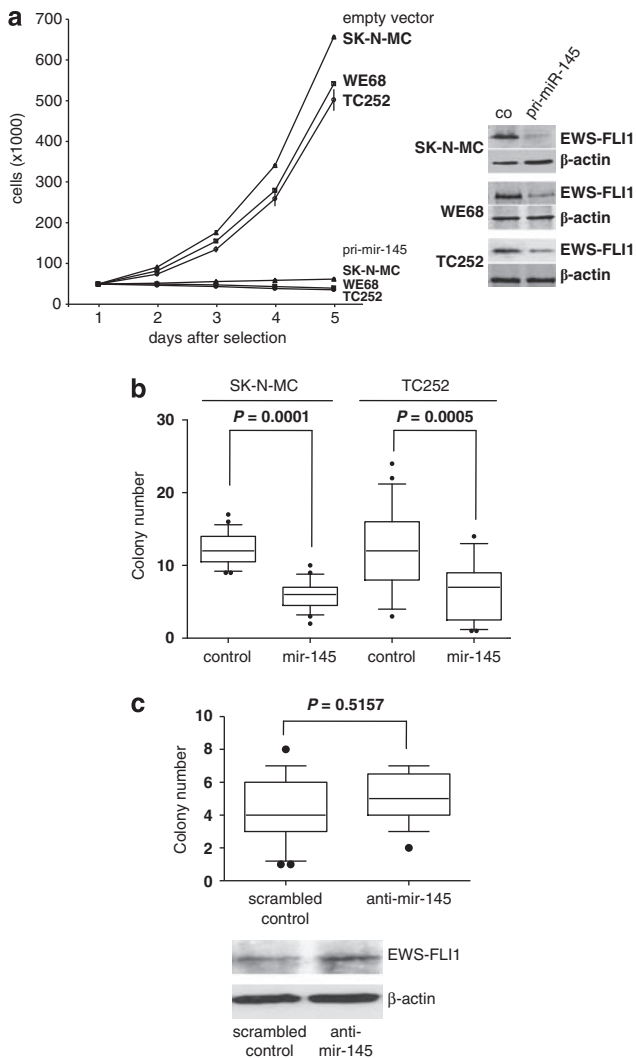
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Figure 4 Ectopic hsa-mir-145 expression suppresses growth of Ewing's sarcoma cells. (**a**) Growth inhibition of SK-N-MC, TC252 and WE68 cells under anchorage-dependent growth conditions. Cells were transfected with either pri-mir-145 expression construct or empty vector and puromycin-selected for 3 days. On day 4 after transfection, cells were split and seeded at 50 000 cells per well. After one day of recovery, cells were counted on a daily basis. (**b, c**) Effect of hsa-mir-145 and of anti-mir-145 on anchorage-independent growth of ESFT cell lines. Experiments studying the consequences of pri-mir-145 expression (**b**) and of anti-mir-145 (**c**) on soft agar colony formation were performed in triplicates and repeated thrice. Results present mean (± s.e.m.) numbers of colonies at seven sites per well for a total of 21 fields. Statistical significance was determined using the unpaired t test. ESFT cells were transfected with pri-mir-145 expression construct or empty vector (**b**), or empty vector plus either anti-mir-145 or control scrambled anti-mir (**c**) and were puromycin selected for 72 h. Cells were seeded in triplicates at 3×10^4 cells/35-mm dish. After resuspension in 0.3% agar in RPMI containing 10% fetal calf serum, cells were plated in 0.6% agar-coated dishes. A top layer containing 0.6% agar was then added. Plates were incubated at $37\,^\circ$C in 5% CO_2. Cells were fed every 3 days by placing three drops of medium on the top layer. Colonies were microscopically counted after 10 days.

In a parallel study investigating the tumorigenic reprogramming of mesenchymal stem cells by EWS-FLI1, Riggi et al. tested specifically the regulation of

hsa-mir-145 as the candidate suppressor of stemness, and arrived at similar conclusions as presented here. Using a 1.5 kb promoter fragment in reporter gene assays, they concluded that EWS-FLI1 directly regulates hsa-mir-145 expression through conserved Ets-binding sites, proximal to the transcription start site (Riggi et al., 2010). However, although our study independently confirms feedback regulation between EWS-FLI1 and hsa-mir-145, we did not detect any EWS-FLI1 binding to the hsa-mir-145 promoter by chromatin immunoprecipitation (Supplementary Figure S1). In fact, genome-wide screening for EWS-FLI1 binding to ESFT chromatin by deep-sequencing of the immunoprecipitated chromatin identified, among a total of ∼16 000 discrete genomic binding regions, the closest hit at a distance of ∼73 kb upstream of the hsa-mir-145 transcription start site (our so far unpublished observations). Thus, the mechanism of hsa-mir-145 repression by EWS-FLI1 remains elusive.

It has recently been demonstrated that primary hsa-mir-145 transcription and processing are activated by wild-type p53 activity (Sachdeva et al., 2009; Suzuki et al., 2009). Though we have previously reported that EWS-FLI1 reduces basal p53 levels by suppression of the NOTCH signaling pathway (Ban et al., 2008), providing a possible mechanism for hsa-mir-145 modulation in Ewing's sarcoma, the highest basal expression of mature hsa-mir-145 and lowest levels of EWS-FLI1 protein were observed in ESFT cell lines that express either a truncated (SK-N-MC) or no p53 at all (A673) (Kovar et al., 1993), and readily allowed for a further significant increase in hsa-mir-145 expression upon silencing of EWS-FLI1. In addition, we did not observe any influence of DNA damage-induced (etoposide and doxorubicin treatment) p53 activation on hsa-mir-145 and EWS-FLI1 expression in wild-type p53 Ewing's sarcoma cell lines (data not shown). Consequently, it is unlikely that EWS-FLI1 regulation of hsa-mir-145 is mediated through a mechanism involving p53. The fact that hsa-mir-145 is involved in the differentiation of several different tissues, and that it is frequently suppressed in many cancers suggest that multiple modes of transcriptional regulation exist for this microRNA. Recent data demonstrate that these mechanisms may be controlled by microenvironmental signaling cues, including growth factors (Quintavalle et al., 2010). It is therefore intriguing to speculate that fine tuning of hsa-mir-145 levels in Ewing's sarcoma, may to some extent, be subjected to the specific tumor microenvironment. We tested platelet-derived growth factor (PDGF) signaling as an example, as PDGF-BB is a known growth factor for Ewing's sarcoma (Uren et al., 2003), dependent on EWS-FLI1-activated phospholipase D2 expression (Nozawa et al., 2005). It was recently demonstrated that PDGF mediates podosome formation of vascular smooth muscle cells by inhibiting hsa-mir-145 (Quintavalle et al., 2010). Although we observed a weak influence of PDGF signaling on hsa-mir-145 expression in ESFT in preliminary experiments, it was variable and not statistically significant (data not shown). Therefore, it is

unlikely that EWS-FLI1 represses hsa-mir-145 expression through the activation of PDGF signaling. Other intrinsic or extrinsic mechanisms may exist regulating the hsa-mir-145/EWS-FLI1 balance that remains to be defined.

The inverse correlation between hsa-mir-145 RNA and EWS-FLI1 protein levels, and its role in Ewing's sarcoma cell growth suggest that subtle variations in the balance between these two molecules may impact tumor growth and progression. Importantly, this may open a new window of opportunity for future microRNA-mediated therapeutic strategies in this disease.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)