Histone Deacetylase Inhibitors Reverse SS18-SSX–Mediated Polycomb Silencing of the Tumor Suppressor Early Growth Response 1 in Synovial Sarcoma


Abstract

Synovial sarcoma is a soft tissue malignancy characterized by the fusion of SS18 to either SSX1, SSX2, or SSX4 genes. SS18 and SSX are transcriptional cofactors involved in activation and repression of gene transcription, respectively. SS18 interacts with SWI/SNF, whereas SSX associates with the polycomb chromatin remodeling complex. Thus, fusion of these two proteins brings together two opposing effects on gene expression and chromatin structure. Recent studies have shown that a significant number of genes are down-regulated by the SS18-SSX fusion protein and that the clinically applicable histone deacetylase (HDAC) inhibitor romidepsin inhibits synovial sarcoma growth. Therefore, we set out to identify direct targets of SS18-SSX among genes down-regulated in synovial sarcoma and investigated if romidepsin can specifically counteract SS18-SSX–mediated transcriptional dysregulation. Here, we report that the tumor suppressor early growth response 1 (EGR1) is repressed by the SS18-SSX protein through a direct association with the EGR1 promoter. This SS18-SSX binding correlates with trimethylation of Lys27 of histone H3 (H3K27-M3) and recruitment of polycomb group proteins to this promoter. In addition, we found that romidepsin treatment reverts these modifications and activates EGR1 expression in synovial sarcoma cell models. Our data implicate polycomb-mediated epigenetic gene repression as a mechanism of oncogenesis in synovial sarcoma. Furthermore, our work highlights a possible mechanism behind the efficacy of a clinically applicable HDAC inhibitor in synovial sarcoma treatment. [Cancer Res 2008;68(11):4303–10]

Introduction

Synovial sarcoma represents 5% to 10% of all soft tissue sarcomas, and although it can occur in many sites and at any age, this malignancy most typically arises in the extremities of young adults (1). Currently, standard treatment is wide local excision with adjuvant radiation. Outcomes vary with institution and patient age, but ~40% recur, 50% metastasize, and 10-year overall survival is 40% (2). Current doxorubicin-based chemotherapy offers only very modest survival benefits in cases of disseminated disease (3).

Synovial sarcoma is defined by a characteristic translocation t(X;18)(p11.2;q11.2) demonstrable in >95% of cases (4). This translocation results in a fusion of SS18 (also known as SYT) on chromosome 18 to either the SSX1, SSX2, or SSX4 gene on the X chromosome, creating a fusion oncogene believed to be the main molecular basis of this disease (5). Although neither SS18 nor SSX has a recognizable DNA-binding domain, SS18 is able to transactivate and SSX is able to repress transcription when targeted to a reporter gene (6, 7). SSX and SS18-SSX have been reported to colocalize with the RING1 and Bmi-1 components of the polycomb (PcG) chromatin remodeling repressor complex and to associate with condensed chromatin and core histones (8, 9). The COOH-terminal portion of SSX (including the SSXRD repression domain retained in the SS18-SSX fusion) is responsible for SSX colocalization with PcG bodies (10), and this correlates with the transforming activity of SS18-SSX in rat 3Y1 cells (11). On the other hand, SS18 (and SS18-SSX) has been reported to directly associate in vitro and colocalize with the BRM (SMARCA2) and BRG1 (SMARCA4) components of trithorax group-related SWI/SNF (9), a global chromatin remodeling transcriptional coactivator complex.

The SS18-SSX fusion thus brings together two protein domains, which associate with opposing chromatin remodeling complexes, and most likely leads to development of the disease by disrupting epigenetic regulation of gene expression (5). SS18 is widely expressed in both embryonic and adult tissues (12), whereas SSX proteins belong to the cancer/testis antigens normally expressed only in cells of germ-line and adult tissues but often reactivated in cancer (13), suggesting that the oncogenic potential of the fusion may come in part from reactivation of SSX functions. SS18 and SS18-SSX show speckled distribution, whereas SSX shows a diffuse nuclear distribution (6, 8–10), implying that the fusion protein may exert its function at sites where SS18 is normally active. The mammalian PcG complex polycomb repressor complex (PRC) 1 can block chromatin remodeling mediated by SWI/SNF complexes (14), raising the possibility that the SSX-associated PcG effect could dominate over SS18-associated SWI/SNF when brought together by the synovial sarcoma oncoprotein.

The initiation of gene silencing by polycomb activity has been shown to involve histone deacetylation (15, 16), and the histone deacetylase (HDAC) inhibitor romidepsin has been recently shown to have a growth-inhibitory effect in synovial sarcoma cell and animal models (17). Thus, if SS18-SSX exerts its function through PcG-mediated repression of tumor suppressor genes, HDAC inhibitors could potentially reverse this process, resulting in a
reactivation of gene expression that leads to growth arrest and apoptosis.

A prominent candidate target for SS18-SSX–mediated repression in synovial sarcoma is the early growth response 1 (EGR1) gene. EGR1 encodes a serum-inducible zinc finger protein that is a critical regulator of proliferation, differentiation, inflammation, and apoptosis (18). EGR1 has been also proposed to have a tumor suppressor function because many human tumor cell lines express little or no EGR1 compared with their normal counterparts and its expression is decreased in non–small cell lung tumors, breast tumors, and gliomas (19–22). Reexpression of EGR1 in human tumor cells suppresses transformation (19) and induces multiple downstream tumor suppressors, including transforming growth factor-β, PTEN, and p53 (23). Primary mouse embryo fibroblasts isolated from EGR1-null mice bypass replicative senescence in culture and fail to arrest after treatment with DNA-damaging agents, effects reversed by reintroducing EGR1 (24). cDNA microarray profiling data (25, 26) and results of a recent in vitro study in a SS18-SSX–inducible system (27) suggest that this tumor suppressor may also be a key player in synovial sarcoma.

Here, we provide evidence that EGR1 is a direct target of SS18-SSX and is down-regulated via repressive histone modifications and recruitment of polycomb proteins to the EGR1 promoter. The growth-inhibitory effect of romidepsin in synovial sarcoma cells is accompanied by reactivation of EGR1 expression through removal of these repressive histone modifications and dissociation of the polycomb repressor proteins from the EGR1 promoter. This not only provides some of the first evidence for a direct target of the SS18-SSX protein but also shows how the transcriptional effects of this fusion oncogene can be reversed with a new class of anticancer drugs.

Materials and Methods

Cells and reagents. The human monophasic synovial sarcoma cell line Fuji and biphasic synovial sarcoma cell line SYO-1, both expressing SS18-SS2, were provided by Dr. Kazuo Nagashima (Hokkaido University School of Medicine, Hokkaido, Japan) and Dr. Akira Kawai (National Cancer Centre Hospital, Tokyo, Japan), respectively. Normal human fibroblasts (HS68) were obtained from SACRI Antibody Services and Cell Bank Facility, University of Calgary (Calgary, Alberta, Canada). All cells were grown under standard incubation conditions (37°C, 95% humidity, 5% CO2) in the following media: Fuji and HS68, RPMI 1640 with 10% fetal bovine serum (FBS); HeLa, RPMI 1640 with 5% FBS; and SYO-1 and HEK293, DMEM with 10% FBS. Romidepsin (NSC 630176, FK228, depsipeptide) was provided through the Developmental Therapeutics Branch of the National Cancer Agency (Bethesda, MD) and dissolved in ethanol. Doxorubicin was purchased from Sigma and dissolved in DMSO.

Oligonucleotide microarray measurements. For each primary tumor specimen, 100 µg total RNA was purified from 200 mg of frozen tumor tissue by Trizol extraction and reverse transcribed using a mixture of oligo(DT) (high-performance liquid chromatography purified; Operon) and random hexamer (Amersham) primers, with incorporation of aminomethyl-dUTP (Ambion). Cy-3 and Cy-5 dyes (Amersham) were used for indirect labeling of cDNA. Reference RNA was from Stratagene (Universal Human Reference RNA). Labeled RNA (30 µg) was hybridized onto Human Exonic Evidence Based Oligonucleotide microarrays. Microarray hybridization and washing was performed using standard procedures (full details are available online).3 Microarrays were scanned on a GenePix 4000 microarray scanner and fluorescence ratios (tumor/reference) were calculated using GenePix software. In this study, expression analysis was limited to the EGR1 gene and to a published list of 55 EGR1 targets (28).

Establishment of SS18-SSX–inducible cell lines (HEK-FUS). The generation of human embryonic kidney (HEK293) cells exhibiting tetracycline-inducible expression of SS18-SSX2 was described elsewhere (27). In brief, HEK293-Trex cells (Invitrogen) were stably transfected with a full-length SS18-SSX2 cDNA cloned into the pcDNA4-T4 vector (Invitrogen) with empty vector as a control. For induction, 1 ng/mL tetracycline (Sigma) was added to the HEK293-Trex empty vector and HEK293-Trex SS18-SSX2 (HEK-FUS) cell lines for 24 h.

Establishment of cell lines stably expressing SS18 and SS18-SSX2 proteins. SS18-SSX2/HisMyc and SS18/HisMyc plasmids were kindly provided by Dr. Marc Landanyi (Memorial Sloan-Kettering Cancer Center, New York, NY). At ~70% confluence, HEK293 cells were transfected with 1 µg of plasmid DNA using Fugene reagent (Roche). Drug selection was started 48 h after transfection with 400 µg/mL Zeocin. Drug-resistant colonies were isolated after 6 wk of selection and protein expression was confirmed by Western blot with anti-Myc tag antibody (Cell Signaling Technology). As a control, HEK293 cells were transfected with vector pcDNA4-Myc-His (Invitrogen).

RNA extraction and real-time quantitative reverse transcription-PCR. Cells were grown as monolayer cultures to ~70% confluence and treated with 0.5, 1, 2.5, and 5 ng/mL of romidepsin or 0.1% ethanol (vehicle control) for 6 or 12 h. RNA was isolated using Trizol according to the manufacturer’s instructions (Invitrogen). RNA (1 µg) was treated with DNasel (Invitrogen) and reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen) in 25 µL reactions. Each quantitative reverse transcription-PCR (qRT-PCR) was done in triplicate using 1 µL of the reverse transcriptase reaction and SYBR Green PCR Master Mix (Applied Biosystems) with the following primers: EGR1, 5′-CAGCTTCCAGGCTGCTC-3′ and 5′-TACATCTCCACTGATTTG-3′; SS18-SSX, 5′-TGACCGATACATGCCCACAG-3′ and 5′-GGGTCCAGACATCTCTCTGTA-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CATCAATGG- AAATCCCCATAC-3′ and 5′-TGACCTCCAGACATCTCTA-3′. Dissociation curve analysis was used to ensure primer specificity and optimal concentration. Changes in expression were quantified using the ABI PRISM 7900HT sequence detection system. All qRT-PCR data were normalized to the expression of GAPDH and fold change in expression was calculated relative to vehicle-treated control. HDAC inhibitors have been shown to affect expression of several housekeeping genes commonly used as endogenous controls in qRT-PCR studies (29). Therefore, we evaluated the effect of romidepsin on the expression of a series of candidate housekeeping genes (G3PDH, TBP, GUSB, RPL13A, RPLP0, PUM1, PBGD, PSMC, SF3A1, GUSB, and MRPL) by semiquantitative PCR. All of the analyzed genes were affected by romidepsin treatment, albeit at different time points. GAPDH was chosen for our qRT-PCR experiments because it was characterized by stable expression in all the cell lines through 12 h of treatment, and its expression was only reduced after 24 h of romidepsin treatment. Because we were unable to find an endogenous control showing stable expression with romidepsin treatment in all the cell lines at 24 h, this and later time points were eliminated from our analyses.

SS18-SSX small interference RNA. For small interference RNA (siRNA), duplex oligos (sense, CAAGAAGCCAGCAAGGATT; antisense, UUCCUCGUGCCGUUUCUGGT) were designed to target the SSX portion of SS18-SSX using the Integrated DNA Technologies RNA interference (RNAi) design tool and synthesized by Integrated DNA Technologies, Inc. At ~60% confluence, SYO-1 cells were transfected with the SS18-SSX siRNA or control siRNA (Qiagen) to a final concentration of 30 nM/L using siLentFect Lipid Reagent (Bio-Rad Laboratories, Inc.). RNA was isolated for qRT-PCR analysis 48 h after transfection.

Chromatin immunoprecipitation analysis. Cells (2 × 105) were washed with PBS and incubated for 15 (in case of modified histone immunoprecipitations) or 30 min (for nonhistone protein immunoprecipitations) at room temperature with 1% formaldehyde in minimal culture medium. After cross-linking, cells were washed with PBS and chromatin was isolated and sheared using the Chip-IT Enzymatic Shearing kit.

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3 Primary microarray data for EGR1 and 21 target genes are included in the article, with full protocols available at http://med.stanford.edu/labs/vanderjijn/Protocols.html.
according to the manufacturer’s recommendations (Active Motif). For the SS18 immunoprecipitation, shearing was performed by sonication as described (27). Protein-DNA complexes were immunoprecipitated using ChIP-IT Chromatin Immunoprecipitation kit (Active Motif) and antibodies against acetylated histone H3 (H3Ac; Upstate), histone H3 trimethylated at Lys4 (H3K4-M3; Abcam), histone H3 trimethylated at Lys27 (H3K27-M3; Upstate), RA2009 (directed against the SS18 moiety of SS18-SSX2; ref. 30), Bmi-1 (Santa Cruz Biotechnology), Ezh2 (Cell Signaling Technology), and rabbit IgG (as the negative control). The recovered DNA was analyzed by PCR with multiple primers spanning the promoter region (−1,500 to +967) of EGR1. In case of anti-SS18 chromatin immunoprecipitation (ChIP) on HEK-FUS cells, the protein-DNA complexes were immunoprecipitated as described previously (27).

Results

EGR1, a down-regulated gene in synovial sarcoma, is a target of the SS18-SSX protein. In our recently published in vitro expression profiling study, EGR1 was identified as one of the genes exhibiting a dramatic decrease in mRNA expression on induction of SS18-SSX expression in the HEK293-FUS cell model, which bears a stably transfected tetracycline-inducible SS18-SSX2 expression construct (27). To confirm that this observation is relevant to primary synovial sarcoma tumor samples, we interrogated microarray expression data from patient specimens and found that EGR1 is down-regulated in synovial sarcoma, as are 21 of 55 published EGR1 targets (Fig. 1; ref. 28). This finding is further confirmed by interrogating published cDNA microarray data from the studies of Baird and colleagues (25) and Nielsen and colleagues (26), who provide searchable online databases of soft tissue expression profiles.6 Considering this evidence for EGR1 down-regulation in inducible cell models and primary tumor samples, and the suspected function of the SS18-SSX synovial sarcoma oncoprotein in transcriptional repression, we tested whether the SS18-SSX protein was present at the EGR1 promoter by ChIP. For this assay, we used an SS18-specific antibody in the HEK293-FUS cell line and compared results after 0 and 24 h of tetracycline treatment. PCR analysis of the ChIP DNA with EGR1 promoter and intron-specific primers indicates that tetracycline-induced expression of the SS18-SSX protein leads to a >50-fold increase in target present at the EGR1 promoter, providing strong evidence for the recruitment of SS18-SSX to the promoter of this gene (Fig. 2A). Next, we confirmed the recruitment of endogenous SS18-SSX by ChIP with the same anti-SS18 antibody from the synovial sarcoma cell line SYO-1 (Fig. 2B). To further confirm the inhibitory effect of SS18-SSX on EGR1 expression, we used RNAi duplex oligos targeting the SSX portion of the fusion mRNA to silence SS18-SSX expression in the SYO-1 cell line. Real-time qRT-PCR analysis of SS18-SSX and EGR1 transcript levels 48 h after transfection with the RNAi duplex revealed a significant decrease in SS18-SSX transcript levels (~30% of control), with a concomitant increase (5-fold) in EGR1 expression (data not shown), thus further supporting SS18-SSX involvement in the repression of EGR1.

Figure 1. EGR1 expression in synovial sarcoma and other nonpleomorphic soft tissue tumor types. Spot information for EGR1 was extracted from oligonucleotide microarray data from 26 primary sarcoma specimens, and both the mean-centered heat map and raw two-channel hybridization images are displayed. The log2 expression ratios of EGR1 relative to Universal Human Reference RNA are shown below the spot images. EGR1 target genes with reduced expression (P < 0.05) in synovial sarcoma are also shown. The color scale represents mean-centered log2 expression ratios, with green denoting a low level of expression. DFSP, dermatofibrosarcoma protuberans; DTF, desmoid-type fibromatosis; GIST, gastrointestinal stromal tumor; SS, synovial sarcoma.

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6 This study references previously published expression data, with searchable online databases at http://watson.nhgri.nih.gov/sarcoma/ and http://genome-www.stanford.edu/sarcoma/.
Romidepsin (FK228), a HDAC inhibitor, restores EGR1 expression in synovial sarcoma cells and in SS18-SSX–expressing HEK293 cells. Independent work has shown (a) that both the SS18-associated SWI/SNF and the SSX-associated Polycomb complexes incorporate HDACs (15, 16, 31) and (b) that the HDAC inhibitor romidepsin (FK228) inhibits growth and induces apoptosis in synovial sarcoma cells (17). Accordingly, we tested the effect of romidepsin on EGR1 expression in two synovial sarcoma cell lines (Fuji and SYO-1) and in HEK293 cells that stably express SS18-SSX2.

First, to assess the specific effect of romidepsin on EGR1 expression in the two synovial sarcoma cell lines, we measured the expression of this gene after 6 and 12 h of romidepsin treatment by qRT-PCR. We found that romidepsin induces EGR1 expression in both synovial sarcoma cell lines in a time- and dose-dependent manner (Fig. 3A). To determine whether this effect is specific to synovial sarcoma cells, we also evaluated EGR1 levels in normal human fibroblasts (Hs68) and in HeLa cells. Before treatment, Hs68 cells display high EGR1 expression levels, which are not affected by romidepsin (Fig. 3A). In contrast, EGR1 expression is low in untreated HeLa cells but is induced by romidepsin treatment (Fig. 3A) at levels comparable with that seen in Fuji cells but less than that seen in SYO-1. Doxorubicin, a common chemotherapy agent used in treatment of synovial sarcoma, had no effect on EGR1 expression in any of the cell lines tested (Fig. 3A). Therefore, it is unlikely that the observed increase in EGR1 after romidepsin treatment is a result of a general cellular response to a cytotoxic agent.

Next, because we previously showed EGR1 to be down-regulated on induction of SS18-SSX expression (27), we determined whether romidepsin affects this SS18-SSX–mediated EGR1 repression. To do so, we compared EGR1 transcript levels in HEK293 cells stably expressing Myc-tagged SS18 or SS18-SSX2 after 6 or 12 h of romidepsin treatment. Expression of SS18 and SS18-SSX2 was confirmed by Western blot using an anti-Myc-tag antibody (data not shown). Expression analysis (by qRT-PCR) yielded no romidepsin-induced effect on EGR1 expression in HEK293 cells expressing SS18 or the vector control (Fig. 3B, black and gray columns). In contrast, romidepsin treatment of SS18-SSX–expressing HEK293 cells did lead to a significantly increased EGR1 expression (Fig. 3B, crosshatched columns). Thus, in a HEK293 background, HDAC inhibitor–induced EGR1 expression is significantly increased in the presence of the fusion oncogene SS18-SSX, whereas the wild-type SS18 confers no such effect. These data also show that HEK293 cells expressing SS18-SSX accurately recapitulate the effects exerted by romidepsin on EGR1 expression observed in synovial sarcoma cell lines expressing endogenous SS18-SSX.

Romidepsin-induced reactivation of EGR1 is associated with activating histone modifications at the EGR1 promoter in synovial sarcoma cells. To determine whether romidepsin affects the histone acetylation status at the EGR1 promoter in synovial sarcoma cells, we used a ChIP assay using antibodies directed against H3Ac. In this assay, we observed significantly increased histone H3 acetylation levels at the EGR1 promoter in romidepsin-treated Fuji and SYO-1 cells compared with vehicle-treated control (Fig. 4A). This increased histone acetylation was observed as early as 6 h after romidepsin treatment, further supporting EGR1 as being a direct target of HDAC inhibitor action in synovial sarcoma cells. By comparison, H3Ac levels at the EGR1 promoter in HeLa cells were low, irrespective of romidepsin treatment (Fig. 4A). This is consistent with the low expression of this gene and indicates that this cell line (which does not express SS18-SSX) uses a different mechanism of romidepsin-mediated EGR1 activation. In human fibroblasts (Hs68), histone H3 acetylation levels of the EGR1 promoter were high, again irrespective of romidepsin treatment, consistent with the high and unchanged expression of this gene in Hs68 (Fig. 3A).

Given the known association of SS18-SSX with trithorax and polycomb homologues, we next investigated whether the observed romidepsin-induced activation of EGR1 is associated with histone H3 trimethylation at Lys4 and/or Lys27 (H3K4-M3 and H3K27-M3, respectively). Trithorax-mediated K4 trimethylation positively regulates transcription by recruiting nucleosome remodeling enzymes and histone acetyltransferases (32–34), whereas polycomb-mediated K27 trimethylation negatively regulates transcription by promoting a compact chromatin structure (35, 36). Histone methylation was assayed by ChIP with antibodies specific to H3K4-M3 and H3K27-M3 on chromatin isolated from romidepsin- and vehicle-treated Fuji, SYO-1, Hs68, and HeLa cells. We observed significant H3K4-M3 levels at the EGR1 promoter in the synovial sarcoma cell lines and in the Hs68 cells and found that this active chromatin mark was not affected by romidepsin treatment in these cells (Fig. 4B). This high level of H3K4-M3 at the EGR1 promoter in Hs68 cells suggests that activation of this gene normally requires H3K4 methyltransferase activity. In HeLa cells, H3K4-M3 was almost undetectable at the EGR1 promoter but was significantly induced by romidepsin treatment (Fig. 4B), consistent with the observed romidepsin-induced increased expression of EGR1 in these cells. The inactive chromatin mark H3K27-M3 was exclusively detected at the EGR1 promoter in untreated synovial sarcoma cells and (importantly) almost completely removed by romidepsin treatment (Fig. 4B). Taken together, we found that, in HeLa cells, romidepsin-mediated EGR1 up-regulation is accompanied by enhanced levels of H3K4-M3 at the EGR1 promoter. In contrast, the romidepsin-mediated EGR1 up-regulation in synovial sarcoma cells is associated with histone H3 trimethylation at Lys4 and/or Lys27 (H3K4-M3 and H3K27-M3, respectively).
sarcoma cell lines is mediated by the removal of the repressive histone mark H3K27-M3.

To determine if SS18-SSX is responsible for these histone modifications at the EGR1 promoter, we again used HEK293 cells stably expressing SS18 or SS18-SSX2. ChIP assays were performed with the same antibodies specific to H3Ac, H3K4-M3, and H3K27-M3, whereas the presence of the Myc-tagged SS18 and SS18-SSX proteins at the EGR1 promoter was confirmed with an antibody recognizing the Myc tag. These experiments showed significant levels of H3K4-M3 at the EGR1 promoter in the vector-transfected HEK293 cells, which were not altered by either SS18 or SS18-SSX expression nor by romidepsin treatment (Fig. 4C). Furthermore, we found that only HEK293 cells expressing SS18-SSX exhibit significant levels of H3K27-M3 at the EGR1 promoter and that this histone modification is removed by romidepsin treatment (Fig. 4C). In contrast, HEK293 cells expressing SS18 displayed increased levels of H3 acetylation, but no H3K27-M3 at the EGR1 promoter, and this status was unaffected by romidepsin treatment (Fig. 4C). Because the Myc-ChIP assays show that both SS18 and SS18-SSX are recruited to the EGR1 promoter, our data suggest that the effect on H3K27-M3 is unique to the SS18-SSX–expressing cells. These findings support a model wherein SS18 is recruited to the EGR1 promoter, but the SSX moiety is required to induce the repressive epigenetic H3K27-M3 mark. The ability of romidepsin to remove this SS18-SSX–mediated repressive histone modification suggests that these changes are dependent on HDAC activity. The presence of H3K27-M3 also suggests the involvement of PcG proteins, known to associate with SSX.

PRC proteins associate with the EGR1 promoter in synovial sarcoma and are removed by HDAC inhibitor treatment. To test whether the H3K27 signatures at the EGR1 promoter are indeed related to PRC activity, we performed ChIP assays using antibodies directed against the Bmi-1 component of the PRC1 and the Ezh2 histone methylase component of PRC2. We found that these polycomb group proteins are indeed present at the EGR1 promoter in synovial sarcoma cell lines expressing endogenous SS18-SSX and in HEK293 cells stably expressing SS18-SSX (Fig. 5). However, HEK293 cells stably expressing wild-type SS18 fail to recruit Bmi-1 or Ezh2 to the EGR1 promoter (Fig. 5A). Treatment with romidepsin removes the Bmi-1 and Ezh2 signals, a finding fully consistent with the observed loss of H3K27 methylation and with the reactivation of EGR1 expression.

Discussion

Repression of EGR1 transcription following induction of SS18-SSX has been previously shown in HEK293 cells (27). Here, we show that EGR1 is also down-regulated in synovial sarcoma primary tumor samples and is reactivated by SS18-SSX2–directed siRNA and by romidepsin treatment of synovial sarcoma cells, thus supporting direct involvement of SS18-SSX and HDACs in EGR1 repression. Furthermore, ChIP assays show that the EGR1 gene is a direct target of the SS18-SSX fusion oncoprotein, one of the first such targets to be identified, and that EGR1 repression is associated with deacetylation of histones, trimethylation of H3K27, and association with polycomb group repressor (PcG) complex proteins. Furthermore, because this process involves HDAC activity, it can be targeted by a class of drugs that have recently become available—the HDAC inhibitors.

Trimethylation of H3K27, a documented substrate of PcG histone methyltransferase activity (37), in synovial sarcoma cells

Figure 3. Effect of romidepsin (FK228) on EGR1 expression. EGR1 expression after romidepsin (FK228) treatment was assessed by qRT-PCR in (A) two synovial sarcoma cell lines (Fuji and SYO-1), normal human fibroblasts (Hs68), and HeLa cancer cells and (B) HEK293 stably transfected with SS18, SS18-SSX2, or empty plasmid. Changes in expression were quantified relative to vehicle-treated (0.1% ethanol) control. Each experiment was done in duplicate and samples were run in triplicates. Bars, 95% confidence interval. Doxo, doxorubicin. A, romidepsin (FK228) treatment up-regulates EGR1 expression in synovial sarcoma cells. B, presence of SS18-SSX2 increases sensitivity of HEK293 cells to romidepsin (FK228)–induced EGR1 expression.
and in SS18-SSX–expressing HEK293 cells implicates PcG activity in SS18-SSX–mediated repression of EGR1. Although PcG colocalization with SS18-SSX was first shown in 1999 (8), no proof of a direct interaction has yet been published. In this work, we show the presence both of SS18-SSX and of the PcG members Bmi-1 and Ezh2 at the promoter of a tumor suppressor gene that is repressed in primary synovial sarcoma samples.

The presence of both activating H3K4-M3 and repressing H3K27-M3 at the EGR1 promoter suggests that, in synovial sarcoma, this gene may be in a “bivalent” state, repressed but poised for activation. This novel chromatin modification pattern overlaying developmental transcription factor genes has been recently described and is associated with low gene expression (38). The presence of the H3K4-M3 mark at the EGR1 promoter in human fibroblasts suggests that its expression may normally be activated by the trithorax group complex. Our results reveal that both SS18 and SS18-SSX are directed to the EGR1 promoter in HEK293 cells, indicating that EGR1 is recognized by native SS18.

Ishida and colleagues (39) have recently shown both SS18 and SS18-SSX to be present at the COM1 promoter in HEK293 cells expressing hemagglutinin-tagged SS18 and SS18-SSX. In their study, SS18-SSX was shown to repress COM1 in HeLa cells stably expressing the fusion oncoprotein. SS18-SSX down-regulated, whereas SS18 up-regulated, COM1 promoter activity of luciferase reporter plasmids, and SS18-SSX blocked activation by SS18. Thus, their study is consistent with ours in supporting a dominant repressive function of the SS18-SSX protein at SS18 target genes. They did not investigate histone modifications nor HDAC inhibitor effects at the COM1 promoter, but it is possible that, similar to EGR1, COM1 is repressed by epigenetic modifications of histones at its promoter. Interestingly, we previously found this gene (also known as P8) to be among the top genes relatively down-regulated in primary synovial sarcoma tumor expression profiles (26).

The dominant function of PcG-type epigenetic changes in synovial sarcoma could also explain a stem cell–like phenotype for this disease (40). PcG proteins have recently been shown to play a major role in the maintenance of the adult stem cell populations (41–44) by keeping differentiation genes silent in embryonic stem cells and marking certain key genes for repressive signals during subsequent development and differentiation (44, 45). Because the repressor function of the fusion protein lies mostly in SSX, which colocalizes with PcG proteins, the oncogenic potential of the fusion is likely due to the reactivation of this SSX function. Indeed, expression of SSX has been recently reported in undifferentiated mesenchymal stem cells and is down-regulated after induction of osteocyte and adipocyte differentiation (46).

If this is the case, drugs restoring expression of differentiation genes, such as HDAC inhibitors (47), represent a very attractive therapy for synovial sarcoma, and identification of genes affected by these inhibitors will help in the search for new therapeutic targets that can aid in the design of even better, more specific...
Figure 5. Involvement of polycomb group (PcG) proteins Bmi-1 and Ezh2 at the EGR1 promoter in synovial sarcoma models and effect of romidepsin (FK228) treatment. HEK293 (A) and synovial sarcoma (B) cells were treated with either vehicle (0.1% DMSO) or 2.5 ng/mL romidepsin (FK228) for 12 h. Binding of PcG proteins to the EGR1 promoter was determined by ChIP using antibodies specific to Bmi-1 and Ezh2. Total chromatin (Input) was used for each ChIP assay as a positive control; anti-rabbit IgG antibody was applied as a negative control. PCR analyses were performed with primers recognizing the promoter region (-449 to -353) of EGR1. A, SS18-SSX2 is required for recruitment of PcG proteins to the EGR1 promoter, and PcG proteins are removed by romidepsin (FK228) treatment. B, romidepsin (FK228) blocks association of PcG proteins with the EGR1 promoter in synovial sarcoma cell lines.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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