EWS-FLI-1 Expression Triggers a Ewing’s Sarcoma Initiation Program in Primary Human Mesenchymal Stem Cells

Nicolò Rigi,1 Mario-Luca Suvà,1 Domizio Suvà,3 Luisa Ciron,1 Paolo Provero,4 Stéphane Tercier,2 Jean-Marc Joseph,3 Jean-Christophe Stehle, Karine Baumer,1 Vincent Kindler,1 and Ivan Stamenkovic1

1Division of Experimental Pathology, Institute of Pathology, and 2Department of Pediatric Surgery, University of Lausanne, Lausanne, Switzerland; 3Department of Orthopedics, University of Geneva, Geneva, Switzerland; and 4Department of Genetics Biology and Biochemistry, University of Turin, Turin, Italy

Abstract

Ewing’s sarcoma family tumors (ESFT) express the EWS-FLI-1 fusion gene generated by the chromosomal translocation t(11;22)(q24q12). Expression of the EWS-FLI-1 fusion protein in a permissive cellular environment is believed to play a key role in ESFT pathogenesis. However, EWS-FLI-1 induces growth arrest or apoptosis in differentiated primary cells, and the identity of permissive primary human cells that can support its expression and function has until now remained elusive. Here we show that expression of EWS-FLI-1 in human mesenchymal stem cells (hMSC) is not only stably maintained without inhibiting proliferation but also induces a gene expression profile bearing striking similarity to that of ESFT, including genes that are among the highest ESFT discriminators. Expression of EWS-FLI-1 in hMSCs may recapitulate the initial steps of Ewing’s sarcoma development, allowing identification of genes that play an important role early in its pathogenesis. Among relevant candidate transcripts induced by EWS-FLI-1 in hMSCs, we found the polycomb group gene EZH2, which we show to play a critical role in Ewing’s sarcoma growth. These observations are consistent with our recent findings using mouse mesenchymal progenitor cells and provide compelling evidence that hMSCs are candidate cells of origin of ESFT. [Cancer Res 2008;68(7):2176–85]

Introduction

Ewing’s sarcoma is the second most common bone malignancy in children and young adults with a peak incidence between the ages of 14 and 20 years. It is associated with specific chromosomal translocations that lead to the formation of fusion genes encoding proteins composed of the transactivation domain of EWS and the DNA binding domain (DBD) of one of five ETS family transcription factors, including FLI1, ERGB, ETVI, ETV4, and FEV. More than 85% of Ewing’s sarcoma family tumors (ESFT) are associated with the chromosomal translocation t(11;22)(q24q12) that generates the EWS-FLI-1 fusion gene (1). The corresponding fusion protein is believed to behave as an aberrant transcription factor that transforms target cells by deregulating their gene expression program.

Among the functions that have been ascribed to the EWS-FLI-1 protein is the regulation of target cell differentiation. EWS-FLI-1 expression induces immunohistologic ESFT features in several immortalized and malignant cell types (2–6) and recent observations have shown that numerous genes involved in neural differentiation and neuroectodermal development that are expressed in ESFT cell lines are regulated by EWS-FLI-1 (7–10). It is also well established that EWS-FLI-1 possesses Oncogenic properties. Its expression can accelerate tumorigenesis of murine NIH 3T3 cells in immunocompromised mice (6, 11), whereas its repression by antisense constructs or specific siRNA sequences in human ESFT cell lines results in decreased cell growth in vitro and tumorigenicity in vivo (12, 13). Introduction of EWS-FLI-1 into heterologous cells and fusion protein expression knockdown in ESFT cell lines have led to the identification of several candidate EWS-FLI-1 target genes that may be implicated in transformation and/or tumor progression (refs. 10, 14; reviewed in ref. 15). However, EWS-FLI-1 function is highly cell context dependent, and the identification of the genes implicated in the initiating phase of ESFT development may elude approaches using established heterologous cell lines and cells derived from late-stage ESFT. Full elucidation of ESFT pathogenesis requires understanding of the tumor initiating program induced by EWS-FLI-1 and identification of primary target cells that are permissive for its expression.

Efforts to identify candidate primary cells that constitute the origin of ESFT and that could help recapitulate the very first steps of tumor formation have been hampered by EWS-FLI-1 toxicity. Thus, introduction of EWS-FLI-1 into mouse embryonic fibroblasts resulted in cell cycle arrest and cell death (16). Mouse embryonic fibroblasts from p19ARF−/− mice transfected with EWS-FLI-1 were observed to maintain EWS-FLI-1 expression but did not form tumors in vivo (16). Only on transformation with SV40 T antigen could EWS-FLI-1-expressing mouse embryonic fibroblasts lacking p19ARF or p53 form tumors in vivo that display histologic features resembling those of human Ewing’s sarcoma (16). Similar observations were made in hTERT-immortalized human primary fibroblasts where EWS-FLI-1 expression induced p53-mediated growth arrest and apoptosis (17). However, at least half of Ewing’s sarcomas seem to have a functional p53 pathway and to retain p19ARF expression (18), suggesting the existence of primary cells that are permissive for EWS-FLI-1 expression without triggering an oncogenic stress type response that results in cell cycle arrest.

Recently, introduction of EWS-FLI-1 into unsorted murine bone marrow–derived cells was observed to result in the formation of tumors displaying various phenotypes including that of Ewing’s sarcoma (19). Work from our own laboratory has shown that primary mouse bone marrow–derived mesenchymal progenitor cells undergo transformation as a result of stable EWS-FLI-1 expression.
(20). Mouse mesenchymal progenitor cells expressing EWS-FLI-1 displayed robust up-regulation of insulin-like growth factor 1 (IGF-1), which is believed to play a major role in ESFT development, and on in vivo injection, formed tumors composed predominantly of sheets of small round cells, consistent with the ESFT phenotype (20). The tumors displayed high sensitivity to IGF-1 receptor (IGF-IR) inhibition, a hallmark of Ewing's sarcoma, as well as expression of Ewing's sarcoma-associated markers including neural specific enolase and CD99 (20).

Based on these observations, we addressed the possibility that human mesenchymal stem cells (hMSC) might provide a permissive cellular environment for EWS-FLI-1. Introduction of the fusion gene into bone-derived hMSCs resulted in its stable expression as well as phenotypic and transcriptional changes that reflect key features of Ewing's sarcoma. Among the observed transcriptional changes was the induction of the polycomb group gene enhancer of zeste homolog 2 (EZH2), which is reported to be highly expressed in Ewing's sarcoma (21) and proposed to be implicated in cancer development, stem cell maintenance, and proliferation (22). Partial suppression of EZH2 in two different Ewing's sarcoma cell lines resulted in a dramatic reduction of their proliferation and tumorigenic potential, suggesting that EZH2 may be an important player in ESFT initiation and growth.

Materials and Methods

Cell culture. Human MSCs were obtained from femoral head bone marrow of seven adult patients undergoing total hip replacement as previously described (23). MSCs were cultured at low confluence in Iscove's modified Dulbecco's medium, 10% FCS, and 10 ng/mL platelet-derived growth factor BB (PeProtech EC) and were tested for multilineage differentiation into adipocytes, chondrocytes, and osteoblasts (24). SK-N-MC and A673 cell lines (American Type Culture Collection) were cultured in RPMI 1640 containing 10% FCS.

Cloning and reverse transcription-PCR. The cDNA clone encoding the human EWS-FLI-1 type 2 fusion gene was amplified from the SK-N-MC Ewing sarcoma cell line, and cloned with or without a V5 tag at its 3'end in the pMSCV Puro retroviral vector (BD Biosciences Clontech) as previously described (20). The EWS-FLI-1 R340N DBD mutant (DBDM) was amplified by PCR using the wild-type (wt) sequence as template, with the following primers: hEWS forward Xhol, CGGCTCGAGCCACCATGGGTCACAGCTACAG; hFLI-1 reverse R340N, ATCATAGTAATAATTGAGGGCCGGCTACAGCTACAG; hFLI-1 reverse R340N, ATCATAGTAATAATTGAGGGCCGGCTACAGCTACAG; hFLI-1 reverse R340N, ATCATAGTAATAATTGAGGGCCGGCTACAGCTACAG. A pMSCV retroviral vector containing a V5 sequence–tagged EWS-FLI-1 cDNA, a V5 sequence–tagged cDNA encoding hFLI-1, a DAKO DAB Kit. Immunohistochemistry and flow cytometry. For in vitro staining, cells were fixed for 20 min at room temperature with 4% paraformaldehyde in 24-well plates. The primary antibodies used were polyclonal rabbit anti-TAU (1:200 dilution; Sigma) and mouse anti-NGFR hybridoma (kindly provided by Dr. Nicole Gross, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland). Paraffin-embedded sections of primary Ewing's sarcoma, SK-N-MC, and A673 derived tumors were stained with rabbit anti-human EZH2 polyclonal antibody (1:50 dilution; Cell Signaling). Horseradish peroxidase staining was done with biotin-conjugated horse anti-mouse or goat anti-rabbit immunoglobulin (Vector Laboratories) and revealed with a DAKO DAB Kit.

Pellets of hMSCs differentiated in the chondrogenic lineage were frozen in optimum cutting temperature compound (Tissue-Tek, Sakura Finetek), cut into 5-μm-thick sections, and stained with anti-collagen type II antibody (clone II-II63 mouse IgG1, 1:2 dilution; Developmental Studies Hybridoma Bank, University of Iowa) as described (23).

Flow cytometry analysis of hMSCs was done as previously described (24). Additional antibodies used in this study included anti-STRO-1 (Invitrogen) and anti-CD106-phycocerythrin (BD Pharmingen).

Affymetrix microarray, quantitative real-time PCR analysis, and EZH2 knockdown are discussed in Supplementary data.

Results

Expression of EWS-FLI-1 alters the hMSC phenotype. Human MSCs were maintained at low confluence and tested for their ability to differentiate into adipocytes, chondrocytes, and osteocytes in response to appropriate cytokines (refs. 23, 24; Fig. 1A, left). In addition to maintaining their differentiation potential, the cells displayed a homogeneous CD45−, CD19−, CD34−, CD11b−, CD80−, CD86−, and CD14-negative and a CD105- and CD90-positive phenotype, consistent with that of MSCs (Fig. 1A, right). These cells were also positive for CD106 and STRO-1 (Supplemental data 1A), as reported in the literature (25). Their in vitro life span was ~3 months (23). A pMSC retroviral vector containing a V5 sequence–tagged EWS-FLI-1 cDNA, a V5 sequence–tagged cDNA encoding the R340N EWS-FLI-1 DBDM, or no extraneous DNA (empty vector) was used to produce the corresponding viruses and infect four independent batches of hMSCs that had undergone 8 population doublings. The EWS-FLI-1 fusion protein bearing the R340N has been shown to lack DNA binding activity while retaining some degree of transforming potential (25). EWS-FLI-1 and DBDM expression was tested by Western blot analysis with the anti-V5 antibody at various time points following infection. Expression of both the wt and DBDM EWS-FLI-1 fusion proteins was observed 24 hours after infection and was found to be stably maintained several weeks later in all samples [Fig. 1B (left) and D (right), data not shown]. Moreover, the fusion protein expression level in two different batches of EWS-FLI-1–infected hMSCs was comparable to that of Ewing sarcoma cell lines, as assessed by Western blot analysis with the anti-FLI1 antibody (Fig. 1B, right).

Human MSCs expressing EWS-FLI-1 (hMSC<sup>EWS-FLI-1</sup>) underwent a marked morphologic change, displaying a round instead of the typically elongated, spindle shape of their wt, empty vector−, and DBDM (hMSC<sup>EWS-FLI-1</sup>)–expressing counterparts (Fig. 1C, whereas their in vitro life span, viability, and proliferation rate remained unaltered as assessed by an MIT assay (Fig. 2A and data not shown). The change in morphology of hMSC<sup>EWS-FLI-1</sup> was consistent with the reported ability of EWS-FLI-1 to modify the differentiation program of mouse mesenchymal progenitor cells (26). Because CD99 is the most reliable, although not specific, marker of ESFT, we addressed the possibility that EWS-FLI-1 expression induces CD99 in hMSCs. Western blot analysis with an
anti-CD99 antibody showed that hMSCs constitutively express low levels of CD99 and that its expression is strongly induced by EWS-FLI-1 (Fig. 1D, left). Interestingly, when hMSCs were tested for their ability to differentiate toward the chondrocytic, osteocytic, and adipocytic lineages 4 weeks after infection, they were observed to conserve their differentiation potential despite expression of EWS-FLI-1 (Fig. 1D, right). Three independent batches of hMSCs expressing EWS-FLI-1 were subjected to lineage-specific, differentiation-inducing culture conditions, and all displayed trilineage differentiation ability.
hMSC_{EWS-FLI-1} display induction of genes implicated in neural differentiation and neuroectodermal development as well as that of known EWS-FLI-1 target genes. Total RNA was extracted 12 days after infection and selection from each of the four EWS-FLI-1–, DBDM–, and empty vector–containing hMSC batches, and the corresponding mRNA was used to perform gene expression profile analysis using the Affymetrix U133 Plus 2.0 Arrays. Statistical analysis, using rank products (27) and a false discovery rate of 1%, revealed 614 and 262 probe sets corresponding to 393 and 174 genes to be respectively induced and repressed in hMSC_{EWS-FLI-1} compared with empty vector–infected counterparts, whereas 104 and 158 genes were respectively up-regulated and down-regulated in hMSC_{DBDM} (Supplementary data 2). Importantly, all four batches of hMSC_{EWS-FLI-1} cells revealed an almost identical gene expression profile, as shown by the heat map in Supplementary data 1B where the expression profile of the four samples is compared with that of cells infected with the R340N DBDM.

Among the most potently induced transcripts in hMSC_{EWS-FLI-1} were genes encoding proteolytic enzymes (primarily matrix

![Figure 2](https://example.com/figure2.png)

**Figure 2.** EWS-FLI-1 does not alter hMSC proliferation and induces a neuroectodermal gene expression profile. **A,** MTT assay analysis of the proliferation rate of hMSCs expressing DBDM or EWS-FLI 1 compared with their empty vector–infected counterparts. **B,** comparison of the induction in hMSC_{EWS-FLI-1} and hMSC_{DBDM} of selected neuroectodermal markers from the list shown in Table 1A, as assessed by real-time PCR. **C,** NGFR and MAPT genes were selected from B to further characterize their induction at the protein level by in vitro staining of hMSC_{EWS-FLI-1} compared with their empty vector– and DBDM-infected counterparts. Magnification, ×200. **D,** the induction of the two candidate EWS-FLI-1 target genes, IGF-I and NKX2-2, was confirmed by real-time PCR. All real-time PCR experiments were normalized to cyclophyllin A and done in triplicate. **Columns,** mean of three independent determinations.
metalloproteinases), adhesion receptors (including CLDN1, ICAM1, PCDH7/17, CEACAM1, and CDH11), and growth factors (including IGF1, TGFα/B, and HBEGF; Supplementary data 1C). Induction of these genes is consistent with the alterations in growth, survival, adhesion/migration, and tissue remodeling potential that may be expected in cells undergoing transformation.

EWS-FLI-1 expression in hMSCs also induced numerous genes that encode neural cell markers or that are implicated in neural crest development and neuronal differentiation, including NPY1R, GRP, MSX1, EGR2, NKK2-2, NGFR, CITED2, CDH11, and MAPT (Table 1A). Because expression of these genes is consistent with the primitive neuroectodermal phenotype of ESFT, six of them, including NPY1R, GRP, MAPT, NGFR, CITED2, and SOX2, were selected for validation of their observed expression change. Up-regulation of all six genes in hMSC_{EWS-FLI-1} was confirmed by quantitative real-time PCR (Fig. 2B). To assess the importance of DNA binding of EWS-FLI-1 in the induction of these six candidate target genes, their expression was assessed in hMSCs infected with the EWS-FLI-1 mutant bearing the R340N loss-of-function counterpart (Fig. 2C). The robust induction of NGFRp75 by EWS-FLI-1 failing to display induction of any of the genes, suggesting that an intact DBD is required for EWS-FLI-1-mediated regulation of their expression (Fig. 2B). To further validate the neuroectodermal phenotype induced by EWS-FLI-1, we assessed NGFR and MAPT protein expression in hMSCs infected with the three retroviruses. Consistent with the gene expression profile, strong induction of both neural markers was observed in hMSC_{EWS-FLI-1}, but not in empty vector– or DBDM-infected counterparts (Fig. 2C). The robust induction of NGFRp75 by EWS-FLI-1 was further confirmed by fluorescence-activated cell sorting (FACS) analysis of the infected cells (Supplementary data 1E).

Many of the reported candidate EWS-FLI-1 target genes were induced in hMSC_{EWS-FLI-1}, including ID2, IGF1, MMP1, TNC, COL11A1, and UPP1 (20, 28–30), as well as the more recently identified targets NROB1 and NKK2-2 (refs. 7, 10; Table 1A). Whereas up-regulation of several of these genes by EWS-FLI-1 has been observed in a variety of cellular backgrounds, induction of IGF1 has thus far been detected only in mouse mesenchymal progenitor cells (20). Ewing’s sarcoma cells are highly sensitive to IGF-IR blockade, consistent with the possibility that IGF-1 may facilitate EWS-FLI-1–mediated transformation and promote ESFT cell survival, particularly in the early phases of ESFT development. The relationship between EWS-FLI-1 and NKK2-2 was recently discovered using an inducible rescue approach in ESFT cell lines (10), and expression of the NKK2-2 gene has been suggested to be relevant to both ESFT differentiation and pathogenesis (10). Quantitative real-time PCR analysis confirmed induction of both IGF1 and NKK2-2 genes in hMSC_{EWS-FLI-1} cells but not in hMSC_{DBDM} cells (Fig. 2D), indicating that regulation of both genes by EWS-FLI-1 requires integrity of the DBD of FLI-1. Transcript levels of a panel of Ewing’s sarcoma discriminating genes in MSC_{EWS-FLI-1} were compared with those of the same genes in a fresh primary Ewing’s sarcoma sample. Several of the transcripts, particularly NPY1R, GRP, EZH2, and IGF1, were found to be expressed at comparable levels in MSC_{EWS-FLI-1} and primary Ewing’s sarcoma cells (Supplementary data 1D).

The expression profile of hMSC_{EWS-FLI-1} closely mimics that of ESFT but not of other bone and soft tissue tumors. To assess the degree of relatedness of the hMSC model to ESFT, we compared the gene expression profile of MSC_{EWS-FLI-1} to those of

| Table 1. EWS-FLI-1–induced differentiation markers and candidate target genes |
|------------------|-------------------|------------------|
| Clone            | M value           | Gene symbol      |
| (A) Neuroectodermal differentiation markers |
| 205440_s_at      | 7.004544088       | NPY1R           |
| 217561_at        | 5.506065541       | CALCA           |
| 203413_at        | 4.293524987       | NELL2           |
| 206915_at        | 4.205361231       | NKK2-2          |
| 214636_at        | 3.662756337       | CALCB           |
| 236088_at        | 3.503590436       | NTMG1           |
| 228038_at        | 3.400729183       | SOX2            |
| 201565_s_at      | 2.792584692       | ID2             |
| 204105_s_at      | 2.571239931       | NRCAM           |
| 205932_at        | 2.652991206       | MSX1            |
| 155448_s_at      | 2.655823127       | TMEM37          |
| 213749_at        | 2.366663943       | NPYX2           |
| 218162_at        | 2.249141903       | OLFML3          |
| 205249_at        | 2.43181602        | EGR2            |
| 230303_at        | 2.39988201        | SYNP3           |
| 208605_s_at      | 2.227463845       | NTRK1           |
| 39966_at         | 2.222217226       | CSF5G           |
| 204869_at        | 2.146557475       | PCSK2           |
| 221933_at        | 1.982400408       | NLGN4X          |
| 203929_s_at      | 1.659102536       | MAPT            |
| 232226_at        | 1.62194636        | LRRC4C          |
| 205858_at        | 1.528171209       | NGFR            |
| 207980_s_at      | 1.461895211       | CITED2          |
| 227933_at        | 1.209141398       | LRRN6A          |
| Candidate EWS-FLI-1 target genes |
| 205440_s_at      | 7.004544088       | NPY1R           |
| 206915_at        | 4.205361231       | NKK2-2          |
| 204475_s_at      | 3.88747653        | MMP1            |
| 201565_s_at      | 2.792584692       | ID2             |
| 209541_at        | 2.366028399       | IGF1            |
| 201645_at        | 1.576769787       | TNC             |
| 37892_at         | 1.434881719       | COL11A1         |
| 203234_at        | 1.460677636       | UPP1            |
| (B) Staeger ESFT signature and hMSC EWS-FLI-1 common genes |
| 205440_s_at      | 7.004544088       | NPY1R           |
| 202746_at        | 6.728787172       | ITM2A           |
| 219908_at        | 4.691662572       | DKK2            |
| 203358_s_at      | 3.103715832       | EZH2            |
| 228636_at        | 2.688202743       | BHLHB5          |
| 1552610_a_at     | 2.163652999       | ZAK1            |
| 205249_at        | 2.243181602       | EGR2            |
| 225871_at        | 2.18874054        | STAP2           |
| 219258_s_at      | 1.988302947       | BCL11B          |
| 206812_s_at      | 1.359914681       | ADRB3           |
| 241946_at        | 1.313478058       | ZDHHC21         |
| 226106_at        | 1.19256861        | BNFL41          |
| 227933_at        | 1.209141398       | LRRN6A          |
| 219825_at        | 1.544188607       | CYP26B1         |

NOTE: (A) Summary of genes implicated in neural differentiation and neuroectodermal development, as well as known EWS-FLI-1 target genes, found to be part of the hMSC EWS-FLI-1 gene expression profile. Log 2 values of fold induction are shown. (B) List of the 14 common genes between hMSC EWS-FLI-1 and the ESFT expression signature published by Staeger et al. (21). Log 2 values of fold induction are shown.
Ewing's sarcoma and other bone and soft tissue tumors in a recently established sarcoma gene expression database (31). A remarkable similarity was found between the gene expression profile of hMSC<sup>EWS-FLI-1</sup> and that of Ewing's sarcoma samples. Of the 225 genes proposed to be ESFT discriminators, 40 were observed to be induced in hMSC<sup>EWS-FLI-1</sup> (Fig. 3C), compared with an expected number of 4.46 (P = 1.95e-25). To determine whether hMSC<sup>EWS-FLI-1</sup> display comparable or greater similarity to ESFT than fibroblast<sup>EWS-FLI-1</sup> (17), direct comparison of each gene expression profile was made to that of native ESFT (31). To do so, only the genes found to be present in all three arrays were used, which resulted in 94 genes in the fibroblast profile list and 235 genes in the MSC profile list. This approach revealed that the 94 genes in the fibroblast profile list, 14 were common to the native ESFT list, for an expected 1.77 (P = 2.0e-9); of 235 genes in the hMSC list, 29 were common to the ESFT list, for an expected 4.43 (P = 3.8 E-16). These observations suggest that fibroblasts provide a more restrictive environment than hMSCs for expression of the target gene repertoire of the EWS-FLI-1 fusion protein (17). Importantly, genes that were induced in hMSC<sup>EWS-FLI-1</sup>, including FVT1, DKK2, MAPT, PRKCB1, JAK1, ITM2A, FRZB, CITED2, STEAP1, STEAP2, and ID2, were among the top discriminators for Ewing's sarcoma (Fig. 3C). Among the 12 other sarcomas analyzed, only dermatofibrosarcoma protuberans displayed a gene expression profile that closely mimics that of ESFT but not other bone and soft tissue tumors. A statistical analysis of the genes common to hMSC<sup>EWS-FLI-1</sup> and a publicly available database of soft tissue tumor gene expression profile [Baird et al.(31)], showing the striking similarity of hMSC<sup>EWS-FLI-1</sup> to ESFT but not to other sarcomas, with the significant P values indicated. B, Venn diagram representation of hMSC<sup>EWS-FLI-1</sup> profile relatedness to ESFT, dermatofibrosarcoma protuberans, and myxoid liposarcoma, based on the number of shared genes in their respective profiles, as showed in A. C, list of the 40 genes found to be shared by the hMSC<sup>EWS-FLI-1</sup> and ESFT gene expression profiles. Asterisks, genes found by Baird et al. to be top discriminators for Ewing’s sarcoma. Log 2 m values of fold induction are shown. D, statistical analysis of the genes common to hMSC<sup>EWS-FLI-1</sup> and the same tumor database used in A, indicating that expression of the EWS-FLI-1 DDDBM in hMSCs does not induce a gene expression profile in the infected cells that can discriminate ESFTs from other sarcomas. 

**Figure 3.** EWS-FLI-1 expression in hMSCs induces a gene expression profile that closely mimics that of ESFT but not other bone and soft tissue tumors. A, statistical analysis of the genes common to hMSC<sup>EWS-FLI-1</sup> and a publicly available database of soft tissue tumor gene expression profile [Baird et al.(31)], showing the striking similarity of hMSC<sup>EWS-FLI-1</sup> to ESFT but not to other sarcomas, with the significant P values indicated. B, Venn diagram representation of hMSC<sup>EWS-FLI-1</sup> profile relatedness to ESFT, dermatofibrosarcoma protuberans, and myxoid liposarcoma, based on the number of shared genes in their respective profiles, as showed in A. C, list of the 40 genes found to be shared by the hMSC<sup>EWS-FLI-1</sup> and ESFT gene expression profiles. Asterisks, genes found by Baird et al. to be top discriminators for Ewing’s sarcoma. Log 2 m values of fold induction are shown. D, statistical analysis of the genes common to hMSC<sup>EWS-FLI-1</sup> and the same tumor database used in A, indicating that expression of the EWS-FLI-1 DDDBM in hMSCs does not induce a gene expression profile in the infected cells that can discriminate ESFTs from other sarcomas. EWS, Ewing’s sarcoma; DFSP, dermatofibrosarcoma protuberans; LIP0, liposarcoma; RMS, rhabdomyosarcoma; MFH, malignant fibrous histiocytoma; OS, osteosarcoma; HPC, hemangiopericytoma; GIST, gastrointestinal stromal tumor; LMS, leiomyosarcoma; SS, synovial sarcoma; MULL, mixed Mullerian; MPNST, malignant peripheral nerve sheath tumor; FIBRO, fibrosarcoma.
profile with marginal similarity to hMSC\textsuperscript{EWS-FLI-1} (P = 0.011) (Fig. 3A and B). The same statistical analysis was applied to the gene expression profile of hMSC\textsuperscript{DBDM}. In contrast to wt EWS-FLI-1, expression of the EWS-FLI-1 mutant form did not confer on MSCs any statistically significant similarity with Ewing sarcoma (Fig. 3D). This observation supports the notion that the lack of shape change displayed by the hMSC\textsuperscript{DBDM} in vitro reflects a different genetic program in these cells, and highlights the importance of DNA binding for the ESFT phenotype–inducing properties of the fusion protein.

The highly discriminating ability of the hMSC\textsuperscript{EWS-FLI-1} gene expression profile for ESFTs was further confirmed by another study, which assessed the transcriptional of a broad range of mesenchymal tumors and identified the CALCB, MAPT, and PRKCB1 genes as prominent Ewing’s sarcoma discriminators (32), all of which we found to be induced in hMSC\textsuperscript{EWS-FLI-1} (Table 1; Supplementary data 2).

Comparison of the genes induced in hMSC\textsuperscript{EWS-FLI-1} with a set of 38 genes (of which only 34 were included on our microarray) found to be up-regulated in ESFT with respect to a wide spectrum of normal tissues and neuroblastomas (21) revealed 14 shared genes (P = 1.12e-015), including the strong ESFT discriminators NPYR1, ITM2A, DKK2, JAK1, STEAP, and EGR2 (Table 1B). Moreover, of a subset of 19 transcripts from the 34-gene set that could clearly distinguish an ESFT cell line (SK-N-MC) from neuroblastoma cell lines (21), 8 were part of the hMSC\textsuperscript{EWS-FLI-1} profile, whereas none of the transcripts in the 34-gene set were identified in the hMSC\textsuperscript{DBDM} expression profile (expected 0.18, P = 1.00). The same study reported that introduction of EWS-FLI-1 into HEK 293 cells induced only one gene of this subset, namely, CCND1, further highlighting the selective permissiveness of hMSCs for EWS-FLI-1 function (21).

**Injection of hMSC\textsuperscript{EWS-FLI-1} into immunocompromised mice.**
Following injection into the subcapsular renal compartment of immunocompromised mice, hMSC\textsuperscript{EWS-FLI-1} did not form tumors, suggesting that despite expressing many of the hallmarks of ESFT, these cells require some additional event to become tumorigenic in mice. This observation is not surprising in the sense that whereas there have been several examples of a single genetic event transforming mouse progenitor cells (20, 33–35), recent evidence suggests that five events are required to transform mouse embryonic fibroblasts and hematopoietic cells but fell short of rendering them tumorigenic in vivo (37, 38).

**EZH2 promotes Ewing’s sarcoma growth.** Among transcripts that were up-regulated in hMSC\textsuperscript{EWS-FLI-1}, we identified the gene encoding EZH2, a member of the polycomb group proteins (Supplementary data 2), which has recently been found to be expressed in ESFT (21). Together with EED and SUZ12, EZH2 forms the polycomb-repressive complex 2, believed to be a key regulator of embryonic development, stem cell renewal, and differentiation (39, 40). EZH2 is the catalytically active component of polycomb-repressive complex 2 and is believed to silence target genes by acting as a methyltransferase specific for Lys\textsuperscript{27} of histone H3 and Lys\textsuperscript{26} of histone H1 (reviewed in ref. 22). Overexpression of EZH2 has been shown to induce a bypass of the cellular senescence program in mouse embryonic fibroblasts and to prevent mouse hematopoietic stem cell exhaustion (41). Conversely, transient knockdown of EZH2 in primary human fibroblasts and a variety of transformed cells inhibited their proliferation in vitro (42). EZH2 expression has been proposed to be controlled by the Rb-E2F pathway and to be a downstream mediator of E2F-dependent proliferation (42). High EZH2 expression that has been observed in a broad range of tumors has thus far been attributed either to Rb loss or gene amplification (42). Its silencing function is speculated to target tumor suppressor genes (43), but the precise mechanism of its action on cell proliferation has thus far not been elucidated.

Because of its role in stem cell maintenance and possibly tumor initiation (42, 44), we addressed the putative implication of EZH2 in ESFT pathogenesis. The EZH2 gene was found to be up-regulated in ESFT compared with normal tissues and neuroblastomas (21), and quantitative real-time PCR analysis confirmed EZH2 induction in hMSC\textsuperscript{EWS-FLI-1} as well as its dependence on EWS-FLI-1 DBD integrity (Fig. 4A and data not shown).

Expression of EZH2 has been reported to progressively decrease during serial passage of primary fibroblasts and hematopoietic stem cells cultured in vitro, resulting in activation of their senescence program (42). We therefore asked whether the elevated EZH2 expression observed in hMSC\textsuperscript{EWS-FLI-1} was the result of true up-regulation or mere maintenance of EZH2 transcripts at the level found in low-passage hMSCs. EZH2 expression levels in two different batches of empty vector–, DBDM–, and EWS-FLI-1–infected hMSCs after 20 doublings were compared by real-time PCR with those in parental wt hMSCs after 8 doublings. A 50% decrease in EZH2 expression was observed in cells infected with empty vector and the DBDM that had undergone 20 population doublings compared with parental wt cells that had undergone 8 doublings. By contrast, hMSC\textsuperscript{EWS-FLI-1}, after 20 doublings, showed a 6- to 7-fold induction of EZH2 expression compared with their 8 population doubling wt counterparts (Fig. 4A). This result clearly shows the ability of EWS-FLI-1 to induce EZH2 in hMSCs above the baseline level of their precursors that had undergone fewer population doublings, and is consistent with a possible role in both senescence prevention and stem cell maintenance in hMSCs.

We next tested Ewing’s sarcoma samples and xenotransplants of Ewing’s sarcoma cell lines for EZH2 expression and found it to be elevated in both primary Ewing’s sarcoma and ESFT cell line–derived tumors grown in immunocompromised mice (Fig. 4B). To address the possible functional implication of EZH2 in Ewing’s sarcoma growth, we relied on a stable shRNA knockdown approach in ESFT cell lines using two distinct RNAi sequences (shRNA1 and shRNA2). Stable EZH2 shRNA expression in A673 and SK-N-MC Ewing’s sarcoma cell lines resulted in >70% reduction of its mRNA level (Fig. 4C, left) and a significant decrease in EZH2 protein expression as assessed by Western blot analysis (Fig. 4C, right). Reduction of EZH2 expression caused a marked decrease in proliferation of the two cell lines in vitro (Fig. 4D). Furthermore, injection of these cells into immunocompromised mice resulted in either no tumor development or strongly reduced tumor growth compared with cells expressing unrelated RNAi sequences (Fig. 5). To exclude possible off-target effects of the shRNA sequences, we repeated these experiments using two additional distinct shRNA sequences and obtained comparable results (data not shown).

Based on the notion that EZH2 may repress p16 and possibly other cell cycle regulators (43), we tested the expression level of several cell cycle control genes in the EZH2-depleted cells by real-time PCR. Both of the ESFT cell lines used in our experiments have a nonfunctional p53 pathway (45). The A673 cell line also lacks the p16\textsuperscript{INKA}-p14\textsuperscript{ARF} locus whereas SK-N-MC cells express an inactive Rb protein but retain expression of wt p16\textsuperscript{INKA}-p14\textsuperscript{ARF} (45).
Whether or not they were mutated, we did not record any significant change in the expression levels of p14ARF, p15, p16INK4A, p18, p21, and p53 following the EZH2 knockdown (data not shown). The observed reduction in proliferation and tumorigenicity can therefore not be explained by an effect on these cell cycle inhibitors and is most likely mediated by a mechanism that has yet to be elucidated.

**Discussion**

EWS-FLI-1 has thus far been found to induce an oncogenic stress type response in primary human cells, leading to cell cycle arrest, and a partial ESFT transcriptome in a variety of heterologous cell lines. Our present observations identify MSCs as the first primary human cell type that, while accurately recapitulating the ESFT gene expression profile, maintains viability and proliferation in response to EWS-FLI-1.

EWS-FLI-1 induced marked rounding of hMSCs in vitro that accompanied robust expression changes of genes implicated in cell differentiation. Consistent with previous studies in tumor cells and immortalized fibroblasts, microarray analysis revealed that numerous genes induced in hMSC$^{EWS-FLI-1}$ are implicated in neural crest development and neuronal differentiation. Among the most differentially expressed transcripts, we found NGFR (p75), which was strongly induced at both the transcription and protein levels, and which, in addition to playing a central role in neural develop-

![EZH2 induction in hMSC$^{EWS-FLI-1}$ and expression in ESFT, supporting tumor cell proliferation.](image)

**Figure 4.** EZH2 is induced in hMSC$^{EWS-FLI-1}$ and expressed in ESFT, supporting tumor cell proliferation. A, EZH2 transcript induction in hMSC$^{EWS-FLI-1}$ compared with empty vector- and DBDM-infected cells at 20 population doublings and parental cells at 8 population doublings. B, immunohistochemical assessment of EZH2 expression in primary ESFT and cell line–derived tumors grown in immunocompromised mice. Magnification, ×100. Inset, staining with isotype-matched unrelated antibody. Magnification, ×200. C, real-time PCR (left) and Western blot analysis (right) of EZH2 expression knockdown following stable shRNA in A673 and SK-N-MC Ewing’s sarcoma cell lines. The experiments were done with two different EZH2-specific (EZH2 shRNA1 and shRNA2) sequences and an unrelated siRNA control. Real-time PCR experiments were normalized to cyclophyllin A and done in triplicate. Columns, mean of three independent determinations; bars, SD. D, A673 and SK-N-MC cell proliferation rate is significantly reduced after the stable knockdown of EZH2 shown in C. Columns, mean of triplicate values of three separate determinations; bars, SD.
ment, is a key marker of neuroectodermal stem cells in both normal tissues and neural crest–derived tumors (46). Among the other genes that constitute part of the neuroectodermal profile of hMSC<sup>ESWS-FLI-1</sup>, SOX2 has been shown to maintain neural progenitor features (47). In contrast to observations in mouse mesenchymal progenitor cells, which were reported to lose osteogenic and adipocytic differentiation potential as a result of EWS-FLI-1 expression, hMSCs expressing EWS-FLI-1 retained at least some degree of trilineage differentiation plasticity. It is plausible that the early-stage neuroectodermal differentiation program induced by EWS-FLI-1 in hMSCs can still be overridden by the supraphysiologic conditions of the <i>in vitro</i> differentiation assays.

Some of the genes that are implicated in neural differentiation have recently been suggested to play an active role in ESFT pathogenesis. Thus, <i>NROB1</i> can promote tumorigenesis of Ewing’s sarcoma cell lines (7), whereas NKK2-2 may provide important functions at specific stages of ESFT development because its repression strongly reduced ESFT cell line tumorigenicity (10). It is noteworthy that both genes were not only strongly induced in hMSC<sup>ESWS-FLI-1</sup> but also remained dependent on EWS-FLI-1 in hMSCs can still be overridden by the supraphysiologic conditions of the <i>in vitro</i> differentiation assays. These observations suggest that they play a role not only during the initial steps of tumor development but also at late stages of tumor growth. Importantly, several of the above genes have been found to be strong discriminators of ESFT, and their expression has been used as an argument that ESFT may be of neuroectodermal origin. However, the present experiments show that expression of genes implicated in neuronal differentiation and neural crest development can be induced by EWS-FLI-1 in the appropriate primary mesenchymal progenitor cell environment. Thus, a hMSC that can undergo partial neuroectodermal differentiation may constitute the origin of ESFT, suggesting that these tumors need not arise from a neuroectodermal precursor to explain their primitive neuroectodermal phenotype.

Several of the genes observed to be up-regulated in hMSC<sup>ESWS-FLI-1</sup> and expressed in ESFT have been proposed to be functionally related to Ewing’s sarcoma development and behavior. Thus, <i>Id2</i>, observed to be induced by EWS-FLI-1 in both hMSCs and mouse mesenchymal progenitor cells (20), may play an important role in promoting cell cycle entry by inhibiting Rb. Induction of fos that was detected as a result of EWS-FLI-1 introduction into hMSCs (Supplementary data 1C) may be of particular interest in light of recent evidence that expression of numerous candidate EWS-FLI-1 target genes requires cooperation between EWS-FLI-1 and activator protein-1 (AP-1) (48). Enhanced expression of fos may explain, in part, the broad ESFT gene repertoire that was up-regulated in hMSC<sup>ESWS-FLI-1</sup>, and may therefore underlie the ability of these cells to recapitulate ESFT features. Moreover, and in contrast to primary fibroblasts, expression of EWS-FLI-1 in hMSCs did not provoke a p53 response, as shown by the absence of p53 target gene induction in their gene expression profile and the corresponding growth arrest. EWS-FLI-1 signals may either fail to activate premature cell senescence and oncogenic stress pathways, including p53 in hMSCs, or trigger mechanisms that neutralize or circumvent them.

Two candidate EWS-FLI-1 target genes that were induced in hMSC<sup>ESWS-FLI-1</sup> and that may prevent cell senescence while promoting survival and proliferation are <i>IGF1</i> and <i>EZH2</i>. We have previously shown that <i>IGF1</i> is induced by EWS-FLI-1 in mouse mesenchymal progenitor cells (20). Coupled to that observation, the present findings suggest that <i>IGF1</i> may be an important EWS-FLI-1 target in MScs. It is noteworthy that mouse mesenchymal progenitor cells and hMSCs are the only cells in which up-regulation of <i>IGF1</i> by EWS-FLI-1 has been reported thus far. Although the direct or indirect nature of the effect remains to be determined, our experiments clearly indicate that the DNA binding ability of EWS-FLI-1 is required for <i>IGF1</i> induction in hMSCs. IGF-IR signaling is observed to be critical for ESFT growth, and its blockade induces growth arrest and apoptosis in ESFT cell lines (49). IGF-1 induction may provide a survival signal that is essential during the initiating phases of tumor development, when the primary cells are subjected to the strong oncogenic stimulus of EWS-FLI-1 expression.

The discovery that <i>EZH2</i> is induced by EWS-FLI-1 in hMSCs may provide an additional explanation about why EWS-FLI-1 does not elicit oncogenic stress–mediated cell cycle arrest in hMSCs. Similar to another polycomb group gene, <i>bmi-1</i>, which cooperates with myc in B-cell and T-cell lymphoma development by inhibiting myc-induced apoptosis through repression of the <i>Cdkn2a</i> locus, <i>EZH2</i> activity is suggested to participate in blocking cellular response to oncogenic stress and maintain stem cell renewal at the expense of differentiation (22). <i>EZH2</i> has also been proposed to act as an oncogene in its own right (42) and to become up-regulated in preneoplastic lesions in the breast (44). Consistent with these scenarios, <i>EZH2</i> repression resulted in reduction of ESFT cell proliferation and tumorigenicity. Combined targeting of IGF-IR and <i>EZH2</i> may provide an attractive therapeutic option in ESFT.

Taken together, our observations provide evidence for the first time that hMSCs can sustain EWS-FLI-1 expression and respond by adopting phenotypic and gene expression hallmarks of ESFT, as would be expected of its putative cell of origin. These results are supported by recent work reporting the appearance of MSC features in Ewing’s sarcoma cell lines subjected to EWS-FLI-1 knockdown (50). As such, hMSC<sup>ESWS-FLI-1</sup> may provide an ideal cellular environment for uncovering new candidate genes whose role in ESFT initiation and subsequent maintenance can be tested. Although tumor initiation by adult hMSCs may require a genetic event in addition to EWS-FLI-1 expression, the overall gene expression and phenotypic changes observed in response to EWS-FLI-1 alone argue that hMSC<sup>ESWS-FLI-1</sup> constitutes a strong candidate precursor of ESFT.
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EWS-FLI-1 Expression Triggers a Ewing's Sarcoma Initiation Program in Primary Human Mesenchymal Stem Cells

Nicolò Riggi, Mario-Luca Suvà, Domizio Suvà, et al.