Development of Ewing's Sarcoma from Primary Bone Marrow–Derived Mesenchymal Progenitor Cells

Nicòlo Riggi,1 Luisa Cironi,1 Paolo Provero,2 Mario-Luca Suvà,1 Konstantinos Kaloulis,3 Carlos Garcia-Echeverría,1 Francesco Hoffmann,4 Andreas Trumpp,4 and Ivan Stamenkovic1

Abstract

Ewing’s sarcoma is a member of Ewing’s family tumors (EFTs) and the second most common solid bone and soft tissue malignancy of children and young adults. It is associated in 85% of cases with the t(11;22)(q24;q12) chromosomal translocation that generates fusion of the 5′ segment of the EWS gene with the 3′ segment of the ETS family gene FLI-1. The EWS-FLI-1 fusion protein behaves as an aberrant transcriptional activator and is believed to contribute to EFT development. However, EWS-FLI-1 induces growth arrest and apoptosis in normal fibroblasts, and primary cells that are permissive for its putative oncogenic properties have not been discovered, hampering basic understanding of EFT biology. Here, we show that EWS-FLI-1 alone can transform primary bone marrow–derived mesenchymal progenitor cells and generate tumors that display hallmarks of Ewing’s sarcoma, including a small round cell phenotype, expression of EFT-associated markers, insulin-like growth factor I dependence, and induction or repression of numerous EWS-FLI-1 target genes. These observations provide the first identification of candidate primary cells from which EFTs originate and suggest that EWS-FLI-1 expression may constitute the initiating event in EFT pathogenesis. (Cancer Res 2005; 65(24): 11459-68)

Introduction

Sarcomas constitute some of the most aggressive adult and childhood malignancies that are believed to originate from as yet poorly defined mesenchymal stem/progenitor cells (1, 2). Currently, sarcomas are subdivided into two broad subclasses based on the genetic mutations that are associated with their development. One subclass is composed of tumors bearing multiple complex chromosomal translocations, duplications, and deletions with the corresponding loss of tumor suppressor genes and amplification/activation of oncogenes. The second subclass, which includes Ewing’s family tumors (EFTs), is composed of tumors associated with specific chromosomal translocations that give rise to fusion genes that are believed to play an active role in transformation (1, 2). Chromosomal translocations associated with EFTs lead to the fusion of the 5′ segment of the EWS gene on chromosome 22 with the 3′ segment of one of the ets family genes on chromosomess 2, 7, 11, 17, and 21, including ERG, t(21;22)(q22;q12); ETV1, t(7;22)(p22;q12); FEV, t(2;22)(q33;q12); and ETV4 (3, 4). However, by far the most common fusion occurs with the FLI-1 gene resulting from the translocation t(11;22)(q24;q12) (5).

The EWS-FLI-1 fusion protein behaves as an aberrant transcriptional regulator (6) whose suppression in EFT cell lines results in decreased cell growth in vitro and tumor-forming capacity in vivo (7). EWS-FLI-1 expression therefore seems required for EFT development, but the mechanisms whereby it induces transformation and/or controls tumor growth and progression remain unknown. Genes whose expression is reported to be induced by EWS-FLI-1 include MYC (8), EAT-2 (9), MMP-3 (10), FRINGE (11), ID2 (12), and CCND1 (13), whereas TGFBR2 (14), CDKN1A (p21/CIP1/WAF1; ref. 15), and p57KIP2 (16) are among those reported to be repressed. However, induction and repression of potential EWS-FLI-1 target genes are dependent on the cellular background (17), as is the physiologic outcome of EWS-FLI-1 expression. EWS-FLI-1 elicits enhanced tumorigenicity in immortalized NIH3T3 cells (18). By contrast, it induces p53-dependent growth arrest in primary human fibroblasts (19) and apoptosis in mouse embryonic fibroblasts (MEF; ref. 20). Deletion of p19ARF, p16INK4A, and p53 attenuates apoptosis and allows stable EWS-FLI-1 expression in MEFs, possibly facilitating subsequent transformation (20). To exert its putative transforming properties, EWS-FLI-1 may therefore require a cellular environment with mutations in genes implicated in growth, senescence, and cell cycle control. However, loss of p19ARF, p16INK4A, and p53 occurs in only a minority of EFTs (21), and the chromosomal translocation leading to EWS-FLI-1 expression may be the only genetic event in a significant fraction of Ewing’s sarcomas (21). These observations suggest the existence of nonmutated primary cells that are permissive for EWS-FLI-1-mediated transformation. In view of the lack of adequate EFT models, identification of such cells constitutes a key step toward elucidating EFT biology (22).

Although EFTs arise in bone and soft tissues, their expression of neuronal markers has kept open the debate as to their potential mesenchymal or neuroectodermal origin. In an effort to identify candidate primary cells from which EFTs originate, we addressed the effects of EWS-FLI-1 expression on the tumorigenic potential and gene expression profile of cells spanning a broad plasticity spectrum, including primary bone marrow–derived mesenchymal progenitor cells (MPC), embryonic stem cells, and spontaneously immortalized embryonic (STO) fibroblasts. Our results indicate that unlike embryonic stem and STO cells, MPCs display permissiveness for EWS-FLI-1-mediated transformation and generate tumors that display hallmarks of Ewing’s sarcoma. Based on these observations, we propose that mesenchymal progenitor cells may constitute a candidate environment from which EFTs originate.
Materials and Methods

Cells and fluorescence-activated cell sorting analysis. The MEF cell line STO and the embryonic stem cell line ES-D3 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Embryonic stem cells were cultured on gelatin-coated plates supplemented with 10 ng/mL leukemia inhibitory factor (LIF; Sigma, St. Louis, MO). MPCs were isolated from bone marrow of wild-type adult C57BL/6 mice according to described procedures (23) and cultured at low density on fibronectin-coated plates (Sigma) in medium containing 2% dialyzed FCS (Sigma), 10 ng/mL epidermal growth factor (EGF; Sigma), 10 ng/mL PDGF-BB (R&D Systems, Minneapolis, MN), and LIF produced by the CHO LIF720D cell line. MPCs were tested by fluorescence-activated cell sorting for mesenchymal stem cell marker expression before and after infection and selection. Phycero- ythrin-conjugated monoclonal antibodies (mAb; BD PharMingen, San Diego, CA) were against CD44, CD45, CD13, CD11b, CD117 (c-kit), CD90 (Thy1), Sca1, and Flk1. SK-N-MC, SK-ES1, and U2OS cell lines (ATCC) were cultured, respectively, in RPMI 1640, McCoy's 5A, and DMEM, all containing 10% fetal bovine serum (FBS).

Differentiation assays. Cells were plated at 1 × 10^5 in 35-mm plates, in medium containing EGF, PDGF-BB, or LIF but with cytokines specific for the different lineages. For adipogenesis, MPCs were allowed to reach 80% medium without EGF, PDGF-BB, or LIF but with cytokines specific for the differentiation. Oil Red-O staining. Cells were plated at 1 × 10^5 in 35-mm plates, in medium containing EGF, PDGF-BB, or LIF but with cytokines specific for the different lineages. For adipogenesis, MPCs were allowed to reach 80% medium without EGF, PDGF-BB, or LIF but with cytokines specific for the

Materials and Methods

Cancer Research

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green intensity (log(Cy5 + log(Cy3))/2. M values were normalized using the library SMA in the statistical software package R by the within-print-tip group lowess normalization procedure (25). Quality control of slide hybridization was done using variables described on the corresponding web site.9

Statistical analysis of the expression data. For each time point and cell line, five m17k microarrays (among which two were dye swaps) were done comparing hEWS-FLI-1-V5 expressing with empty vector control cells. Expression data for each time point and cell line were analyzed with standard one-sample, one-sided t tests applied to the logarithm of the ratio of the expression levels of the hEWS-FLI-1-V5 and the control sample. The null hypothesis is that the mean of such a logarithm is 0, and the alternate hypothesis is that the mean is >0 or <0 for the one-sided test used to identify induced or repressed clones. The one-sample t test was shown in a recent study to be more effective than the two-sample test due to the correlation between expression levels measured on the same array (26).

The use of a standard statistical test (as opposed to methods based on cutoff on fold change) allowed us to estimate the false discovery rate (FDR) of the lists of induced and repressed genes. This was done using the Benjamin-Hochberg method (27).

Functional analysis of the lists of induced and repressed genes. The annotations of the clones included in the array to Gene Ontology (28) terms were downloaded from the mouse 17k deconvolution file of the Swiss Institute for Experimental Cancer Research. To conform to the hierarchical structure of the Gene Ontology terms, annotation of each clone must be considered not only according to the term to which it is directly associated but to all its ancestors in the Gene Ontology graph. The prevalence of each Gene Ontology term in the lists of induced and repressed clones was evaluated and compared with its prevalence among all the clones present on the microarray. When overrepresentation was found, its statistical significance was determined with Fisher’s exact test.

Real-time quantitative reverse transcription-PCR. cDNA was obtained using a Moloney murine leukemia virus reverse transcriptase and RNase H minus (Promega, Madison, WI). Typically, 500 ng of template total RNA and 250 ng of random hexamers were used per reaction. Real-time PCR amplification was done using a Taqman Universal PCR mastermix and Assays-On-Demand gene expression products in an ABI Prism 7700 instrument (Applied Biosystems, Foster City, CA). Relative quantitation of target, normalized with an endogenous control (cyclophyllin A), was done using a comparative Cq or a standard curve method (Applied Biosystems). Probes used included mouse Igf1, Igf1 binding protein 3, Igf1 binding protein 5, Acam, Jam2, Lum, Birc2, Emb, Yap, and TGF-βRII. The use of a standard statistical test (as opposed to methods based on cutoff on fold change) allowed us to estimate the false discovery rate (FDR) of the lists of induced and repressed genes. This was done using the Benjamin-Hochberg method (27).

Results

Expression of EWS-FLI-1 in embryonic stem cells, mesenchymal progenitor cells, and STO fibroblasts. The type 2 EWS-FLI-1 fusion gene, which is associated with Ewing’s sarcoma bearing the poorest prognosis (29), was RT-PCR amplified from total DNA derived from the SK-N-MC Ewing’s sarcoma cell line, and the corresponding cDNA was inserted into the pMSCV retroviral vector containing sequences encoding the v5 tag at the 3’ end. After sequence verification, the construct was transfected into Eco293 packaging cells, and the resulting retrovirus was used to infect embryonic stem cells, MPCs, and STO cells. In all cases, bulk cultures of infected cells were used for subsequent experiments.

MPCs were derived from the bone marrow of wild-type C57BL/6 mice as described in Materials and Methods and characterized by a CD45-negative, CD11b-negative, Fik-negative, CD44-positive, Thy1-positive, CD117 weakly positive, CD13 weakly positive, and Sca1 strongly positive phenotype (Fig. 1A). Although mesenchymal progenitor/stem cells can differ in cell surface receptor expression according to the microenvironment from which they are derived (30) and the mouse strain of origin (31), the phenotype displayed by the MPCs used in the present work is consistent with that described in other studies (30, 32). In addition, these cells display strong Rex-1 and weak Oct-4 expression (Fig. 1B), reflecting their progenitor status (29) and differentiate into adipocytes, osteocytes, and neurons in response to appropriate stimuli (Fig. 1C). Their differentiation potential, therefore, encompasses the mesenchymal and neuronal attributes of EFTs.

Expression of the EWS-FLI-1 fusion protein in each cell type was assessed by RT-PCR and Western blot analysis using the anti-V5 antibody, 24, 48, and 72 hours following infection. In all three cell types, expression of EWS-FLI-1 could be detected by Western blot analysis at each of the time points (Fig. 2A-C). By day 14, however, the fusion protein was no longer detectable in embryonic stem and STO cells, despite the presence of the corresponding transcripts, as assessed by RT-PCR (Fig. 2D). In contrast, expression of the EWS-FLI-1 fusion protein in MPCs persisted several weeks following infection (Fig. 2D; data not shown) and displayed the expected nuclear localization (Fig. 2E).

EWS-FLI-1 expression induces tumorigenicity in mesenchymal progenitor cells. Embryonic stem and STO cells as well as MPCs infected with EWS-FLI-1 (MPC-EWS-FLI-1) or empty vector were injected s.c. into severe combined immunodeficient (SCID) mice 5 days following infection, when the fusion protein was detectable in all three cell types. Expression of EWS-FLI-1 in embryonic stem and STO cells had no effect on the intrinsic ability of these cells to form teratomas and slowly growing spindle cell tumors, respectively (Supplementary Data 3A). By contrast, although MPCs infected with empty virus failed to produce tumors, all 12 mice injected with MPC-EWS-FLI-1 developed tumors

9 http://intranet.isrec.ch/microarrays/nccr_isrec.html
10 http://intranet.isrec.ch/microarrays/arrays_users.html
within 6 weeks (Fig. 3A). MPC\textsuperscript{EWS-FLI-1} tumors (MPC\textsuperscript{EWS-FLI-1-T1}) were composed predominantly of small round cells with dense homogeneous nuclei, characteristic of EFT, occasionally organized into nests surrounded by fibrotic strands and spindle cells (Fig. 3B). Reinjection of the tumor cells into SCID mice resulted in development within 2 to 4 weeks of tumors (MPC\textsuperscript{EWS-FLI-1-T2}) composed of sheets of small round cells, virtually identical to tumors formed by injection of the human EFT cell lines SK-ES1, SK-N-MC, and A673 (Fig. 3B; data not shown). Tumors resulting from an additional round of injection (MPC\textsuperscript{EWS-FLI-1-T3}) emerged more rapidly than their predecessors but displayed the same histologic phenotype (data not shown). Tumor cells were positive for the EFT-associated markers CD99 and NSE (ref. 33; Fig. 3C) and retained EWS-FLI-1 protein expression as determined by Western blot analysis of corresponding lysates (Fig. 3D).

To exclude the possibility that the observed MPC\textsuperscript{EWS-FLI-1} tumor phenotype was a reflection of intrinsic MPC histogenetic properties irrespective of the transforming agent, MPCs stably expressing the oncogenes ErbB2, SYT-SSX-1, and Bcl-2 (Fig. 2F) were tested for tumorigenicity in SCID mice. Overexpression of ErbB2 is implicated in the formation of a variety of tumor types (34), whereas expression of SYT-SSX-1, the fusion protein associated with synovial sarcoma, can enhance tumor formation by immortalized fibroblasts (35). By suppressing apoptosis, Bcl-2 overexpression is believed to facilitate oncogenic mutations in hematopoietic cells, and chromosomal translocations involving Bcl-2 are associated with the pathogenesis of hematopoietic malignancies (36).

Neither MPC\textsuperscript{ErbB2} nor MPC\textsuperscript{SYT-SSX-1} cells formed tumors in mice (Fig. 3A), suggesting that the oncogenic effect of EWS-FLI-1 does not reflect indiscriminate MPC susceptibility to transformation. Of the 12 mice injected with MPC\textsuperscript{Bcl-2}, only four developed tumors that became detectable >4 months following injection. The late emergence of these tumors in only a fraction of the mice is consistent with the occurrence of oncogenic mutations favored by Bcl-2-promoted survival. MPC\textsuperscript{Bcl-2} tumors were composed of CD99- and NSE-negative spindle cells with large clear nuclei, bearing no resemblance to their MPC\textsuperscript{EWS-FLI-1} counterparts (Fig. 3B-C). These observations support the notion that MPC\textsuperscript{EWS-FLI-1} tumor histogenesis is a function of EWS-FLI-1 expression.

To ensure that induction of tumorigenic properties in MPCs was not due to altered EWS-FLI-1 activity associated with the V5 tag or to potential genetic events secondary to in vitro selection,
MPC<sub>EWS-FLI-1</sub> tumorigenicity experiments were repeated using fresh, independently isolated batches of MPCs, V5-tagged and untagged EWS-FLI-1 constructs, different selection markers, and variable selection periods. Neither the V5 tag, the duration of selection in vitro nor the type of selection marker used affected the observed transformation. Altogether, six sets of tumorigenicity experiments were done in 36 test and 36 control mice using three independent MPC batches. In each set of experiments, all of the mice injected with MPC<sub>EWS-FLI-1</sub> developed tumors within 4 to 6 weeks (data not shown), whereas none of the mice bearing vector-only-expressing MPCs developed tumors even several months after injection.

Mesenchymal progenitor cells and MPC<sub>EWS-FLI-1</sub> tumors maintain p53 function and p19<sub>ARF</sub> expression. Because loss of p53 function seems to be a prerequisite for the maintenance of EWS-FLI-1 expression in MEFs (20) and because induction of a p53 response precludes primary human fibroblast transformation by EWS-FLI-1 (19), we assessed the p53 status in MPCs and MPC<sub>EWS-FLI-1</sub> tumors. Sequence analysis of PCR-amplified p53 from MPC RNA revealed no mutations. Treatment of MPC<sub>EWS-FLI-1-T1</sub> and MPC<sub>EWS-FLI-1-T2</sub> cells with 5-fluorouracil, a potent activator of p53 (37), revealed induction of the p21 protein and several other p53 targets, as assessed by microarray analysis (Fig. 4A and B), confirming the preservation of a functional p53 pathway.

MPCs displayed weak constitutive expression of p19<sub>ARF</sub>, as detected by RT-PCR (Fig. 4C). Although the physiologic signals that regulate p19<sub>ARF</sub> are only partly understood, myc overexpression has been observed to induce p19<sub>ARF</sub> in MEFs, where its action is believed to provide a p53-dependent safeguard against transformation (38). Similar to its effect in MEFs, overexpression of myc in MPCs induced p19<sub>ARF</sub> (Fig. 4C), indicating that p19<sub>ARF</sub> is responsive to oncogenic signals in these cells. Consistent with this observation, p19<sub>ARF</sub> expression was observed to be induced in MPC<sub>EWS-FLI-1-T1</sub> and MPC<sub>EWS-FLI-1-T2</sub> cells (Fig. 4D), and both p16<sub>INK4A</sub> and p19<sub>ARF</sub> displayed wild-type sequence in MPCs, MPC<sub>EWS-FLI-1-T1</sub> and MPC<sub>EWS-FLI-1-T2</sub> cells (data not shown). Thus, EWS-FLI-1 can transform MPCs despite the presence of wild-type p16<sub>INK4A</sub>/p19<sub>ARF</sub> and intact p53 function.

Immunohistochemical staining of the tumor sections with anti-Ki67 and anti-TdT antibodies confirmed the high proliferative and low apoptotic index of MPC<sub>EWS-FLI-1</sub> tumors (Supplementary Data S3B).

Mesenchymal progenitor cells, embryonic stem, and STO cells display different responses to EWS-FLI-1 expression. To address the response of MPCs to EWS-FLI-1 at the molecular level, we compared the gene expression profile of MPCs infected with EWS-FLI-1 containing retrovirus to that of MPCs infected with empty virus 24, 48, and 72 hours after infection. RNA obtained from two independently EWS-FLI-1-infected and two corresponding empty vector–infected cell batches and expression of 17,000 cDNA clones was compared using the NIA-17k mouse cDNA array (24). Five microarrays were used to assess expression profile changes at each time point. Expression data for each clone present on the microarray were analyzed as described in Materials and Methods.

Twenty-four hours following infection with EWS-FLI-1 retrovirus, 1,046 and 779 genes were, respectively, induced and repressed in MPCs with a FDR as low as 1% (Supplementary Data S1). The induced transcripts included genes annotated to Gene Ontology terms of chaperone activity, structural molecule activity, ribosomes, protein biosynthesis, ATP binding, biosynthesis, macromolecule biosynthesis, ribonucleo-complex, and adenyl nucleotide...
binding (Supplementary Data S1). Interestingly, 72 hours following EWS-FLI-1 expression marked induction of insulin-like growth factor-1 (IGF-I) and IGF binding proteins 3 and 5 (IGFBP-3 and IGFBP-5; Fig. 5A-B) was observed. Neither ErbB2, SYT/SSX, nor Bcl-2 induced IGF-I in MPCs (data not shown), supporting the notion that the observed IGF-I induction was EWS-FLI-1 specific. In contrast to its effect in MPCs, EWS-FLI-1 expression in embryonic stem and STO cells resulted, respectively, in decreased and <2-fold increased IGF-I expression (Fig. 5B), whereas IGFBP-3 and IGFBP-5 expression remained unaltered (data not shown). IGF-I receptor (IGF-IR) expression was observed in all three cell types (Fig. 5C) and was unaffected by EWS-FLI-1 (data not shown).

EWS-FLI-1–induced mesenchymal progenitor cell tumors are insulin-like growth factor-1 dependent. Association of IGF-I/IGF-IR signaling with cancer is well established (39). EFTs are among tumors that not only express IGF-I (13) but also require IGF-I/IGF-IR signals for growth and survival (40–42) and are highly sensitive to IGF-IR inhibition (40, 42). Accordingly, >90% of MPC<sub>EWS-FLI-1-T1/T2</sub> cells dissociated from surgically removed tumors died in response to IGF-IR inhibition (Fig. 5C-D) by the small molecular weight kinase inhibitor NVP-AEW541, a pyrrolo[2,3-<i>d</i>]pyrimidine derivative that is highly selective for IGF-IR (43). Human EFT SK-N-MC cells displayed similar sensitivity to IGF-IR blockade, whereas MPC<sub>EWS-FLI-1-T1</sub> cells harvested before injection into mice and osteosarcoma U2OS cells displayed lower sensitivity and resistance, respectively (Fig. 5C-D). Although IGF-I/IGF-IR signaling may be essential for EFT cell survival, additional pro-oncogenic events are believed to be necessary to generate and promote EFT growth (40–42). Consistent with this view, IGF-I overexpression alone was not sufficient to initiate MPC tumorigenicity, as MPCs displaying a 400-fold increase in IGF-I expression following infection with IGF-I retrovirus (data not shown) failed to form tumors in mice (Fig. 3A).

**Figure 3.** Growth and histology of MPC<sup>EWS-FLI-1</sup> tumors. **A,** tumor growth in SCID mice injected with MPCs expressing EWS-FLI-1V5, SYT/SSX1, ERBB2, IGF-I, or empty vector. Three mice were sacrificed after 6, 9, 12, and 15 weeks, and tumors were weighed. Two of the tumors removed 15 weeks after injection were dissociated and the cells assessed for EWS-FLI-1V5 expression (D). These cells (MPC<sup>EWS-FLI-1-T1.1/T1.2</sup>) were reimplanted, and the resulting tumors were removed 4 weeks later. Second-round tumor-derived cells (MPC<sup>EWS-FLI-1-T2.1/T2.2</sup>) were subjected to another round of in vivo growth. Points, mean tumor weight; bars, SD. B, H&E staining of indicated tumors showing spindle cell morphology of MPC<sup>BCL2</sup> tumors, which contrasts with the small round cell morphology of MPC<sup>EWS-FLI-1</sup>–tumors and tumors generated by the human EFT cell line SK-ES1. C, CD99 and NSE expression in MPC<sup>BCL2</sup> and MPC<sup>EWS-FLI-1</sup> tumors. Magnification: ×200. D, Western blot analysis of EWS-FLI-1V5 expression in MPC<sup>EWS-FLI-1-T1</sup> and MPC<sup>EWS-FLI-1-T2</sup> cells.
gene expression profile of cells derived from two independent first-round tumors (MPC<sup>EWS-FLI-1-T1/T1</sup>) to that of MPC<sup>EWS-FLI-1</sup> cells harvested immediately before injection into mice (Supplemental Data S2). MPC<sup>EWS-FLI-1-T1/T1</sup> displayed altered expression of several known EWS-FLI-1 target genes, including induction of MYC (8), MMP-3 (11), ID2 (12), and CCND1 (13) and repression of CDKN1A (p21; ref. 15; Fig. 5E).

Additional potentially relevant genes whose expression was induced in tumor-derived cells were yes-associated protein (YAP), baculoviral IAP containing 2 (BIRC2), EMBGIN, IGFBP-3; several chemokines, including CCL2; proteolytic enzymes, including CATHEPSIN C; and the neuronal inducer NECEDIN (Supplemental Data S2). Repressed transcripts included LUMICAN and several genes encoding adhesion receptors, most notably α5 and α6 integrins, Jam-2 and Jam-3, and ALCAM (Supplemental Data S2). Real-time PCR comparison of expression of ALCAM, Jam-2, LUMICAN, BIRC2, EMBGIN, and YAP in MPC<sup>EWS-FLI-1</sup> and MPC<sup>EWS-FLI-1-T1</sup> confirmed the changes suggested by microarray analysis (Fig. 6).

Comparison of MPC<sup>EWS-FLI-1-T1</sup> and MPC<sup>EWS-FLI-1-T2</sup> transcripts by cDNA microarray analysis revealed similar gene expression profiles, with nevertheless reduced expression of the EWS-FLI-1 target gene TRGBRII in the latter (Fig. 6).

Discussion

Our observations constitute the first demonstration of primary cell transformation by EWS-FLI-1, leading to the formation of tumors that display hallmarks of EFT, including a small round cell phenotype, expression of CD99 and NSE, IGF-I dependence, and changes in expression of several known EWS-FLI-1 target genes.

EWS-FLI-1 expression has been observed to block differentiation of p19<sup>ARF</sup>−/− mice along osteogenic and adipogenic lineages (44), but its transforming effect in neither these cells nor their wild-type counterparts had been tested thus far. The observation that EWS-FLI-1 transformed several independently isolated batches of primary MPC derived from wild-type mice and displaying a normal diploid karyotype (data not shown) argues in favor of its expression being the initiating event in MPC<sup>EWS-FLI-1</sup> tumor formation. Moreover, our results indicate that neither p19<sup>ARF</sup> nor p53 constitute a barrier to the transforming potential of EWS-FLI-1 in MPCs, and that similar to the majority of EFTs, MPC<sup>EWS-FLI-1</sup> tumors retain wild-type p16<sup>INK4A</sup>/p19<sup>ARF</sup> and a functional p53 pathway.

The ability of a single oncogenic event to induce tumor formation may seem to diverge from the widely held view that malignant transformation of cells, particularly those which give rise to solid tumors, requires at least three genetic events. However, the complexity of mutual events necessary for tumor formation may depend, at least in part, on the nature of the cells from which the tumor originates. Thus, the self-renewal capacity intrinsic to progenitor cells, which is advantageous to malignant growth, may reduce the number of mutations necessary to achieve transformation and render the cells more sensitive to the transforming potential of selected oncogenic agents. Consistent with this notion, single chromosomal translocations that generate aberrant transcription factors may suffice to initiate some types of leukemia from hematopoietic progenitor cells (45–47), and expression of the myxoid liposarcoma–associated FUS/TLS-CHOP fusion protein in adipocyte precursor cells is able to induce the corresponding tumors in transgenic mice (48). EFTs may resemble these malignancies in that their initiation may require a single genetic event in the appropriate progenitor cell environment.

The timing of EWS-FLI-1 expression may also be important in EFT pathogenesis. Most EFTs arise in bone at puberty, which coincides with robust activation of IGF-I/IGF-IR signaling triggered by a growth hormone spurt, suggesting that IGF-I may participate in the early stages of EFT development. The EWS-FLI-1-mediated induction of IGF-I in MPCs observed in the present work may recapitulate events that precede EFT formation and help establish

![Figure 4](image-url)

**Figure 4.** Induction of p53 target genes in T<sub>1</sub> and T<sub>2</sub> tumor derived cells treated with 5-fluorouracil (5-FU) and the presence of p19<sup>ARF</sup> transcript in MPC wild type (wt) and their tumor-derived counterparts. **A,** Western blot analysis of p21 induction in MPC<sup>EWS-FLI-1-T1/T2</sup>-derived cells treated with 50 μg/mL 5-fluorouracil or solvent (DMSO) for 16 hours, confirming the preservation of a functional p53 pathway. **B,** induction of p53 target genes in the same cells as in (A), as assessed by cDNA microarray analysis. **C,** semiquantitative RT-PCR analysis confirming the presence of a p19<sup>ARF</sup> transcript in wild-type MPC and its induction in the same cells overexpressing c-myc: No induction by c-myc is observed in p19<sup>ARF</sup>−/− MPCs. **D,** presence of p19<sup>ARF</sup> in MPC<sup>EWS-FLI-1-T1/T2</sup>-tumor–derived cells, as assessed by RT-PCR.

**Table 1.**

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conditions that facilitate transformation by EWS-FLI-1 target gene products. Sustained IGF-I overproduction (42) or the recently observed down-regulation of IGFBP-3 (49), which sequesters IGF-I and limits its bioavailability, may provide at least two possible mechanisms for maintaining IGF-I levels required for subsequent EFT survival. Selection of one mechanism over the other may depend on the cellular context (17) and possibly the location and duration of tumor growth.

Several EWS-FLI-1 target genes that are potentially implicated in transformation and tumorigenesis and that may cooperate with
IGF-I, including MYC, ID2, CCND1, MMP-3, CDKN1A, and TGFBRII, displayed altered expression in MPC EWS-FLI-1 tumors. Most of these genes have been identified as EWS-FLI-1 targets individually in different cells, consistent with cell type–specific EWS-FLI-1 target gene expression patterns (17). The potential physiologic consequences relative to EFT development of their combined expression changes in a single cell population have therefore not been fully appreciated. Id proteins are general inhibitors of differentiation and stimulators of proliferation and are implicated in the development of neuroectodermal tumors (50). Id2 overexpression in neuroblastoma cells has been shown to mediate cellular transformation and maintenance of the malignant phenotype (50, 51). Ewing's sarcoma is also associated with elevated expression of Id2, which is a target of myc oncproteins, IGF-I and EWS-FLI-1, raising the possibility that EWS-FLI-1, c-myc, and IGF-I may cooperate in maintaining abnormal Id2 expression in EFT (50). Id proteins are recruited by myc to bypass the tumor suppressor function of the retinoblastoma protein (Rb; refs. 50, 52). Concomitant induction of cyclin D1 and down-regulation of p21WAF1/CIP1, a key inhibitor of cyclin-dependent kinases, may facilitate proliferation by accelerating S-phase entry and decreasing G1 arrest efficacy, respectively. It would therefore seem reasonable to suggest that activation of proliferation checkpoints by the combined action of IGF-I, myc, Id2, cyclin D1, and repression of p21WAF1/CIP1 may provide a basis for EWS-FLI-1-mediated transformation. Products of other known EWS-FLI-1 target genes found to display altered expression in MPC EWS-FLI-1 tumors may subsequently contribute to tumorigenesis. Thus, in agreement with recent observations on EFT cells (14), decreased expression of TGFBRII correlated with increased rapidity of MPC EWS-FLI-1 tumor growth and transition from a histologic phenotype characterized by tumor cell nests surrounded by fibrotic strands to one dominated by sheets of tumor cells.

At least one of the newly identified EWS-FLI-1 target gene products (i.e., IGF-I) may provide clues as to how EWS-FLI-1 might transform cells that maintain functional p19ARF and p53. Induction of myc by EWS-FLI-1 may be expected to up-regulate p19ARF, leading to p53 activation and apoptosis. In MPCs, however, induction of IGF-I precedes that of myc. Given that a major role of IGF-I is to promote cell survival by activating the phosphatidylinositol 3-kinase/Akt pathway, early induction of IGF-I may provide a mechanism to prevent p19ARF- and p53-mediated apoptosis. This notion is supported by observations that in the presence of constitutively active Akt, myc can induce lymphoma formation and immortalize primary cells despite the presence of wild-type p19ARF and p53 (53). IAP and YAP may further promote survival of transformed cells, explaining, at least in part, tumor growth in the face of a functional p19ARF and p53 axis. Induction of MMP-3, MMP-13, and CATHEPSIN C may promote surrounding stromal tissue remodeling, whereas down-regulation of integrins, ALCAM and lumican may augment tumor cell motility, further contributing to the malignant phenotype.

The present model should provide the means to test the physiologic relevance of individual EWS-FLI-1 target genes in Ewing's Sarcoma and M PCs

![Figure 6](https://www.aacrjournals.org/11467/)

**Figure 6.** Comparative gene expression profile analysis between MPC EWS-FLI-1 before injection into mice and MPC EWS-FLI-1-T1. A, genes displaying differential expression in MPC EWS-FLI-1-T1 that are relevant to transformation and tumor development. B, histogram representation of induction and repression of selected transcripts from the list shown in (A), as assessed by real-time PCR normalized to cyclophyllin A. All experiments were done in triplicate. Columns, means of three independent determinations; bars, SD. C, repression of the TGFB-RRI transcript in MPC EWS-FLI-1-T2 compared with MPC EWS-FLI-1-T1 derived cells as assessed by cDNA microarray and real-time PCR analysis.

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**Table 1**

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MPC.EWS-FIL1-1 tumor pathogenesis and determine the chronology of molecular events leading from EWS-FLI-1-mediated primary cell transformation to tumor growth and progression. Moreover, extraplation of mechanisms underlying mouse tumor development to human cancer must be made with caution, the similarity of MPC.EWS-FIL1-1 tumors to EFTs supports the premise that key aspects of their pathogenesis are likely to be shared.

Taken together, our observations have identified primary bone marrow–derived mesenchymal progenitor cells as a permissive environment for EWS-FLI-1-mediated transformation. Their location in the bone, where the majority of EFTs arise, and their ability to form tumors with molecular, morphologic, and immunohistochemical features of EFTs render MPCs strong candidates for the origin of these tumors. Moreover, our results provide experimental support to the long-suspected notion that EWS-FLI-1 expression in permissive cells can constitute the initiating event in the pathogenesis of Ewing’s sarcoma.

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References

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Nicolò Riggi, Luisa Cironi, Paolo Provero, et al.


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