The Ews/Fli-1 Fusion Gene Changes the Status of p53 in Neuroblastoma Tumor Cell Lines

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ABSTRACT

One hallmark of Ewing’s sarcoma/peripheral neuroectodermal tumors is the presence of the Ews/Fli-1 chimeric oncogene. Interestingly, infection of neuroblastoma tumor cell lines with Ews/Fli-1 switches the differentiation program of neuroblastomas to Ewing’s sarcoma/peripheral neuroectodermal tumors. Here we examined the status of cytoplasmically sequestered wt-p53 in neuroblastomas after stable expression of Ews/Fli-1. Immunofluorescence revealed that in the neuroblastoma-Ews/Fli-1 infectant cell lines, p53 went from a punctate-pattern of cytoplasmic sequestration to increased nuclear localization. Western blot analysis revealed that PARC was down-regulated in one neuroblastoma cell line but not expressed in the second. Therefore, decreased PARC expression could not fully account for relieving p53 sequestration in the neuroblastoma tumor cells. Neuroblastoma-Ews/Fli-1 infectant cell lines showed marked increases in p53 protein expression without transcriptional up-regulation. Interestingly, p53 was primarily phosphorylated, without activation of its downstream target p21⁰⁰⁰⁰. Western blot analysis revealed that whereas MDM2 gene expression does not change, p14⁰⁰⁰⁰, a negative protein regulator of MDM2, increases. These observations suggest that the downstream p53 pathway may be inactivated as a result of abnormal p53. We also found that p53 has an extended half-life in the neuroblastoma-Ews/Fli-1 infectants despite the retention of a wild-type sequence in neuroblastoma-Ews/Fli-1 infectant cell lines. We then tested the p53 response pathway and observed that the neuroblastoma parent cells responded to genotoxic stress, whereas the neuroblastoma-Ews/Fli-1 infectants did not. These results suggest that Ews/Fli-1 can directly abrogate the p53 pathway to promote tumorigenesis. These studies also provide additional insight into the relationship among the p53 pathway proteins.

INTRODUCTION

The p53 tumor suppressor gene is mutated in >50% of all cancers (1). It is a sequence-specific DNA binding protein able to activate transcription leading to either cell cycle arrest or apoptosis in response to DNA damage or other cellular perturbations (2). After genotoxic stress to cells, p53 degradation is relieved by several mechanisms including p53 phosphorylation disrupting binding to the E3 ligase oncoprotein MDM2 or p14⁰⁰⁰⁰ binding/sequestration of MDM2 (3,4). This allows the tumor suppressor protein to be phosphorylated and stabilized in the nucleus to up-regulate the cyclin/cyclin-dependent kinase inhibitor p21⁰⁰⁰⁰ leading to a G₁ arrest (3). If DNA is damaged beyond repair, some cells remain stably arrested, whereas others enter apoptosis or programmed cell death (5,6). This important response pathway reduces the occurrence of mutations, decreasing the risk for carcinogenesis.

Ewing’s sarcoma/peripheral neuroectodermal tumors are pediatric soft tissue sarcomas characterized in >95% of cases by the expression of the balanced translocation t(11;22)(q24;q12) resulting in the Ews/Fli-1 chimeric fusion protein (7–9). A small fraction of primary Ewing’s sarcoma/peripheral neuroectodermal tumor patients have been reported as having p53 mutations (10–12) resulting in poor prognosis (13). It has been reported previously that loss of the p53-ARF pathway in mouse embryonic fibroblasts (MEFs) is sufficient for Ews/Fli-1 stable expression (14). It has also been reported recently that exogenous expression of Ews/Fli-1 protein in primary human fibroblasts causes the induction of p53 resulting in cell cycle arrest, whereas abrogation of the p53 pathway prevents growth suppression (15). These results suggest that p53 loss of function is a necessary event for Ews/Fli-1 to cause tumorigenesis.

We wanted to explore what effects Ews/Fli-1 may have on p53 status in neuroblastoma tumor cell lines. Neuroblastomas are common pediatric solid cancers that are found primarily in young children (16,17). One distinguishing marker of neuroblastoma cell lines is cytoplasmically sequestered wt-p53 (18). Although p53 mutations in neuroblastomas are rare (19), the cytoplasmic-sequestration of the protein is believed to abrogate the response pathway (20,21). However, our laboratory and others have reported previously that some neuroblastoma cell subtypes are able to elicit a p53 response after DNA damage (22,23). We have established by retroviral infection neuroblastoma cell lines stably expressing the Ews/Fli-1 chimeric oncogene. We observed that Ews/Fli-1, an aberrant transcription factor, regulates many genes in the neuroblastoma cell lines switching the differentiation program to Ewing’s sarcoma/peripheral neuroectodermal tumors (24).

We report here that Ews/Fli-1 expression in neuroblastoma tumor cells greatly modifies the localization and expression of p53 in neuroblastoma cells. We also monitored whether p53, its upstream effectors, or downstream effectors showed altered levels of expression in our neuroblastoma-Ews/Fli-1 infectants. We observed that whereas p53 and p14⁰⁰⁰⁰ proteins were overexpressed relative to the parental and vector control cell lines, p21⁰⁰⁰⁰ and MDM2 protein levels did not change. We also show that the p53 protein overexpression was not due to transcriptional up-regulation. We used the Affymetrix GeneChip microarray and Northern blot analysis to monitor p53 pathway protein gene expression profiles. We sequenced the p53 gene in both the neuroblastoma parent and neuroblastoma-Ews/Fli-1 infectant cell lines and found that it was wild type. We also found that the p53 in our neuroblastoma-Ews/Fli-1 infectants shows increased Ser-15 phosphorylation and stability; however, it was unable to elicit a DNA damage response. These results show that Ews/Fli-1 expression in neuroblastoma cell lines abrogates the p53 pathway. They suggest that in order for Ews/Fli-1 to elicit its oncogenic effects, p53 function must be compromised. We believe that our observations provide a valuable insight into the role of Ews/Fli-1 in some Ewing’s sarcoma/peripheral neuroectodermal tumors and propose a novel mechanism for p53 inactivation by this oncogene.

MATERIALS AND METHODS

Cell Lines. The neuroblastoma cell lines used in this study, LAN5 and NGP9A Tr1 (a HPRT-deficient tumorigenic clone of the NGP cell line; ref. 25)
were grown in RPMI 1640 supplemented with 10% fetal bovine serum. We infected LAN 5 or NGP9A Tr1 with either LXS neomycin vector or Ews/Fli-1 virus to generate Ews/Fli-1-expressing neuroblastoma cell lines as described (24). We designated the Ews/Fli-1-expressing LAN5 infectants as LEWS, and the LXS controls are LEWS-N. We designated the Ews/Fli-1-expressing NGP9A Tr1 infectants as NEWS and the LXS controls as NEWS-N. The numbers after these designations indicate different single cell clonal cell lines. The A673 is a human peripheral neuroectodermal tumor/Ewing’s sarcoma cell line (26). All of the cells were grown in 5% CO2 at 37°C.

**Immunofluorescence.** Cells were grown to 90% to 95% confluence on Lab-Tek II glass chamber slides (Nalge Nunc, International, Rochester, NY), washed with 1× PBS and fixed in cold acetone for 5 minutes. Fixed cells were washed with PBS and blocked in 5% bovine serum albumen for 40 minutes at 37°C and probed with primary p53 (DO-1) mouse monoclonal (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies in 2% bovine serum albumen for 1.5 hours. The neuroblastoma parent and vector control cell lines were probed with 1:100 primary antibody dilution, whereas the neuroblastoma-Ews/Fli-1 infectant cells were probed with 1:1,000 primary antibody dilution. This proved necessary due to the ~10 fold increase in p53 protein levels in the infectant cell lines. Without this lower concentration of antibody, it was difficult to determine the subcellular localization of the p53 protein in the infectants. Cells were washed and probed with secondary 1:250 rhodamine-based antimouse IgG CY3 conjugate (Sigma) for 30 minutes. The cells were mounted with 1:1 PBS/glycine and with 4,6-diamidino-2-phenylindole 0.1 μg/mL and viewed under the Axiostick Leitz Microscope.

**Western Blot Analysis.** Total cellular protein from near-confluent cell lines was extracted using 8 mol/L Urea extraction buffer (8 mol/L Urea, 0.1 mol/L NaH2PO4, and 10 mmol/L Tris (pH 8.0)) and fractionated by SDS-PAGE using NuPage Bis-Tris (Invitrogen Life Technologies, Inc. Carlsbad, CA) gels. We then transferred proteins to Immobilon-P filters and probed with antibodies according to the manufacturer’s protocol. Proteins were visualized using enhanced chemiluminescence (ECL, Amersham, Piscataway, NJ). Primary antibodies included p53 (DO-1) from Santa Cruz Biotechnology, p21/WAF1 (Ab-1) and MDM2 (AB-1) from Oncogene, PARP (VIC-5) from Roche (Basel, Switzerland), p14ARF and PARC from Dr. Xue Yong at University of North Carolina-Chapel Hill (Chapel Hill, NC), Phospho-p53 (Ser-15) from Cell Signaling (Beverly, MA), and actin from Sigma. Secondary antirabbit and antimouse antibodies were obtained from Amersham Biosciences.

**Northern Blot Analysis.** Expression of p53 mRNA was determined by standard Northern blot analysis. Total cellular RNA was extracted using the Qiagen RNeasy mini kit. Ten micrograms of total RNA were run on 1% agarose/formaldehyde gels and were gravity transferred to positively charged nylon membranes. The membranes were stained for ribosomal 28 s proteins for equal loading. p53 probes were 32P-radiolabeled by random primer extension, and were grown to near confluence. Cycloheximide was added to the medium at a final concentration of 100 μmol/L. Cells were washed with 1× PBS, trypsinized, and pellets were harvested at 8- and 24-hour postexposure time points. The cell pellets were then washed with 1× PBS, and protein was extracted using 8 mol/L Urea buffer. Poly(ADP-ribose) polymerase (PARP) and p53 protein expression was then analyzed using Western blot.

**yIrradiation.** Cells were seeded on 100-mm plates and grown to 70% to 80% confluence. Cells were then treated with 5-Gy173 Cs γ-rays (Gammacell 40). The cells were then placed back into 37°C/5%CO2 incubators and harvested at 0-, 2-, 6-, 12-, and 24-hour time points. The cells were lysed, and total protein was extracted using 8 mol/L Urea buffer. p53, p21WAF1, and ser-15 phosphorylated p53 protein expression was analyzed using Western blot. Cells were also harvested for flow-cytometric analysis at 0-, 2-, 6-, and 18-hour time points. The cells were trypsinized, washed in PBS, fixed in 70% EtOH, and then stained with propidium iodide solution (20 g/mL propidium iodide (Molecular Probes), 200 g/mL RNaseA (Sigma), and 0.1% Triton X-100 (Sigma) in PBS). Cells were analyzed for DNA content on a FACSscan flow cytometer (Becton Dickinson, San Jose, CA). Cell cycle distribution was determined using ModFit LT software (Verity, Topsham, ME).

**RESULTS**

**p53 Protein Accumulates in the Nucleus of the Neuroblastoma-Ews/Fli-1 Infectants, Whereas PARC Gene Expression Is Suppressed.** Neuroblastoma tumor cells are characterized by the cytoplasmic sequestration of wt-p53 protein (18, 19). This sequestration is believed to abrogate the tumor suppressive properties of the p53 gene (20). Stable expression of the Ews/Fli-1 fusion gene in neuroblastoma tumor cell lines relieved the cytoplasmic sequestration of p53 and caused the protein to accumulate in the nucleus (Fig. 1A). We also found that p53 protein levels increased in the neuroblastoma-Ews-Fli-1 infectants evident in the primary antibody dilution used to visualize the p53 localization. To observe the location of p53 in the infectants, we had to reduce the concentration of primary p53 (DO-1) antibodies from a dilution of 1:100 to 1:1,000. These results suggest that Ews/Fli-1 expression relieves the cytoplasmic sequestration of p53.

**PARC is a Parkin-like ubiquitin ligase that forms a complex with p53, causing the tumor suppressor protein to be cytoplasmically sequestered in neuroblastoma tumor cell lines (27).** Inactivation of PARC presumably relieves the cytoplasmic sequestration of p53 and induces nuclear accumulation (27). By Western blot analysis, we observed a significant reduction in PARC expression in the LEWS cell lines (Fig. 2A). However, we did not find PARC expression in the NGP cell line (Fig. 2A). Therefore, suppression of PARC expression could account for the loss of p53 cytoplasmic sequestration in the LEWS cell lines. However, a different mechanism must cause this event in the NEWS cell lines.

**p53 Pathway Protein Expression Profiles Change in the Neuroblastoma-Ews/Fli-1 Infectants.** The immunofluorescence studies suggested that p53 levels increased in the nucleus of the neuroblastoma-Ews/Fli-1 infectants. This observation led us to monitor the expression of proteins in the p53 pathway by Western blot in the neuroblastoma-Ews/Fli-1 infectant cell lines (Fig. 2). Interestingly, p53 and p14ARF protein were markedly overexpressed in the neuroblastoma-Ews/Fli-1 infectants, whereas p21WAF1 and MDM2 levels remained unaltered. These results suggest that Ews/Fli-1 causes aberrant expression of p53 without induction of its downstream targets.

**p53 Protein Half-Life Is Abnormally Extended in the Neuroblastoma-Ews/Fli-1 Infectants.** The immunofluorescence studies suggested that p53 levels increased in the nucleus of the neuroblastoma-Ews/Fli-1 infectants. This observation led us to monitor the expression of proteins in the p53 pathway by Western blot in the neuroblastoma-Ews/Fli-1 infectant cell lines (Fig. 2). Interestingly, p53 and p14ARF protein were markedly overexpressed in the neuroblastoma-Ews/Fli-1 infectants, whereas p21WAF1 and MDM2 levels remained unaltered. These results suggest that Ews/Fli-1 causes aberrant expression of p53 without induction of its downstream targets.
Northern blot. As shown in Fig. 3A, p53 was not transcriptionally up-regulated in the neuroblastoma-Ews/Fli-1 infectant cell lines. Therefore, we determined whether the p53 protein overexpression is due to abnormal or attenuated degradation by protein half-life assays. Using cycloheximide, we found that the p53 half-life is extended beyond normal degradation from 20 to 30 minutes to at least 5 hours (Fig. 3B). These results demonstrate that Ews/Fli-1 affects the stability of the p53 protein, presumably through an MDM2-independent mechanism (see Fig. 2A).

The p53 Gene Is Wild-Type and the Protein Is Hyper-Phosphorylated in the Neuroblastoma-Ews/Fli-1 Infectants. Prolonged stabilization of p53 can be due to post-translational modifications or mutant protein phenotypes. Whereas the two neuroblastoma cell lines used in this study express wild-type p53, it was possible that the neuroblastoma-Ews/Fli-1 infectant cell lines had accumulated mutations. Therefore, we determined the sequence of the p53 gene in these cell lines and found no mutations in the neuroblastoma parent, vector, or neuroblastoma-Ews/Fli-1 infectant cell lines (data not shown).

Because p53 protein overexpression in the neuroblastoma-Ews/Fli-1 infectant cell lines was not due to genetic mutations, we wanted to monitor p53 post-translational modifications as the potential cause of the observed abnormal p53 stabilization. The ATM protein kinase phosphorylates p53 on Ser-15 after genotoxic stress (28, 29) resulting in p53 stabilization. We found by Western blot that the p53 in the neuroblastoma-Ews/Fli-1 infectants is phosphorylated on Ser-15 (Fig. 3C). These results provide a possible explanation for the p53 protein overexpression in the neuroblastoma-Ews/Fli-1 infectant cell lines.

![Fig. 1. Localization of p53 by immunofluorescence. Cell lines were grown to near confluence on chamber slides, fixed, and probed with p53 (D0-1) primary antibodies for 1.5 hours. The neuroblastoma parent and vector control cell lines were probed with 1:100 primary antibody, whereas neuroblastoma-Ews/Fli-1 infectant cell lines were probed with 1:1,000 primary antibody. The cells were then probed with secondary 1:250 rhodamine-based antimouse IgG CY3 conjugate, mounted, and viewed under the Axioskop Leitz Microscope. DAPI, 4',6-diamidino-2-phenylindole](image)

![Fig. 2. p53 Protein expression in neuroblastoma-Ews/Fli-1 Infectants. Western blot analysis of p53 protein expression in neuroblastoma (LAN5 and NGP), neuroblastoma-vector controls (LEWS-N and NEWS-N), and neuroblastoma-Ews/Fli-1 infectant cell lines was carried out as described in Materials and Methods. A. neuroblastoma parental and neuroblastoma-Ews/Fli-1 infectant cell line blots were probed with anti-PARC and anti-MDM primary antibodies, stripped, and reprobed with an antiaction antibody as a loading control. B. neuroblastoma parental and neuroblastoma-Ews/Fli-1 infectant cell line blots were probed with anti-p53, p21, and p14ARF primary antibodies, stripped, and reprobed with an antiaction antibody as a loading control.](image)
overexpression and stabilization in the neuroblastoma-Ews/Fli-1 infectants, because Ser-15 phosphorylation blocks MDM2 binding to p53 (30). These observations suggest a novel mechanism by which Ews/Fli-1 may abrogate p53 function and subsequently its DNA damage response pathway.

p53 Is Nonfunctional in the Neuroblastoma-Ews/Fli-1 Infectants. Following DNA damage caused by various genotoxic stresses, p53 protein rapidly becomes stabilized and accumulates in its active form in the nucleus (31). Active p53 then transactivates downstream targets resulting in cell cycle arrest or apoptosis (31). If p53 is nonfunctional, it is unable to elicit a tumor-suppressive response increasing the risk for cancer (32, 33). Our results showing a stabilized, phosphorylated, and nuclear p53 protein without a concomitant increase in p21WAF1 protein levels suggested a dysfunctional p53 protein in the neuroblastoma-Ews/Fli-1 infectants. Although the steady-state p21WAF1 levels were not increased in the neuroblastoma-Ews/Fli-1 infectant cell lines, the p53 protein may still remain functional in response to genotoxic stress. Therefore, we first monitored the p53 response pathway in the neuroblastoma and neuroblastoma-Ews/Fli-1 infectant cell lines by exposing the cells to staurosporine, a protein kinase inhibitor that induces apoptosis (34). By measuring PARP cleavage (35), we found that the apoptosis response in the neuroblastoma-Ews/Fli-1 infectants is absent or delayed beyond 24 hours postexposure (Fig. 4). We did see an induction of p53 in the Lan5 cells in previous assays (data not shown). However, the Western blot depicting apoptosis in this experiment showing PARP cleavage along with dramatic p53 and actin protein loss after continued exposure to staurosporine in the Lan5 and LEWS-N cells probably arises from substantial death by this time point. However, we do show the activation of p53 protein along with PARP cleavage at 24 hours in the NGP and NEWS-N cells indicating that these tumor cells lines have a slightly slower apoptosis response. More interestingly, the neuroblastoma-Ews/Fli-1 infectants do not respond to staurosporine treatment, providing additional evidence that ectopic expression of Ews/Fli-1 fusion protein in neuroblastoma cells abrogates the p53 apoptosis-response pathway.

Cells exposed to ionizing radiation show activation and accumulation of p53 protein (28, 36, 37). The activated p53 protein up-regulates the cyclin/kinase inhibitor p21WAF1 causing cell cycle arrest at the G1 checkpoint (36, 38). We exposed the neuroblastoma and neuroblastoma-Ews/Fli-1 infectant to 5-Gy137 Cs rays and took total cellular protein samples at different postexposure time points to analyze p53 function. We show by Western blot that the neuroblastoma parental cells increase p53 protein levels and activate p21WAF1 after exposure to 137Cs irradiation, whereas the neuroblastoma-Ews/Fli-1 infectant p53 protein levels do not change, and p21WAF1 is not induced (Fig. 5). However, the p21WAF1 response appears muted, because it occurs between 4 and 10 hours after p53 induction. Interestingly, despite the increase of phosphorylation on Ser-15, the p53 protein in the neuroblastoma-Ews/Fli-1 infectant p53 remains nonfunctional. More interestingly, these results show that the upstream p53 pathway in the neuroblastoma-Ews/Fli-1 infectants remained intact as revealed by increased phosphorylation of p53 after γ irradiation. These results

Fig. 3. Altered p53 half-life in neuroblastoma-Ews/Fli-1 infectants. A, Northern blot analysis of p53 expression in neuroblastoma, neuroblastoma-vector controls, and neuroblastoma-Ews/Fli-1 infectant cell lines. Ten μg of total RNA was fractionated by 1% agarose gel electrophoresis in the presence of formaldehyde and transferred by capillary action to a nylon membrane. A 32P-labeled cDNA probe for p53 was hybridized to the blot and bands visualized by autoradiography. 28S RNAs were used as loading controls. B, Cell lines were grown to near confluence onto 100-mm plates and then exposed to cycloheximide at a final concentration of 4 μg/mL. Cells were harvested at 0, 0.5, 1, 2, and 5-hour time points. Protein was extracted and Western blot analysis of p53 protein expression in normal human fibroblast (NHF), neuroblastoma parent, and neuroblastoma-Ews/Fli-1 infectant cell lines were carried out as described in Materials and Methods. C, Cell lines were grown to near confluence onto 100-mm plates and harvested. Total protein was extracted and Western blot analysis of Ser-15 phosphorylated p53 protein expression neuroblastoma parental and neuroblastoma-Ews/Fli-1 infectant cell lines were carried out as described in Materials and Methods.
suggest that Ews/Fli-1 may be causing a p53 protein conformational change in a direct or indirect manner.

The p53 downstream target p21WAF1 is responsible for arrest at the G1 checkpoint after exposure to ionizing irradiation (38–40). It has been reported that neuroblastoma cell lines with functional p53 that transactivate p21WAF1 still have attenuated cell cycle arrests due to p21WAF1 dysfunction (23, 41). We assessed whether p53 could induce cell cycle arrest after exposure to 5-Gy 137Cs rays by flow cytometric analysis. We observed a significant difference between the responses of the two neuroblastoma parent cell lines and their vector controls. Whereas none of these cell lines displayed an obvious G1 arrest even 18 hours after X-irradiation exposure, the LAN5 cell line showed no evidence of a G2 arrest (Fig. 6). In contrast, the NGP cell line showed an attenuated cell cycle arrest at the G2-M checkpoint (Fig. 7). These results were surprising, because Western blot analysis showed a significant p21WAF1 induction after irradiation (Fig. 5). However, they agree with previously reported observations that suggest that p21WAF1 in neuroblastoma cell lines is dysfunctional (42). More interestingly, Figs. 6 and 7 show that neuroblastoma-Ews/Fli-1 infectant cell lines display a quicker G2-M arrest even without p53 function (Fig. 5). These results suggest that whereas Ews/Fli-1 is able to abrogate p53 protein function, it may also up-regulate a p53-independent pathway to elicit a G2-M arrest.

**DISCUSSION**

The p53 tumor suppressor gene is one of the most frequently mutated genes associated with cancer. This tumor suppressor gene has been reported to play a role in the control of cell growth after genotoxic stress, most notably through cell cycle arrest and apoptosis (43). Although much research has been done to understand this pathway, therapies targeting the p53 gene to treat or cure cancer have yet to be realized. Understanding how this pathway is abrogated in certain cancers may help develop therapies targeted at the potential underlying tumor causative agents, such as the Ews/Fli-1 chimeric gene found in Ewing’s sarcoma/peripheral neuroectodermal tumors.

There have been a limited number of reports that have investigated the effects of Ews/Fli-1 expression on the p53 pathway response (14, 15). A recent report showed that Ews/Fli-1 expression in normal human fibroblasts resulted in the up-regulation of p53 with subse-
quent arrest (15). Therefore, the Ews/Fli-1 protein must overcome this suppressor pathway in order for Ewing’s sarcoma/peripheral neuroectodermal tumor to develop. Mutations of the p53 gene could provide the mechanism by which Ewing’s sarcoma/peripheral neuroectodermal tumors progress. However, only a small percentage of primary Ewing’s sarcoma/peripheral neuroectodermal tumors have p53 mutations (10–12). So the question remains, how do Ewing’s sarcoma/peripheral neuroectodermal tumors overcome p53 tumor suppressor function? We offer a novel mechanism for how Ewing’s sarcoma/peripheral neuroectodermal tumors are able to bypass p53 tumor suppressor function. We propose that Ews/Fli-1 abrogates wild-type p53 function by converting the conformation of the protein into an inactive form. Whether binding to Ews/Fli-1 stabilizes p53 or another protein up-regulated by the fusion gene remains unknown. However, the resulting loss of p53 function may lead to increased genetic instability and tumorigenicity due to Ews/Fli-1 expression.

Nakatani et al. (44) reported recently that p21WAF1 is a direct target of Ews/Fli-1 resulting in p21 gene suppression. However, we did not see suppression of p21WAF1 transcription in the neuroblastoma-Ews/Fli-1 cell lines. It is unclear whether the Ews/Fli-1 protein in our cells acts directly at the p21WAF1 promoter. Therefore, studies using chromatin immunoprecipitation assays need to be performed to address whether Ews/Fli-1 suppresses gene transactivation by p53 by interfering at p53-responsive promoters. The potential association of the fusion gene with CBP/p300 may provide another mechanism by which Ews/Fli-1 could inhibit p53 activity in the neuroblastoma-Ews/Fli-1 infectants. Phosphorylation of p53 at Ser-15 stimulates the interaction of the tumor suppressor gene with its coactivators p300 and CBP (45), resulting in acetylation of the COOH terminus of p53, and increases sequence-specific binding to downstream targets including p21WAF1 (46). The EWS gene has been reported to physically and functionally interact with CBP and p300 (47), specifically via amino acids 83 to 227 (48), which are contained in the EWS portion of the Ews/Fli-1 fusion gene. Another Ews fusion gene, Ews-ATF-1, has been reported to associate with CBP/p300 and interfere with p33-mediated transactivation function (49). The Ews/Fli-1 chimeric oncogene may be associating with CBP/p300, thereby competing with and suppressing p53 transactivation of p21WAF1.

Lessnick et al. (15) reported that expression of Ews/Fli-1 in human fibroblasts results in the up-regulation of p53 transcription followed by growth arrest. However, the increased p53 protein levels in our neuroblastoma-Ews/Fli-1 infectants were due to protein stabilization without transcriptional up-regulation. More importantly, our Ews/Fli-1-expressing neuroblastoma tumor cell lines did not undergo growth arrest but seemed to grow faster when compared with the neuroblastoma parent cell lines (data not shown). Several critical differences between these studies could account for the divergent results. The studies reported by Lessnick et al. (15) used normal cell lines to monitor p53 response to Ews/Fli-1 expression, whereas we used...
tumor cells. Normal cells maintain tumor-suppressive function and present evidence of p53 protection against tumorigenesis, whereas the cellular background of neuroblastoma tumor cells may provide a more conducive system to monitor the consequence of Ews/Fli-1 expression on p53. The cellular background is another important variable that could account for the different observations (50). Whereas Lessnick et al. (15) used fibroblast cells to monitor the p53 pathway after Ews/Fli-1 expression, we used neuronal tumor cells. Ewing’s sarcoma/peripheral neuroectodermal tumors express neuronal markers (51, 52), suggesting a neuronal origin of these tumors. Therefore, it may be important to study the effects of Ews/Fli-1 expression in a cellular context consistent with the environment of the naturally occurring Ews/Fli-1 translocation and activation. Therefore, more studies need to be performed using normal neuronal cells to fully assess the consequence of Ews/Fli-1 expression on the p53 pathway. However, both studies substantiate the hypothesis that p53 abrogation cooperates with Ews/Fli-1 to promote development of Ewing’s sarcoma/peripheral neuroectodermal tumors.

We also performed flow cytometric analysis to monitor the integrity of cell cycle arrest mediated by p53. Although the p53 protein is functional in the neuroblastoma parent cells, our results reveal that the neuroblastoma parent cells have both an attenuated G2 and G1-S arrest, which is probably a consequence of p21 WAF1 dysfunction as reported previously (23, 42). More interestingly, the neuroblastoma-Ews/Fli-1 infectants have restored a quicker and more robust G2 arrest without a G1-S arrest. Cells can undergo cell cycle arrest at the G2 checkpoint via the pRb-pathway (3). The neuroblastoma-Ews/Fli-1 infectants appear to have an intact pRb pathway evident in the response of the cellular pRb becoming hypophosphorylated after γ irradiation (data not shown). Hypophosphorylated pRb activates the tumor suppressor properties of pRb allowing the protein to bind and inhibit cell cycle promoting transcription factors (53, 54). Because the p53 tumor suppressor pathway in the neuroblastoma-Ews/Fli-1 infectant cells appear to be dysfunctional due to Ews/Fli-1 fusion protein expression, these cells may divert their primary response pathway to their intact pRb tumor suppressor pathway. Our findings also indicate that the upstream signaling pathway, mediated by ATM activity, remains intact and unaffected by Ews/Fli-1 expression. Although the neuroblastoma-Ews/Fli-1 infectant cells did not respond to γ irradiation, the Ser-15 phosphorylated p53 appeared to increase slightly in the neuroblastoma-Ews/Fli-1 infectants without p21 WAF1 induction. Thus, the abrogation of the pathway appears to begin and end at p53.

Whereas it has been suggested that cooperativity of p53 pathway abrogation along with Ews/Fli-1 expression is necessary for transformation by this chimeric oncogene, more studies need to be aimed at investigating this hypothesis. Our results offer insight into how Ewing’s sarcoma/peripheral neuroectodermal tumors may arise as cancer. We have reported that stable expression of Ews/Fli-1 can abrogate p53 pathway function in neuroblastoma cells, thereby providing a system to study the transformative properties of the fusion gene. However, it is still unclear how Ews/Fli-1 abrogates the p53 pathway in these cells. Initial immunoprecipitation studies suggest that Ews/Fli-1 does not cause a conformational change by directly binding to p53 (data not shown). Therefore, another protein potentially up-regulated by Ews/Fli-1 may bind to the p53 protein causing the observed dysfunction. Alternatively, other protein modifications such as phosphorylation, acetylation, and/or ubiquitination may have affected p53 in the neuroblastoma-Ews/Fli-1 infectants. These studies also offer insight into the p53 pathway in neuroblastoma tumor cells. We have shown in these two neuroblastoma cell lines that the p53 is functional, although the downstream pathway may be somewhat compromised due to an attenuated cell cycle arrest. The apoptosis pathway in the neuroblastoma parent cells is intact and may provide clues and insight into the development of therapies taking advantage of this pathway. Understanding the p53 properties in these cell lines could potentially be used to give cancer patients more detailed prognosis with a personally designed treatment regimen.

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