

The *Ews/Fli-1* Fusion Gene Switches the Differentiation Program of Neuroblastomas to Ewing Sarcoma/Peripheral Primitive Neuroectodermal Tumors

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ABSTRACT

Neuroblastoma (NB) and the Ewing sarcoma (ES)/peripheral primitive neuroectodermal tumor (PNET) family are pediatric cancers derived from neural crest cells. Although NBs display features of the sympathetic nervous system, ES/PNETs express markers consistent with parasympathetic differentiation. To examine the control of these differentiation markers, we generated NB × ES/PNET somatic cell hybrids. NB-specific markers were suppressed in the hybrids, whereas ES/PNET-specific markers were unaffected. These results suggested that the *Ews/Fli-1* fusion gene, resulting from a translocation unique to ES/PNETs, might account for the loss of NB-specific markers. To test this hypothesis, we generated two different NB cell lines that stably expressed the *Ews/Fli-1* gene. We observed that heterologous expression of the *Ews/Fli-1* protein led to the suppression of NB-specific markers and *de novo* expression of ES/PNET markers. To determine the extent of changes in differentiation, we used the Affymetrix GeneChip Array system to observe global transcriptional changes of genes. This analysis revealed that the gene expression pattern of the *Ews/Fli-1*-expressing NB cells resembled that observed in pooled ES/PNET cell lines and differed significantly from the NB parental cells. Therefore, we propose that *Ews/Fli-1* contributes to the etiology of ES/PNET by subverting the differentiation program of its neural crest precursor cell to a less differentiated and more proliferative state.

INTRODUCTION

Neuroblastoma (NB) and the Ewing sarcoma (ES)/peripheral primitive neuroectodermal tumor (PNET) family are pediatric cancers of neural crest origin that differentiate along a neuroendocrine lineage (1–6). NB and ES/PNET belong to the family of small blue round cell tumors that includes Wilm's tumor, hepatoblastoma, and rhabdomyosarcoma (4, 7, 8). In addition to their small blue round cell appearance under light microscopy, NB and ES/PNET share other morphological and histological features including prominent nucleoli, high nucleus:cytoplasm ratio, and the formation of Homer Wright rosettes (9). However, significant clinical and phenotypic features define these tumors.

NB is one of the most common solid tumors in children, with the majority of patients presenting under age 6 years (2, 10). The tumors are usually localized to the adrenal medulla or the sympathetic ganglia of the peripheral nervous system (2). Aggressive NB tumors often show loss of heterozygosity for polymorphic markers in 1p36 (2, 11) and gain of 17q21–25 (12). NBs generally show

increased expression of sympathetic pathway enzymes tyrosine hydroxylase and dopamine- β -hydroxylase, MycN amplification, markers of adrenal chromaffin-related differentiation such as chromogranin A (CgA), and overexpression of neuroendocrine markers β 2-microglobulin, and synaptophysin (9, 13, 14).

ES/PNET, a common pediatric soft tissue sarcoma, usually occurs in adolescents between the ages of 10 and 20 years (8). The most frequent sites of ES/PNET are the pelvic bones, femur, tibia, and humerus (8, 15). A unique cytogenetic feature common to ES/PNETs is the presence of a balanced translocation joining the DNA-binding domain of an Ets or Ets-like transcription factor family member (16, 17) to the NH₂-terminal region of the *Ews* gene (22q12; Refs. 18 and 19). The most common *Ews/Ets* translocations are *Ews/Fli-1* t(11;22)(q22;q12) and *Ews/Erg* t(21;22)(q12;q12) (19–22). In contrast to NB, C-Myc amplification, overexpression of the surface glycoprotein MIC2, and increased levels of parasympathetic pathway enzymes such as choline acetyltransferase (23) characterize ES/PNET (15).

The apparent sympathetic lineage of NB *versus* the seemingly parasympathetic lineage of ES/PNET suggests that these two tumors have retained expression of the regulatory pathways that distinguish these two differentiation programs. Whether these programs act in a hierarchical or an independent fashion remains an open question. To examine the control of NB and ES/PNET differentiation programs, we generated NB × NB, NB × ES/PNET, and ES/PNET × ES/PNET somatic cell hybrids. Our results showed suppression of the NB-specific markers in the NB × ES/PNET hybrids, whereas the ES/PNET-specific markers remained expressed.

We then investigated the mechanism that accounts for the observed gene expression changes in the NB × ES/PNET hybrids. An obvious difference between the two tumor types is the ES/PNET genetic marker *Ews/Fli-1*, a reported aberrant transcription factor (16–18, 21, 24). We retrovirally infected NB parental cell lines with *Ews/Fli-1* and established stably expressing clones. We found that the introduction of the *Ews/Fli-1* chimeric gene into the NB parents recapitulated the results observed in NB × ES/PNET hybrids. We next used the Affymetrix GeneChip microarray system (25–27) to assess the extent of the transcriptional changes of genes regulated as a result of infecting NB parent cell lines with *Ews/Fli-1*. This analysis confirmed our previous results. These data suggest that *Ews/Fli-1* is capable of suppressing sympathetic, neural, and adrenal chromaffin pathways in NB and inducing the phenotypic traits associated with ES/PNET. They also raise the possibility that parasympathetic differentiation precedes sympathetic differentiation during normal development. The microarray analysis revealed many genes that may be regulated by *Ews/Fli-1*, allowing for the proper diagnosis and distinction from similar tumors such as NBs and ES/PNETs.

MATERIALS AND METHODS

Cell Lines and Somatic Cell Hybridization. The NB cell lines used in this study were LAN 5, NGP9A Tr1, OLIVA, and SK-N-SH. The ES/PNET cell lines used were A673 BrdU, SK-N-MC, TC32, and TC106. Parental cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum. Polyethylene glycol was used to generate somatic whole cell fusions from the parental

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cell lines as described previously (28). Individual clones were isolated and expanded for further characterization. We use the prefix HHy to signify a human hybrid derived from two human cell lines. We then assign a numerical identifier (*e.g.*, 66) with individual clones from different dishes identified by their plate number. An Arabic letter designates the order in which colonies were removed from the same dish (*e.g.*, P2B). Hybrid cell lines were routinely passaged on basal RPMI 1640 supplemented with 20% FCS. The A673 × NGP9A Tr1 hybrids were denoted HHy66 and selected for growth in RPMI 1640 supplemented with 20% FCS and hypoxanthine-aminopterin-thymidine. SK-N-MC × A673 hybrids were denoted HHy73; A673 × LAN 5 hybrids were denoted HHy82; and LAN 5 × NGP9A Tr1 hybrids were denoted HHy87. These hybrids were selected for growth in RPMI 1640 supplemented with 20% FCS, hypoxanthine-aminopterin-thymidine, and 600 µg/ml G418. All cells were grown in 5% CO₂ at 37°C and routinely monitored for *Mycoplasma* infection.

Generation of NB Ews/Fli-1-Infectant Cell Lines. We used the Calcium Phosphate Transfection System (Life Technologies, Inc.) and standard protocols to generate LXS neomycin vector only and Ews/Fli-1 virus (18, 29, 30) from human embryonic kidney 293-T cells. After 24–48 h, supernatant from the infected cells was added to subconfluent LAN 5 or NGP9A Tr1 cells in the presence of Polybrene (4 µg/µl). After a 2-h exposure with intermittent shaking, the medium was removed and replaced with fresh growth medium. After 48 h, stable infectants were selected for growth in growth medium with G418 (600 µg/ml). After approximately 2 weeks of selection, individual colonies were isolated and expanded for characterization. We designated the Ews/Fli-1-expressing LAN 5 infectants as LEWS and the LXS controls as LEWS-N. We designated the Ews/Fli-1-expressing NGP9A Tr1 infectants as NEWS and the LXS controls as NEWS-N.

Reverse Transcription (RT)-PCR. We verified the expression of Ews/Fli-1 by RT-PCR. Parental and infectant total RNA was extracted using the RNeasy Mini Kit (Qiagen), and cDNA was generated using the Omniscript RT Kit (Qiagen). The cDNA was annealed with Fli-1 primer 11.3 [5'-ACTC-CCCCTGGTCCCCTCC-3' (19, 31)] and Ews primer 22.3 [5'-TCCTA-CAGCCAAGCTCCAGGTC-3' (19, 32)] and amplified by PCR. The resulting products were fractionated on agarose gels and visualized by ethidium bromide staining.

Northern Blot Analysis. Expression of CgA, MycN, and C-Myc mRNA was determined by standard Northern blot analysis. Total cellular RNA was purified by the guanidinium thiocyanate extraction method (33) and by using the Qiagen RNeasy Mini Kit. Ten µg of total RNA were run on 1% agarose/formaldehyde gels and gravity transferred to positively charged nylon membranes. Probes were ³²P radiolabeled by random primer extension, hybridized to membranes, and subsequently rinsed to remove nonspecific radioactivity. Hybridization bands were visualized by autoradiography.

Immunocytochemistry. MIC2 antibody was obtained from DAKO Corp. (Carpenteria, CA). Immunocytochemical staining followed the manufacturer's protocols. Immunostaining was preceded by a 15-min peroxide pretreatment (DAKO), followed by antigen retrieval with AR 10 Antigen Solution (Biogenex), and sequential 1-h treatments with avidin, biotin, and protein blocking reagent from Biogenex. The primary antibody was applied overnight at 4°C. The substrate was visualized by application of 3-amino-9-ethylcarbazole MIC2. Cells were counterstained with hematoxylin, rinsed with deionized water, mounted with a coverslip, and photographed at ×40 magnification with a Zeiss axioplan microscope.

Western Blot Analysis. Total cellular protein from near-confluent cell lines was extracted using 8 M urea extraction buffer [8 M urea, 0.1 M NaH₂PO₄, and 10 mM Tris (pH 8)] and fractionated by SDS-PAGE using Tris-glycine (Fisher Scientific, Pittsburgh, PA) or NuPage Bis-Tris (Invitrogen Life Technologies, Inc., Carlsbad, CA) gels. We then transferred proteins to Immobilon-P filters and probed them with antibodies according to the manufacturer's protocol. Proteins were visualized using enhanced chemiluminescence (Amersham). Primary antibodies included ShcA from Transduction Laboratories, MycN (Ab-1) from CalBiochem, actin from Sigma, and C-Myc (9E10) from Santa Cruz Biotechnology. ShcC expression was assessed as described previously (34). Secondary antirabbit and antimouse antibodies were obtained from Amersham Biosciences, and antigoat secondary antibody was obtained from Santa Cruz Biotechnology.

Microarray Analysis. Total RNA was extracted from NB parental cell lines, NB vector control cell lines, NB Ews/Fli-1 infectant cell lines, and

ES/PNET cell lines using the RNeasy Mini Kit (Qiagen). Equal amounts of RNA from NB parental cell lines, along with their respective vector control cell lines, each respective set of NB Ews/Fli-1 infectant cell lines, and the ES/PNET cell lines TC32, A673, and TC106, were pooled to a concentration of 0.6 µg/µl. The pooled LAN 5 Ews/Fli-1 infectants were named LFBC; the pooled NGP9A Tr1 Ews/Fli-1 infectants were named NBFBC; and the pooled ES/PNET cell lines were named ES-TAT. Each sample set was run on duplicate Affymetrix GeneChip HU95A.ver2 microscope slides in the Affymetrix Core Facility (University of North Carolina-Chapel Hill). The data output was analyzed using GeneSpring 5.0 software (Silicon Genetics). The data were normalized and subjected to Student's *t* test (*P* ≤ 0.05), and subsequent gene lists were generated based on a 100 minimal hybridization signal with at least a 5-fold difference of lower or higher expression of the NB Ews/Fli-1 infectants and the ES/PNET cell lines compared with the gene expression signals of the parental cell lines. Clustering was performed to generate a hierarchical tree of all 12,625 genes.

RESULTS

NB × ES/PNET Hybrid Cell Lines Show Suppression of NB Tumor Markers and No Change of ES/PNET Markers. We initially generated the NB × ES/PNET hybrid series HHy66 by somatic whole cell fusion. We confirmed their origin by total chromosome content (35) and dinucleotide repeat polymorphisms. Each of the HHy66 clonal cell lines retained nearly the sum total of chromosomes from both parents (Table 1) and at least one polymorphic marker from each parent (data not shown). We next examined the expression of two markers of neural differentiation, CgA, which is expressed in NB, and C-Myc, which is highly expressed in ES/PNET. Northern blot analysis of three independent hybrid cell lines showed down-regulation of the NB-specific marker CgA and retention of the ES/PNET-specific marker C-Myc (Fig. 1). These data suggested that suppression of NB markers may occur in the presence of the ES/PNET differentiation program. To rule out the caveat of a nonspecific effect of cell fusion, we generated additional sets of hybrid cell lines. As a control for nonspecific changes in the expression of NB-specific markers, we fused two NB cell lines, NGP9A Tr1 and LAN 5 (HHy87). HHy73, a fusion of the ES/PNET cell lines A673 BrdU and SK-N-MC (36), served as a control for nonspecific changes in ES/PNET. Finally, we

Table 1 Characteristics of NB^a and ES/PNET parental cell lines and hybrids

Cell line	Tumor type/parental cell line	Modal chromosome no. ^{b,c}	Ref. no.
LAN 5	NB	46 (46)	88
NGP9A Tr1	NB	46 (46)	89
A673 BrdU	ES/PNET	63, 67 (61–67)	36 and 90
SK-N-MC	ES/PNET	46 (46)	91
HHy73	SK-N-MC × A673 BrdU ^{d,e}	Ideal modal no. ^c (107–113)	36
P1		88 (78–91)	
P2		98 (88–104)	
P3		100 (92–103)	
HHy82	A673 BrdU ^{d,e} × LAN 5	Ideal modal no. ^c (107–113)	
P1		65, 109 (65–109)	
P2		109 (62–109)	
P3		93, 95 (82–107)	
HHy87	LAN 5 × NGP9A Tr1 ^f	Ideal modal no. ^c (92)	
P1		70 (51–77)	
P6		61, 67 (61–77)	
HHy66	NGP9A Tr1 ^f × A673 BrdU ^d	Ideal modal no. ^c (107–113)	
P2		101, 104 (88–105)	
P3		93 (89–118)	

^a NB, neuroblastoma; ES, Ewing sarcoma; PNET, primitive neuroectodermal tumor; P, plate number.

^b Modal chromosome number for each cell line.

^c Range of chromosome number for each cell line.

^d TK⁻ parent.

^e Neomycin⁺ parent.

^f Hypoxanthine-guanine phosphoribosyltransferase⁻ parent.

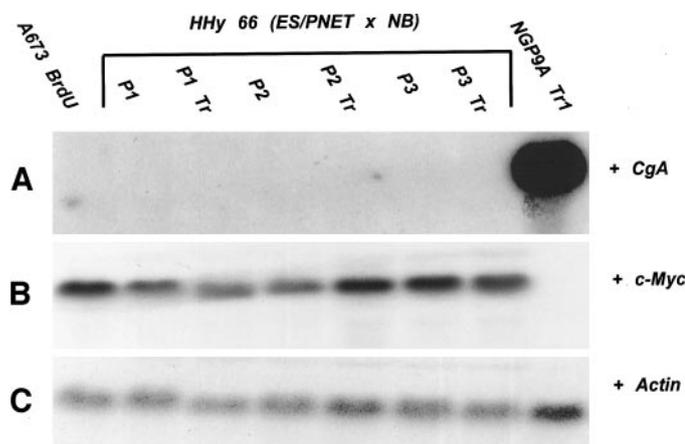


Fig. 1. Expression of chromogranin A (CgA) and C-Myc in neuroblastoma \times Ewing sarcoma/peripheral primitive neuroectodermal tumor hybrid HHy66. Ten μ g of total RNA were fractionated by 1% agarose gel electrophoresis in the presence of formaldehyde and transferred by capillary action to a nylon membrane. A 32 P-labeled cDNA probe for neuroblastoma-specific sympathetic marker chromogranin A was hybridized to the blot (A). The blot was then stripped and rehybridized with a 32 P-labeled cDNA probe for C-Myc (B) and a 32 P-labeled cDNA γ -actin (C) to verify RNA loading and integrity.

hybridized the LAN 5 cell line with the A673 BrdU cell line to generate a different NB \times ES/PNET hybrid cell line, HHy82. In agreement with the HHy66 hybrids, HHy73, HHy82, and HHy87 retained nearly the sum total of chromosomes from each parent (Table 1). We again used dinucleotide repeat polymorphisms or RFLPs to confirm the origin of parental chromosomes in HHy72, HHy82, and HHy87 (data not shown).

We next examined the expression of two NB-specific differentiation markers, two ES/PNET-specific markers, and two nonspecific neuroendocrine markers by immunocytochemistry. Table 2 summarizes the expression of all markers in the parental and hybrid cell lines. Both NB parents expressed CgA and MycN, whereas the ES/PNET parents did not. The NB \times NB hybrids continued to express CgA and MycN, whereas there was a down-regulation of CgA and MycN expression in the NB \times ES/PNET hybrids. These results indicated that gene(s) in the ES/PNET parents were able to suppress MycN and CgA expression. Expression of ES/PNET-specific markers MIC2 and Ews/Fli-1 were also examined in the hybrids. The expression pattern of the ES/PNET-specific markers in the parent and hybrids showed the opposite pattern. The ES/PNET parents A673 BrdU and SK-N-MC and the ES/PNET \times ES/PNET hybrids were positive for MIC2. The NB parents did not stain for MIC2, whereas the NB \times ES/PNET hybrids continued to express MIC2. We also determined expression of the Ews/Fli-1 gene by RT-PCR. As expected, the NB parents and NB \times NB hybrids did not express the fusion gene, whereas the ES/PNET parents, ES/PNET \times ES/PNET hybrids, and the NB \times ES/PNET hybrids retained expression. These data further

support the hypothesis that the ES/PNET differentiation program is dominant over the NB differentiation program.

We also examined the expression of two general neuroendocrine-specific differentiation markers, β 2-microglobulin and synaptophysin, that are overexpressed in both NBs and ES/PNETs (9, 15). All parental and hybrid cell lines expressed both markers, with the exception of SK-N-MC, which lacked synaptophysin protein. Therefore, the ES/PNET genes did not alter expression of more generalized neuroendocrine markers in most cases. These results clearly demonstrate the loss of NB-specific markers and the retention of ES/PNET markers in NB \times ES/PNET hybrid cell lines. Thus, the latter cells express gene(s) that can coordinately regulate differentiation in a dominant fashion in the NB cell lines.

Ews/Fli-1 Can Be Stably Expressed in NB Parent Cell Lines. Suppression of NB-specific differentiation markers in the NB \times ES/PNET hybrids implies that genes in ES/PNET can regulate the differentiation program in the NB parents. The chimeric protein Ews/Fli-1 warranted further investigation because of its continued expression in NB \times ES/PNET hybrids (data not shown). Ews/Fli-1 is known to function as a transcription factor and can induce expression of some neuronal and hematopoietic differentiation markers in non-neuronal cells (16, 18, 37–41). Furthermore, a recent study has shown that the *Ews/Fli-1* gene can suppress transforming growth factor β receptor II expression in NB cell lines (42).

To test the effects of Ews/Fli-1 on the expression of differentiation markers in the NB cell lines, we infected the LAN 5 and NGP9A Tr1 cell lines with a retrovirus containing a type IV *Ews/Fli-1* gene (18, 29). We also infected cells with a neoR vector as a control. We chose six independent clones for further characterization and confirmed the expression of the Ews/Fli-1 transgene by RT-PCR. All six NB Ews/Fli-1 infectants expressed the chimeric gene by RT-PCR (Fig. 2).

NB-Specific Markers Are Suppressed in NB Ews/Fli-1 Infectants. MycN expression, a classic marker of NB, was extinguished in the NB \times ES/PNET hybrid cells. Therefore, we first examined expression of this gene in the Ews/Fli-1-expressing NB cell lines. We

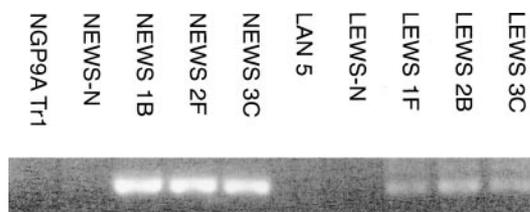


Fig. 2. Expression of Ews/Fli-1 in neuroblastoma (NB) parents and NB Ews/Fli-1 infectants by reverse transcription-PCR. Expression of the Ews/Fli-1 transcript in the NB and Ewing sarcoma/peripheral primitive neuroectodermal tumor and in the NB parents stably expressing a type IV Ews/Fli-1 construct was determined by reverse transcription-PCR. LEWS and NEWS are the LAN 5 and NGP9A Tr1 parents stably expressing the Ews/Fli-1 construct. Isolation of RNA and primers used are described in "Materials and Methods." The product was run on a 1% agarose gel and stained with ethidium bromide for UV visualization. The PCR product was verified by automated sequencing.

Table 2 Expression of differentiation markers NB,^a ES/PNET parents, and NB \times ES/PNET hybrids

Differentiation marker	LAN 5	NGP9A Tr1	HHy82 P1	HHy82 P2	HHy82 P3	HHy66 P2	HHy66 P3	A673 BrdU	SK-N-MC
CgA	+ ^b	+	–	–	–	–	–	–	–
MycN	+	+	–	–	–	–	–	–	–
ShcC	+	+	–	–	–	–	–	–	–
Ews/Fli-1	–	–	+	+	+	+	+	+	+
MIC2	–	–	+	+	+	+	+	+	+
ShcA	–	+	+	+	+	+	+	+	+
β 2M	+	+	+	+	+	+	+	+	+
SYNA	+	+	+	+	+	+	+	+	–

^a NB, neuroblastoma; ES, Ewing sarcoma; PNET, primitive neuroectodermal tumor; P, plate number; CgA, chromogranin A; SYNA, synaptophysin; β 2M, β 2-microglobulin.

^b +, positive expression in parent or hybrid; –, negative expression in parent or hybrid.

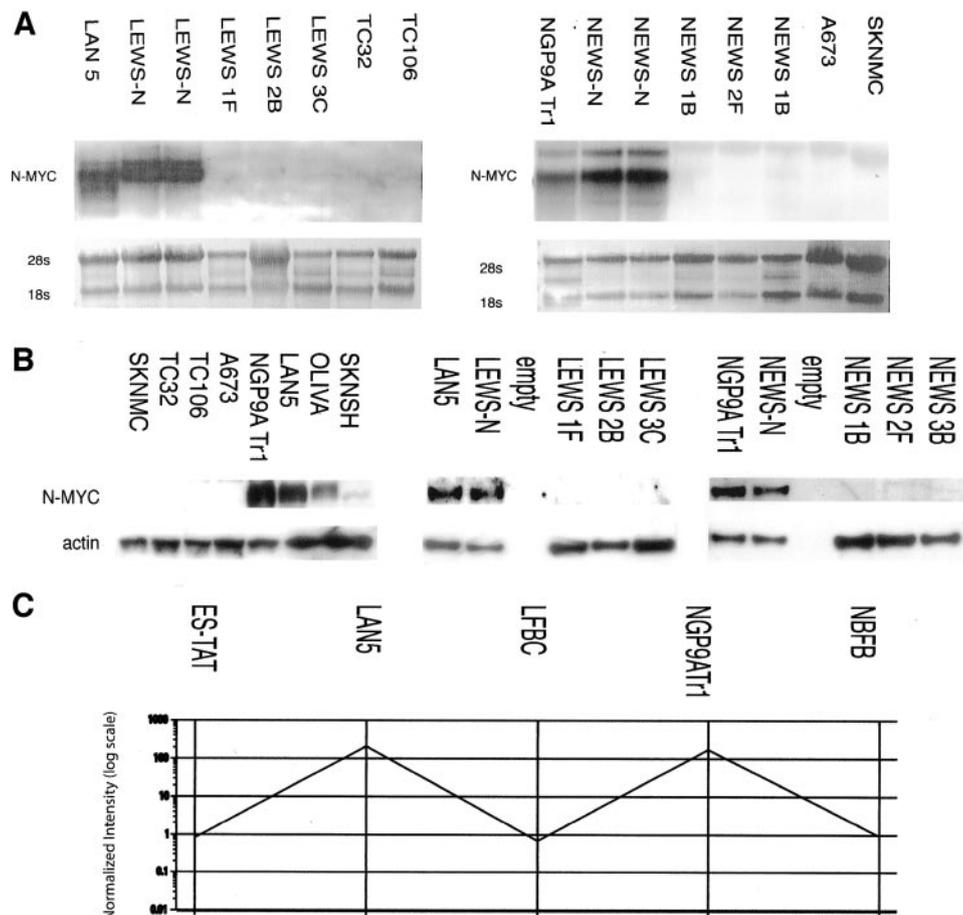


Fig. 3. MycN expression in neuroblastoma (NB) Ews/Fli-1 Infectants. *A*, Northern blot analysis of MycN expression in NB, NB Ews/Fli-1 infectants, and Ewing sarcoma/peripheral primitive neuroectodermal tumor cell lines. Ten μg of total RNA were fractionated by 1% agarose gel electrophoresis in the presence of formaldehyde and transferred by capillary action to a nylon membrane. A ^{32}P -labeled cDNA probe for MycN was hybridized to the blot, and bands were visualized by autoradiography. 28S and 18S RNAs were used as loading controls. *B*, Western blot analysis of MycN protein expression in Ewing sarcoma/peripheral primitive neuroectodermal tumor, NB, and NB Ews/Fli-1 infectant cell lines was carried out as described in "Materials and Methods." The blots were first probed with an anti-MycN antibody, stripped, and reprobed with an anti-actin antibody as a loading control. *C*, GeneSpring 5.0 hybridization expression graph for MycN from Affymetrix GeneChip data output. Normalized intensity was plotted on a logarithmic scale.

found that Ews/Fli-1 expression was sufficient to suppress MycN expression in NB cells (Fig. 3). Northern blot analysis revealed that MycN mRNA was suppressed to undetectable levels in the infectants (Fig. 3A). A Western blot panel of ES/PNET cell lines and NB cell lines reveals that NB but not ES/PNET cell lines overexpress MycN protein, confirming that MycN is characteristic to NB and not ES/PNET (9). NB Ews/Fli-1 infectants do not express MycN protein (Fig. 3B), suggesting that Ews/Fli-1 can regulate MycN gene expression. Microarray analysis show that MycN is suppressed at least 5-fold in NB Ews/Fli-1 infectant cell lines (Fig. 3C), further confirming the down-regulation of MycN by Ews/Fli-1. Suppression of CgA also occurs in the NB parents infected with Ews/Fli-1, but CgA remains expressed in the NB parents infected with the neomycin vector (data not shown). Taken together, these results demonstrate that the Ews/Fli-1 gene dramatically inhibits expression of the NB-specific proteins at the level of transcription and implicate the Ews/Fli-1 gene as the operative agent for alterations in the NB \times ES/PNET hybrids.

ES/PNET-Specific Markers Are Up-Regulated NB Ews/Fli-1 Infectants. The results in the hybrid cells could arise from two different mechanisms. The Ews/Fli-1 protein could merely suppress expression of the NB-specific genes in the hybrid cells rather than regulate the differentiation program of the ES/PNET cell lines. Alternatively, expression of Ews/Fli-1 could underlie the differentiation program found in the ES/PNETs. If Ews/Fli-1 only suppresses NB-specific genes, we should not observe ES/PNET-specific gene expression in the NEWS and LEWS cell lines. C-Myc and MIC2 overexpression are characteristic markers of ES/PNETs (8, 15). Because we did not observe a change in C-Myc expression in the NB \times ES/PNET hybrids, we investigated its expression in NB Ews/Fli-1 infectants. We have reported previously that the expression of Ews/Fli-1 in the

NGP9A Tr1 cell line resulted in increased expression of C-Myc (43). Ectopic expression of Ews/Fli-1 in the LANS 5 NB cells also caused overexpression of C-Myc (Fig. 4). Northern blot analysis revealed that C-Myc mRNA was up-regulated in the NB Ews/Fli-1 infectants (Fig. 4A). A Western blot panel of ES/PNET cell lines and NB cell lines also confirmed that ES/PNET but not NB cell lines overexpress C-Myc protein [Fig. 4B (8, 15)]. In agreement with the Northern blot data, the NB Ews/Fli-1 infectants overexpress C-Myc protein, whereas the parental NB cell lines do not (Fig. 4B). Microarray analysis showed that C-Myc expression is at least 5-fold higher in NB Ews/Fli-1 infectant cell lines as compared with NB parental cell lines (Fig. 4C), further confirming the up-regulation of C-Myc by Ews/Fli-1.

We used immunohistochemistry to ascertain the level of MIC2 expression in the LEWS and NEWS cell lines. The MIC2 expression pattern in the NB Ews/Fli-1 infectants is virtually identical to the pattern observed in the ES/PNET parents (Fig. 5A). We again verified this result by microarray, and the MIC2 gene was up-regulated at least 5-fold in NB cells infected with Ews/Fli-1 (Fig. 5B). These results strongly suggest that Ews/Fli-1 is capable of altering the differentiation program in other neural crest cell derivatives and is responsible for some of the phenotypic traits associated with ES/PNET.

NB Ews/Fli-1 Infectants Reveal Suppression of Shc Adaptor Proteins. We also examined the expression of potential neuroendocrine signal transduction pathways in the parental and Ews/Fli-1-infected cell lines. The mammalian adaptor proteins ShcA and ShcC interact with receptor tyrosine kinases such as the Trk family (TrkA, TrkB, and TrkC) to mediate, in part, the effects of these receptors (34, 44–46). The ShcA gene encodes for three proteins of 46, 52, and 66 kDa. Similar in structural motif to ShcA, the ShcC gene encodes for

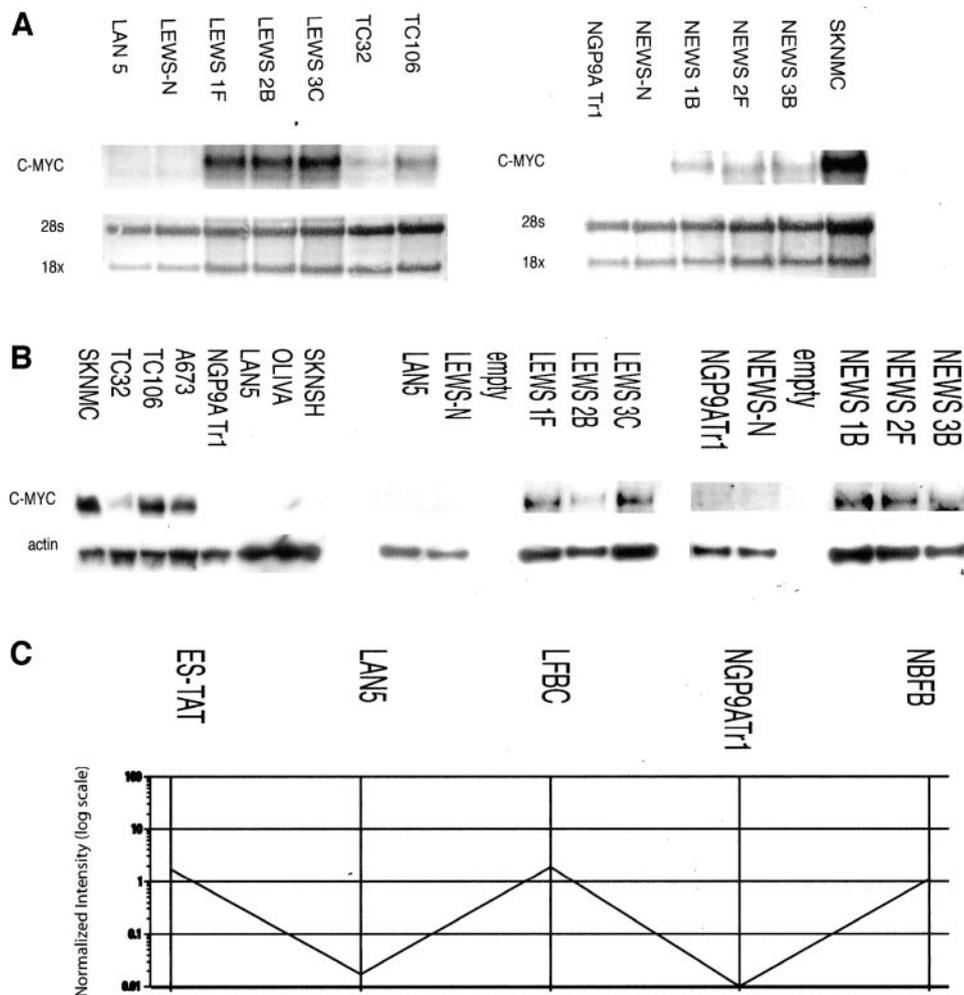


Fig. 4. C-Myc expression in neuroblastoma (NB) Ews/Fli-1 infectants. **A**, Northern blot analysis of C-Myc expression in NB, NB Ews/Fli-1 infectants, and Ewing sarcoma/peripheral primitive neuroectodermal tumor cell lines. Ten μ g of total RNA were fractionated by 1% agarose gel electrophoresis in the presence of formaldehyde and transferred by capillary action to a nylon membrane. A 32 P-labeled cDNA probe for C-Myc was hybridized to the blot, and bands were visualized by autoradiography. 28S and 18S RNAs were used as loading controls. **B**, Western blot analysis of C-Myc protein expression in Ewing sarcoma/peripheral primitive neuroectodermal tumor, NB, and NB Ews/Fli-1 infectant cell lines was carried out as described in "Materials and Methods." The blots were first probed with an anti-C-Myc antibody, stripped, and reprobed with an anti-actin antibody as a loading control. **C**, GeneSpring 5.0 hybridization expression graph for C-Myc from Affymetrix GeneChip data output. Normalized intensity was plotted on a logarithmic scale.

two isoforms of 55 and 69 kDa (47, 48). We have shown previously that ShcC is restricted to expression in the brain, and in particular, expression in the nervous system is restricted to neurons (34, 49, 50). Although ShcA proteins are found in the brain during embryonic development, expression appears restricted to the olfactory epithelium postnatally (48). ShcA and ShcC are differentially expressed in ES/PNETs and NBs (Fig. 6A). ES/PNETs express abundant ShcA proteins, whereas NBs show reduced expression of ShcA. Furthermore, we have detected abundant ShcC expression in NB tumors, with little expression in ES/PNETs (Fig. 6A). We also observed in the NB \times ES/PNET hybrids that ShcC expression was suppressed, providing more evidence and support for the theory that the ES/PNET phenotype is dominant to the NB with regard to Shc expression (data not shown). We examined the expression pattern of ShcA and ShcC by Western blot in the NB Ews/Fli-1 infectants to determine whether suppression or expression of these proteins corresponded to the dominance of the ES/PNET differentiation pathway in the hybrids. Fig. 6B shows that all NB neoinfectants express the p55^{ShcC} isoform but not the p69^{ShcC} isoform seen in the NB parents (Fig. 6A). In contrast, the LAN 5 and NGP9A Tr1 Ews/Fli-1 infectants do not express any of the ShcC isoforms (Fig. 6B). All NB parents stably expressing an Ews/Fli-1 construct express two of the three ShcA isoforms. The LEWS infectants express the p52^{ShcA} and the p46^{ShcA} isoform seen in the ES/PNET parents and the NB parent NGP9A Tr1. The LEWS-N and NEWS-N infectants express all three ShcA isoforms. The minor differences in Shc expression in LEWS-N and NEWS-N may be due to clonal variation. In addition, the LAN 5 and NGP9A Tr1 Ews/Fli-1

infectants do not express lower levels of the p95 catalytic inactive form of the TrkB receptor (51, 52) and the brain-derived neurotrophic factor ligand found in NB cell lines (data not shown). Table 3 summarizes the expression of NB and ES/PNET differentiation markers in the infectants. The pattern of loss of NB marker expression and gain of ES/PNET marker expression clearly follows the hybrid data in Table 2. These results establish that expression of the Ews/Fli-1 gene causes profound changes in the differentiation programs of neural crest-derived tumors.

Microarray Analysis Reveals That NB Cell Lines Stably Expressing Ews/Fli-1 Resemble ES/PNET Cell Lines. Gene microarray analysis can be a useful tool in comparing the expression of thousands of genes in a single sample specimen (25, 27, 53). We used the Affymetrix GeneChip system (26, 54, 55) to compare and contrast genetic expression profiles of the cell lines used in this study. We performed microarray analysis on NB parental cell lines, NB Ews/Fli-1 infectant cell lines, and ES/PNET cell lines. We ran each sample in duplicates and analyzed the data output using GeneSpring 5.0 analysis software. Interestingly, we discovered that hundreds of genes were regulated in NB cells after expression of Ews/Fli-1.

First, we performed a hierarchical cluster of all 12,625 genes found on the microarray chip. The hierarchical tree branched together similar expression patterns between each sample type and revealed that the infectant gene hybridization expression patterns were similar to those of the ES/PNETs (Fig. 7). Secondly, we narrowed our search to genes that were not only down-regulated in the NB Ews/Fli-1 infectants but were also expressed at low levels in ES/PNET cells when

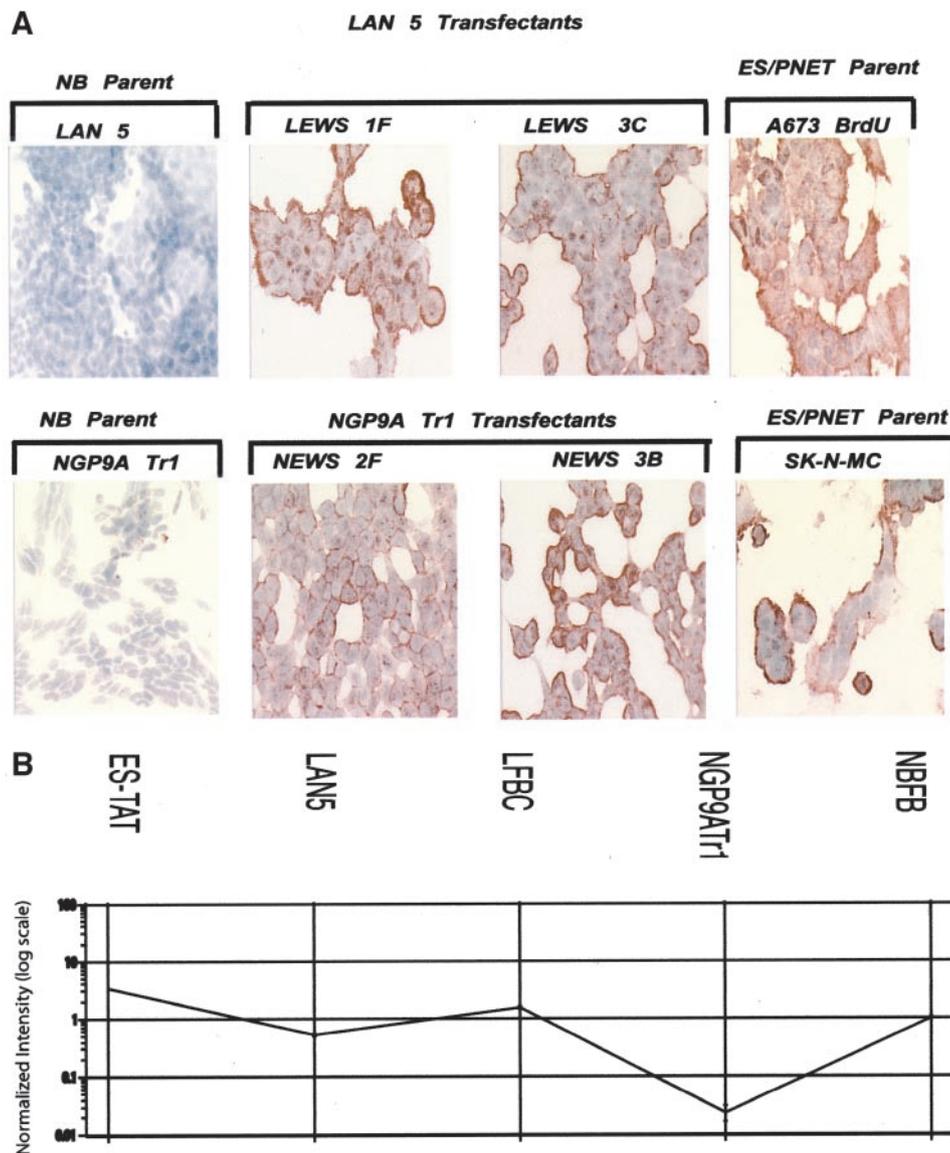


Fig. 5. MIC2 expression in neuroblastoma (NB) Ews/Fli-1 infectants. A, immunohistochemistry staining for MIC2 expression in NB parent and NB EWS/Fli-1 infectant cell lines. Cells were fixed on glass chamber slides in acetone/methanol/formalin (10:10:1). Cells were then probed with MIC2 primary antibody, washed, and probed with a biotinylated streptavidin-horseradish peroxidase-conjugated secondary antibody. The substrate was visualized by application of 3-amino-9-ethylcarbazole. The cells were then counterstained with hematoxylin and photographed at $\times 40$ magnification with a Zeiss Axioplan microscope. B, GeneSpring 5.0 hybridization expression graph for MIC2 from Affymetrix GeneChip data output. Normalized intensity was plotted on a logarithmic scale.

compared with the NB parental cell lines. We used the software to normalize the samples and filter out genes whose hybridization expression signals were higher in the NB parental cells as compared with the NB Ews/Fli-1 infectants. We also performed statistical analysis to look at gene expression comparisons that passed the Student's t test with a $P \leq 0.05$. We then filtered out genes that were at least 5-fold higher in the NB parental cell lines as compared with the NB Ews/Fli-1 infectants and the ES/PNET cells. We looked at only common genes whose hybridization signals were lower in both NB Ews/Fli-1 and ES/PNET cell lines after subsection to the same filtered conditions. We then clustered the data, and we found that 119 genes were down-regulated or had lower expression levels with a minimal hybridization expression signal of 100 and with a difference of at least 5-fold signal expression in the NB Ews/Fli-1 infectants and ES/PNET as compared with the NB parental cell lines. Minimal hybridization expression signal is a value assigned to mRNA expression after normalization and can be used as a restriction in the GeneSpring software to include only those samples that express at least the arbitrary value chosen. In Table 4, we list the 30 genes that reveal the most dramatic differences. It is evident that Ews/Fli-1 suppresses the expression of many different genes.

Lastly, we narrowed our search to genes that were not only up-

regulated in the NB Ews/Fli-1 infectant cells but were also highly expressed in ES/PNET cells when compared with the NB parental cell lines. We filtered the genes and subjected them to the same statistical analysis as mentioned previously. We looked at genes with 100 minimal hybridization expression signals and at least 5-fold higher expression signals in the NB Ews/Fli-1 infectants and ES/PNETs as compared with the NB parental cell lines. It was revealed that 95 genes were up-regulated or had higher expression levels in the NB Ews/Fli-1 infectants and ES/PNETs as compared with the NB parental cell lines. Table 5 lists 30 genes that reveal the most dramatic differences. Thus, Ews/Fli-1 expression causes global changes in gene expression by both induction and suppression of many genes.

DISCUSSION

We undertook a study to examine the control of parasympathetic and sympathetic marker expression in two pediatric cancers of neural crest origin, NB and ES/PNET. Through use of somatic cell hybrids, our study shows that several sympathetic, neural, and neuroendocrine markers present in NB are suppressed in the NB \times ES/PNET hybrids. The suppression of the mature adrenal medullary phenotype of NB when fused to ES/PNET appears similar to the observations of Weiss,

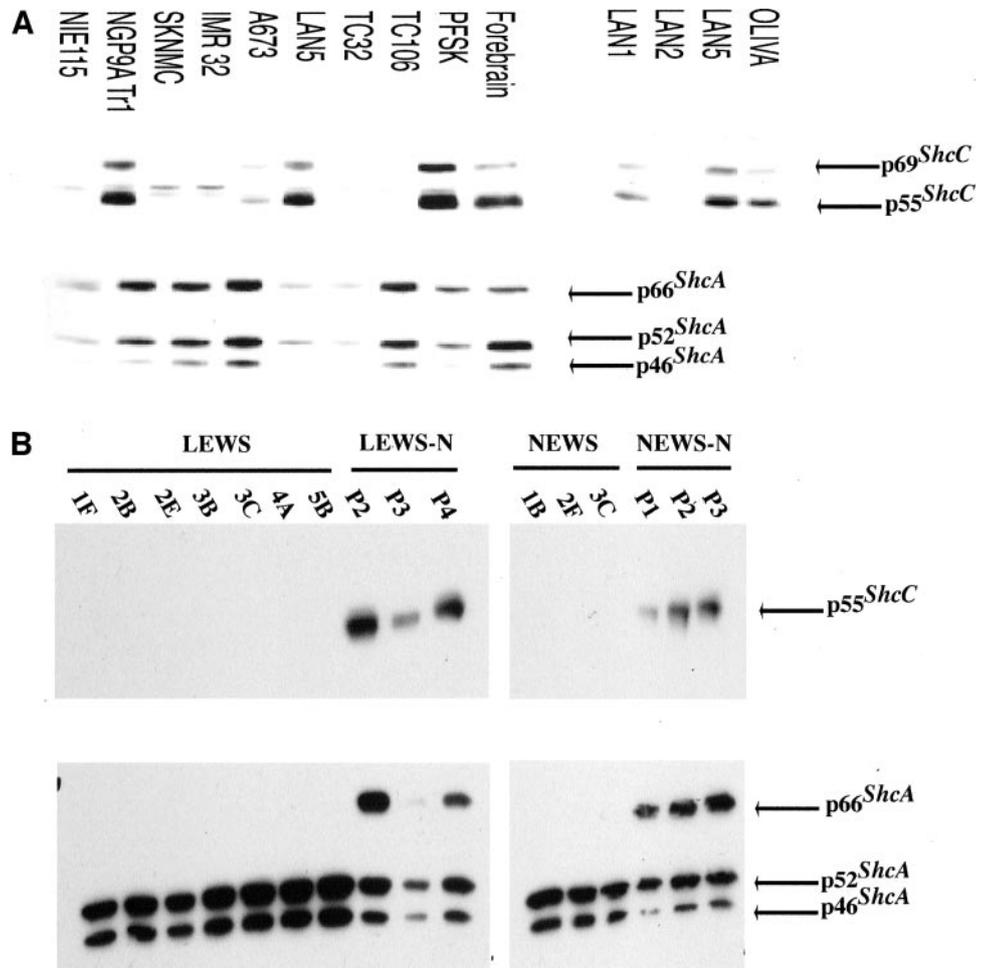


Fig. 6. Expression of ShcA, ShcC, and TrkB and brain-derived neurotrophic factor in neuroblastoma (NB) and Ewing sarcoma/peripheral primitive neuroectodermal tumor parents and NB infectants. *A*, polyclonal ShcA antibodies were used according to manufacturer's protocol. Polyclonal ShcC antibodies were isolated as described previously (34). For Shc antibodies, protein lysates were separated on 10% SDS-PAGE gels (NB and Ewing sarcoma/peripheral primitive neuroectodermal tumor parents) and 8% gels (NB cells stably expressing the Ews/Fli-1 construct). *B*, NB parents stably expressing a Ews/Fli-1 construct were run on SDS-PAGE gels, probed with the same antibodies, and visualized using enhanced chemiluminescence.

Fournier, and colleagues (56–59). If our studies follow the paradigm of these earlier investigations, the neuronal lineage of NB represents a more differentiated state than the neuronal lineage of ES/PNET. Furthermore, if these tumors recapitulate normal differentiation, our results would also imply that differentiation of the parasympathetic nervous system precedes that of sympathetic nervous differentiation from a common progenitor. However, a linear model of ES/PNET and NB differentiation is not supported by the localization of the tumors. NB tumors have a sympathetic and neuroendocrine phenotype and localize to regions in the body such as the adrenal chromaffin and the sympathetic trunk (2). Most ES/PNETs are localized to the humerus, tibia, and femur (8), which does not correspond to the origin of parasympathetic neurons in development (60). In addition, the peak incidence of ES/PNETs occurs many years after the peak incidence of

NB. Thus, the parasympathetic phenotype and other phenotypic traits associated with ES/PNET may result from activation of a dormant differentiation program by the Ews/Fli-1 gene.

Results from the NB × ES/PNET hybrids and NB Ews/Fli-1 infection studies showed that the *Ews/Fli-1* gene could account for the observed alterations in gene expression. Thus, Ews/Fli-1 regulates expression of CgA, MIC2, ShcC, and other genes at the level of transcription. Although we do not know the mechanism of its action, previous studies may provide a clue. Sp1 binding sites are present in a number of genes overexpressed in NB of adrenal chromaffin lineage but not in ES/PNET. These include CgA, neuropeptide Y, and tyrosine hydroxylase (61). In addition, Gaetano *et al.* (61) showed that CgA shares several other regulatory sequences in common with neuropeptide Y and tyrosine hydroxylase. Gaetano *et al.* (61) demonstrated that Sp1 expression increased the expression of CgA in NB cell lines of neuroendocrine lineage. Therefore, Ews/Fli-1 may suppress transcription of CgA by suppressing Sp1 activation. Jensen *et al.* (62) showed that E2F antagonizes expression of genes that have Sp1 binding sites. Thus, Ews/Fli-1 may bind to other sites to suppress Sp1-mediated CgA expression in the hybrids and infectants.

Although E2F may suppress transcription of Sp1-mediated genes, it is also capable of initiating transcription of genes in conjunction with Sp1 and other transcription factors. For example, Izumi *et al.* (63), showed that E2F, the Ets/Notch-related gene GA-binding protein, and Sp1 cooperatively promote expression of the catalytic subunit of mouse DNA polymerase α . Molecular analysis of the structure of the MIC2 gene shows that it contains Sp1 binding sites (64). Thus, a

Table 3 Expression of differentiation markers in neuroblastoma infectants

Differentiation marker	LEWS				NEWS				
	LAN5	L1F	L2B	L3C	LEWS-N	NGP	N2F	N3B	NEWS-N
CgA ^a	+ ^b	-	-	-	+	+	-	-	+
MycN	+	-	-	-	+	+	-	-	+
ShcC	+	-	-	-	+	+	-	-	+
Ews/Fli-1	-	+	+	+	-	-	+	+	-
MIC-2	-	+	+	+	-	-	+	+	-
ShcA	-	+	+	+	+	+	+	+	+
TrkB	++	+	+	+	++	++	+	+	++
BDNF	++	+	+	+	++	++	+	+	++

^a CgA, chromogranin A; BDNF, brain-derived neurotrophic factor.

^b +, positive expression in parent or infectant; ++, highly positive in parent relative to infectant; -, negative expression in parent or infectant.

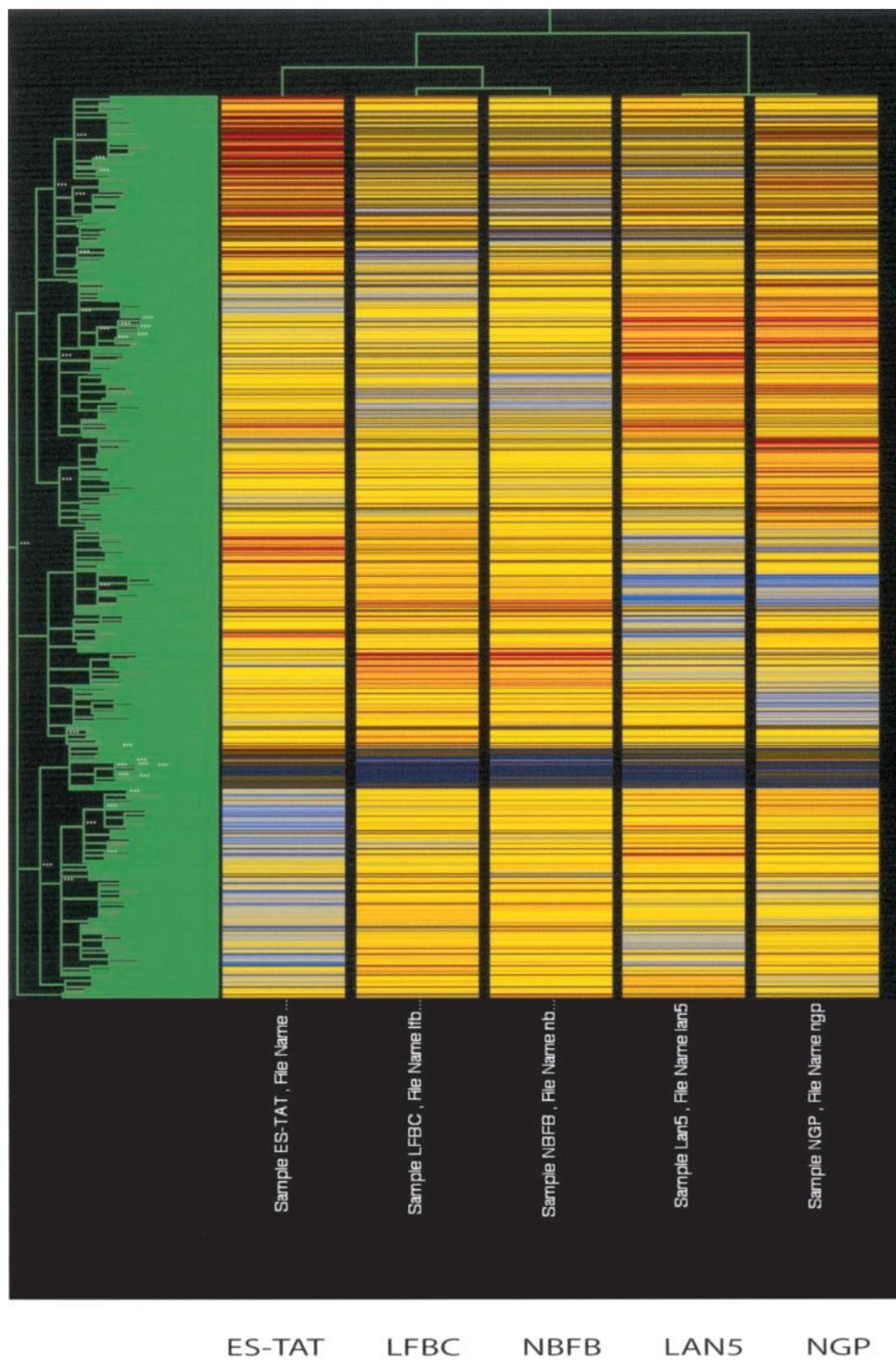


Fig. 7. Hierarchical gene tree of all 12,625 gene expression hybridization signals in Ewing sarcoma/peripheral primitive neuroectodermal tumors, neuroblastoma Ews/Fli-1 infectants, and neuroblastomas. Total RNA was extracted using RNeasy Mini Kit (Qiagen) and hybridized to Affymetrix HU95A.ver2 GeneChips in the Affymetrix Core Facility (University of North Carolina-Chapel Hill) as described in "Materials and Methods." Data from Affymetrix GeneChip Array system were analyzed using GeneSpring 5.0 software. The genes were uploaded into the software and then filtered. A hierarchical gene tree was constructed of all 12,625 genes, and similar expression patterns were branched between each sample type.

possible mechanism for MIC2 expression in the infectants may involve the Ets-like factor Ews/Fli-1, Sp1, and E2F cooperatively promoting the transcription of MIC2. Expression of MIC2 in the NB Ews/Fli-1 infectants contradicted the results of Dworzak *et al.* (65) which indicated that a correlation did not exist between Ews/Fli-1 and MIC2 expression in Ewing's tumor \times mouse intertypic hybrids. Instability of the human chromosomes in the intertypic hybrids may explain the lack of Ews/Fli-1 expression in these hybrids. In our stable human \times human hybrids, Ews/Fli-1 expression was verified by RT-PCR, and MIC2 expression was verified by immunocytochemistry. The molecular mechanisms for the different transcriptional effects of Ews/Fli-1 remain to be determined.

Expression of C-Myc in the NB \times ES/PNET hybrids appears consistent with the results of Bailly *et al.* (21) showing up-regulation of the C-Myc promoter by Ews/Fli-1. Dauphinot *et al.* (66) showed that Ews/Fli-1 up-regulated the expression of C-Myc in the NGP9A Tr1 infectants and down-regulated p57^{Kip2} expression. Thus, the mechanism by which Ews/Fli-1 alters transcription and whether it occurs in a cell cycle-dependent manner warrants further investigation because others have shown that Sp1 and E2F interact with some proteins in a cell cycle-dependent manner (62, 67, 68).

Immunocytochemical and Northern blot analyses consistently showed that NB-specific differentiation markers and neuroendocrine markers that are absent in ES/PNET are suppressed in NB \times ES/

Table 4 The 30 genes showing the greatest decrease in expression in NB^a Ews/Fli-1 infectants

Gene description/name ^b	GenBank accession no.	Average raw hybridization signals ^c				
		ES-TAT	LAN 5	LFBC	NGP	NBFB
1. MycN	Y00664	65.4	15.8K ^d	50.6	11.7K	74.1
2. AF1q	U16954	1.8K	12.4K	1.0K	18.1K	1.1K
3. Human (dlk)	U15979	173.0	7.4K	81.2	6.5K	91.9
4. sFRP	AF056087	132.0	8.2K	227.0	5.4K	456.0
5. P311 HUM	U30521	981.0	7.9K	924.0	6.8K	830.0
6. Secretogranin I (chromogranin B)	Y00064	5.1	5.2K	15.0	3.7K	16.5
7. KIAA0531	AB011103	84.7	8.9K	486.0	5.4K	864.0
8. CAGR1	U38810	9.4	7.7K	65.4	4.3K	70.2
9. GS3955	D87119	322.0	5.5K	238.0	3.9K	404.0
10. hGATA3	X58072	31.3	5.1K	149.0	3.0K	152.0
11. Dihydropyrimidinase-related protein-1	D78012	466.0	5.7K	67.6	4.4K	117.0
12. Autotaxin	L35594	137.0	5.3K	191.0	1.9K	179.0
13. Secretogranin II	M25756	211.0	5.7K	8.0	5.0K	30.8
14. RGS5	AB008109	44.9	5.8K	55.0	8.0K	52.6
15. Islet-1	U07559	562.0	5.4K	436.0	6.5K	457.0
16. GATA2	M68891	415.0	4.7K	758.0	7.2K	1.3K
17. Neurofilament-66	S78296	548.0	5.3K	415.0	6.7K	595.0
18. Peripherin (PRPH)	L14565	911.0	4.4K	451.0	6.0K	542.0
19. HUMCD24B	L33930	18.6	3.4K	63.1	3.0K	134.0
20. Aromatic amino acid decarboxylase (dde)	M76180	35.9	5.3K	50.8	5.1K	54.0
21. Doublecortin (DCX)	AJ003112	46.8	3.8K	9.0	3.2K	17.0
22. Chromogranin A (CHGA)	U03749	8.8	3.2K	5.0	5.6K	5.6
23. APCL	AB012162	246.0	2.8K	186.0	1.6K	228.0
24. Tropomodulin	M77016	21.5	3.0K	67.0	2.1K	118.0
25. KIAA0318	AB002316	125.0	2.8K	32.6	2.3K	48.6
26. Neuronal growth protein 43 (GAP-43)	M25667	48.0	3.1K	104.0	1.7K	30.4
27. Neural adhesion molecule L1	U52112	87.1	3.0K	76.0	1.5K	185.0
28. PLE21	D26158	362.0	1.9K	186.0	1.9K	206.0
29. Dopamine β -hydroxylase type a	X13255	47.4	2.4K	50.9	728.0	61.4
30. FEV	Y08976	289.0	1.8K	182.0	1.6K	169.0

^a NB, neuroblastoma.^b Genes are listed in descending order of change in the ratio of hybridization signal of NB parent cell lines versus the NB-Ews/Fli-1 infectant cell lines.^c Hybridization signals represent averages of normalized intensity duplicate microarray chips.^d K = 1 × 1000.

PNET hybrids. However, Pagani *et al.* (7) showed that expression of the neuroendocrine markers CgA and secretogranin II is detected in several ES/PNETs and cell lines by the more sensitive RT-PCR technique. Detection of CgA, secretogranin II, and other markers in ES/PNET would support current hypotheses of the neural crest origin of NB and ES/PNET. Pagani *et al.* (69) showed previously that ES/PNETs can show either mesenchymal or neuroendocrine lineage. Therefore, further investigation into the abilities of different Ets family member/Ews fusion proteins in soft-tissue sarcoma cell lines may provide invaluable information in deciphering the genes controlling parasympathetic, sympathetic, neuroendocrine, and mesenchymal differentiation of neural crest cells.

Does the Ews/Fli-1 fusion protein activate a dormant differentiation program or extinguish the neuronal differentiation program in ES/PNET? Evidence from previous reports could support either possibility. Ets family members participate in the regulation of developmental programs such as hematopoiesis and neuronal development (70, 71). Therefore, an aberrant Ets protein such as Ews/Fli-1 could certainly affect the regulation of other differentiation programs such as neuroendocrine development. On the other hand, we have shown that the Ews/Fli-1 gene can alter expression of the TrkB, ShcC, and ShcA proteins. Our results showed concomitant loss of TrkB, brain-derived neurotrophic factor, and ShcC expression in the Ews/Fli-1-expressing NB cell lines. Therefore, we are examining whether disruption of these pathways by other means in NB cell lines may mimic the effects of Ews/Fli-1 expression. It seems that Ews/Fli-1 may be changing the NB cells to a less differentiated state. This may explain why there are few reported cytogenetic changes in ES/PNETs as compared with NBs, which have many different reported chromosomal aberrations including chromosomes 1–5, 9, 11, 14, and 17 (12, 72–76). It has been reported that Ews/Fli-1 also blocks the differentiation of pluripotent marrow stromal cells, providing further evidence that it is the causative agent of the poorly differentiated state of ES/PNETs (77).

Previous reports have shown that Ews/Fli-1 can regulate a number of genes in other cell line models. Our data both dispute and support some of the observations reported (data not shown). Transforming growth factor β signaling is essential for normal development and plays an important role in proliferation, differentiation, and apoptosis (43, 78). Hahm *et al.* (42) and Athanasiou *et al.* (79) demonstrated the down-regulation of transforming growth factor β receptor II expression by the Ews/Fli-1 gene. However, we observed that transforming growth factor β receptor II expression is higher in our ES/PNET cell lines compared with the NB cell lines with increased expression in the NB Ews/Fli-1 infectant cells. Cyclin D1, an important regulator of cell cycle progression, is overexpressed in several types of neoplasia (80, 81). Whereas Matsumoto *et al.* (82) and Wai *et al.* (83) have shown that cyclin D1 is up-regulated by Ews/Fli-1, our microarray data reveal that the primary ES/PNET cell lines express cyclin D1 at levels similar to those seen in the NB parental cell lines, with decreased expression in the NB Ews/Fli-1 infectants. However, we do observe a 40–100-fold increase in cyclin D2 expression (Table 5). We believe that these differences reflect the introduction of the Ews/Fli-1 gene into a nonneural cell line such as NIH/3T3 or HeLa.

We do find agreement with the reports of Nishimori *et al.* (84) and Fukuma *et al.* (85) that Ews/Fli-1 up-regulates Id2, a basic helix-loop-helix protein that negatively regulates the myogenic regulatory transcription factor family and plays a role in apoptosis (86). We have reported previously that p57^{Kip2}, a cyclin kinase inhibitor (87), is down-regulated by Ews/Fli-1 by Western blot analysis (66). We have confirmed these results by our microarray analysis. The p57^{Kip2} is expressed at low levels in ES/PNETs and down-regulated transcriptionally as a result of Ews/Fli-1 expression in our NB cell lines. Thus, the mechanism by which Ews/Fli-1 alters transcription of these genes and the issue of whether it occurs in a cell cycle-dependent manner warrant further investigation.

Whereas existing studies have identified unique molecular and

Table 5 The 30 genes showing the greatest increase in expression in NB^a Ews/Fli-1 infectants

Gene description/name ^b	GenBank accession no.	Average raw hybridization signals ^c				
		ES-TAT	LAN 5	LFBC	NGP	NBFB
1. Sequence-specific DNA-binding protein (AP-2)	M36711	380.0	28.2	1.6K ^d	7.6	1.7K
2. Keratin 18	M26326	3.0K	4.2	761.0	4.0	1.2K
3. Glypican-4 (GPC4)	AF030186	260.0	4.2	684.0	4.1	680.0
4. Ubiquitin gene	U49869	16.8K	1.1K	18.8K	13.8	21.2K
5. MHC class I molecule (MICB)	U65416	1.4K	10.4	821.0	9.0	1.3K
6. C-Myc	V00568	1.6K	101.0	1.3K	104.0	878.0
7. Selenoprotein P	Z11793	138.0	2.7	171.0	1.6	191.0
8. Homeodomain protein DLX-2	L07919	425.0	10.4	1.2K	7.8	872.0
9. KIAK0002 (cyclin D2)	D13639	611.0	31.8	1.2K	16.8	1.6K
10. HNMP-1	U87947	4.3K	10.4	674.0	14.3	1.4K
11. Lipocortin	X05908	3.3	15.2	107.0	9.3	380.0
12. Prostate apoptosis protein (PAR4)	U63809	421.0	12.4	445.0	3.9	260.0
13. Nidogen	M30269	161.0	7.6	1.2K	15.4	865.0
14. Serin protease	D87258	126.0	13.6	317.0	9.7	560.0
15. MN1	X82209	275.0	16.8	270.0	6.7	353.0
16. RDC-1 POU domain-containing protein	X64624	1.3K	16.2	662.0	25.6	1.2K
17. Retinol-binding protein	M11433	311.0	12.2	643.0	7.6	374.0
18. RP3	U02556	1.3K	73.0	678.0	22.6	669.0
19. HIC	AF054589	276.0	8.4	314.0	6.9	257.0
20. Malic enzyme 1	AL049699	489.0	50.8	229.0	6.7	184.0
21. Decay-accelerating factor	M31516	170.0	14.8	592.0	24.0	836.0
22. Cystathionine γ -lyase	S52028	396.0	39.2	317.0	20.4	403.0
23. LHA-E	X56841	286.0	32.8	365.0	19.6	503.0
24. Gadd45 β	AF078077	152.0	20.2	381.0	32.6	772.0
25. HLA class I locus heavy chain	X58536	4.2K	147.0	4.8K	181.0	5.7K
26. Mitogen-responsive phosphoprotein DOC-2	U53446	274.0	9.1	121.0	6.5	197.0
27. Connective tissue growth factor	X78947	326.0	21.0	134.0	11.4	293.0
28. IPL	AF001294	534.0	88.0	825.0	75.0	2.1K
29. Uracil-DNA glycosylase	X52486	238.0	6.3	161.0	7.7	212.0
30. ALL1 responsive protein ARP1c (ARP1)	AF048722	111.0	5.7	228.0	8.7	238.0

^a NB, neuroblastoma.

^b Genes are listed in ascending order of change in the ratio of hybridization signal of NB parent cell lines versus the NB-Ews/Fli-1 infectant cell lines.

^c Hybridization signals represent averages of normalized intensity duplicate microarray chips.

^d K = 1×1000 .

cytogenetic markers for NB and ES/PNET, this information does not distinguish between genes essential for the expression of each tumor phenotype within this group and genes arising from remnants of the more primitive neural crest or neuroectodermal cells. Identification of the genes controlling differentiation of the sympathetic and parasympathetic lineages in neural crest-derived tumors will enable investigators to address this problem. The availability of the NB \times ES/PNET hybrids and NB/Ews/Fli-1 infectants should help identify suppressed or up-regulated genes associated with each phenotype. Novel diagnostic and prognostic factors for neural crest-derived pediatric cancers may be discovered by identifying genes that contribute to the aggressive and undifferentiated characteristics of ES/PNETs or the less aggressive and more differentiated phenotypes of some NB subtypes. Finally, increased survival of NB or ES/PNET patients may occur if the differentiation programs of these tumors could be altered to a state associated with prognostically favorable cancers (15).

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