Activation of trkA Induces Differentiation and Inhibits the Growth of JK-GMS Askin Tumor Cells

Gi-Jin Kim, Chong Jai Kim, So Young Cho, In Pyung Chung, Sun-Hwa Park, Min Jung Lee, and Je G. Chi

Department of Pathology (CJK, SYC, IPC, MJL, JGC), Seoul National University College of Medicine, and Graduate School of Biotechnology (G-JK) and Institute of Human Genetics (S-HP), Department of Anatomy, College of Medicine, Korea University, Seoul, Korea

SUMMARY: Peripheral primitive neuroectodermal tumor (PNET) and Ewing's sarcoma (ES) constitute a unique group of small round cell tumors in childhood and young adults that are characterized by the same chromosomal translocation t(11;22)(q24;q12). Recently, the expression of neurotrophin receptors has been found in various human tumors including PNET/ES, but the functional significance of these receptor expressions has not been documented in PNET/ES. In the present study, we investigated the biologic effects of trkA neurotrophin receptor activation by nerve growth factor (NGF) in a newly established Askin tumor cell line, JK-GMS, which constitutively expresses a high level of trkA. The activation of trkA induced differentiation and inhibited the growth of JK-GMS cells, which was characteristically associated with down-regulation of c-myc and N-myc mRNA expression. NGF did not exert significant changes in two different PNET/ES cell lines, CADO-ES1 and RD-ES, which did not express detectable levels of trkA. The biologic effects mediated by NGF were abrogated by treatment of the cells with K-252a, and the treatment with brain-derived neurotrophic factor did not affect the biologic behavior of JK-GMS cells, indicating that the effects are trkA specific. The results observed were quite similar to those of neuroblastoma cells, another childhood tumor of neural crest origin. Overall findings strongly suggest that the trkA-mediated signaling pathway plays a crucial role in controlling the basic biologic properties of JK-GMS cells. (*Lab Invest* 2002; 82:221-229).

Peripheral primitive neuroectodermal tumor (PNET) and Ewing's sarcoma (ES) belong to a group of major solid tumors of childhood and young adults, the so-called small round cell tumors. Although there have been debates on their histogenesis, PNET and ES are currently regarded as tumors of the same spectrum, which share a common chromosomal translocation, t(11;22)(q24;q12), and the elevated expression of CD99 (Bhagirath et al, 1995; McKeon et al, 1988; Scotlandi et al, 1996; Stephenson et al, 1992). PNET/ES cells are believed to originate from the neural crest and share histogenetic similarities with neuroblastoma, another major solid tumor in childhood (Ladanyi et al, 1990; Lipinski et al, 1987).

Neurotrophins (NTs) and their receptors play a key role in modulating the biology of both the central and peripheral nervous systems and broadly affect the basic properties of neuronal cells such as growth, differentiation, and survival (Snider, 1994). Three types of high-affinity NT receptors have been reported to date, which include the trkA, trkB, and trkC tyrosine

kinase receptors (Klein et al, 1991; Lamballe et al, 1991; Soppet et al, 1991). These receptors are expressed and believed to play a significant role in various types of human cancer (McGregor et al, 1999; Miknyoczki et al, 1999; Oelmann et al, 1995), moreover, the type of the receptor expressed is a major prognostic factor in neuroblastoma (Brodeur et al, 1997).

In the context that PNET/ES cells show both biochemical and morphologic evidence of neural differentiation (Noguera et al, 1992), it is highly likely that NTs and the receptors are of functional significance. A previous analysis of PNET/ES tumors demonstrated selective expression of trkA mRNA and protein in the cells showing neural differentiation (Nogueira et al, 1997), whereas trkB and trkC expression was found in limited numbers of undifferentiated tumor cells. In addition, the induction of c-fos, after nerve growth factor (NGF) treatment, was documented in ES cell lines (Thomson et al, 1989). These findings led us to believe that the activation of trkA may play a pivotal role in controlling the basic properties of PNET/ES cells, and for this reason we investigated the biologic consequences of trkA activation in the newly established Askin tumor cell line JK-GMS.

JK-GMS constitutively expressed high level of trkA, and the activation of trkA by NGF resulted in differentiation and growth inhibition; but NGF treatment did not significantly affect the biologic features of the other PNET/ES cell lines CADO-ES1 and RD-ES,

Received December 6, 2001.

This research was supported in part by Grant 04-1999-0720 from the Seoul National University Hospital Research Fund and in part by Grant KOHTRF-99-12 of the Korean Human Technology Research Foundation.

Address reprint requests to: Dr. Chong Jai Kim, Department of Pathology, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul, 110-799, Korea. E-mail: cjkim@plaza.snu.ac.kr

which do not express significant levels of *trkA*. The modulation of basic properties of PNET/ES cells by *trkA* activation suggested that the *trkA*-mediated signaling pathway would be a potential therapeutic target in the treatment of PNET/ES.

Results

Characteristics of Askin Tumor Cell Line JK-GMS

Both the original tumor and the tumor formed in the nude mouse showed identical histopathologic features. Both tumors were composed of compactly arranged sheets of small round cells with scant cytoplasm. The cells had indistinct nucleoli and cell borders (Fig. 1A). Ultrastructurally, the cells were poorly differentiated, monotonous, and had few cytoplasmic organelles and glycogen particles (Fig. 1B). On continuous culture, the cells were bipolar and adherent, with a doubling time of 38.4 hours. Screening of *c-myc* and *N-myc* mRNA expression showed readily detectable levels of both.

On cytogenetic analysis, the modal number of JK-GMS had a near hypodiploidy, and there were seven

marker chromosomes. The frequency of marker chromosomes was 82% to 100%. Final karyotyping was achieved by comparing the G-banding, Rx-FISH, and chromosome paintings. The representative karyotype is shown in Figure 1 (C and D) and was 42-45,XY,-4,der(4;5)(p10;q10),del(9)(p23),t(11;22)(q24;q12),der(11)t(8;11)(q13;p13),der(13)t(13;16)(q22;p13),der(16)t(16;?)(q12;?)[cp67]. The cells gained 8q13-qter and lost 4q, 5p, 9p23-pter, 11p13-pter, and 13q22-qter.

Effects of *trkA* Activation, Retinoic Acid (RA), and dibutyryl-cAMP (*dbcAMP*)

Screening of *trkA* expression in JK-GMS cells revealed a readily detectable level of *trkA* mRNA expression, which was comparable to that of the RA-treated SH-SY5Y human neuroblastoma cell line (Fig. 2A). *TrkA* was phosphorylated by NGF treatment, and a barely detectable level of phosphorylated *trkA* was even present in control cells (Fig. 2B). NGF treatment induced *c-fos* mRNA (Fig. 2C).

To analyze the biologic effects of NGF on growth and differentiation, the cells were treated with 100

Figure 1.

Morphologic characteristics of the tumor and karyotypes of JK-GMS. A, The tumor formed in a mouse is composed of compactly arranged small round cells with scanty cytoplasm (hematoxylin and eosin, $\times 200$). The cells strongly express CD99 (inset). B, Ultrastructurally, the cells have round nuclei with euchromatin and a single nucleolus. Cytoplasmic organelles are sparse, and glycogen particles are noted ($\times 6,000$). C, G-band karyotype of JK-GMS cells. Arrows indicate the breakpoints of structural aberrations. M1 = der(5)t(4;5)(p10;p13); M2 = del(9)(p21); M3 = t(11;22)(q24;q12); M4 = der(11)t(8;11)(q11;p13); M5 = der(13)t(13;16)(q22;p13); M6 = der(16)t(16;?)(p10;?); M7 = t(11;22)(q24;q12). D, Rainbow-FISH karyotype of JK-GMS cells.

Figure 2.

Expression of trkA and the receptor is activated by nerve growth factor (NGF). A, Expression of trkA mRNA in different cell lines. Ten micrograms of total RNA was subjected to electrophoresis in a 1.2% formaldehyde agarose gel and transferred to a nitrocellulose membrane. The membrane was hybridized with human trkA cDNA. The expression level in JK-GMS was similar to that of an RA-treated SH-SY5Y human neuroblastoma cell line, whereas other peripheral primitive neuroectodermal tumor and Ewing's sarcoma (PNET/ES) cell lines, CADO-ES1 and RD-ES, did not express significant levels of trkA. B, Western blot of NGF-treated cells with phosphospecific anti-trkA antibody (E6; Santa Cruz). A low level of phosphorylated trkA is expressed even in the absence of NGF treatment, which suggests the presence of an autocrine/paracrine loop of NGF and trkA. The expression level of phosphorylated trkA is also comparable to that of RA-treated SH-SY5Y neuroblastoma cells. C, NGF treatment induces c-fos demonstrable on RT-PCR in JK-GMS cells.

ng/ml of NGF, 5 μ M RA, and 100 nM dbcAMP. The cells underwent neuronal differentiation characterized by the extension of long neurites and the up-regulation of neuronal markers such as bcl-2 and 200-kD neurofilament, and the number of viable cells decreased (Fig. 3A). The degree of growth inhibition and differentiation was inversely related to serum concentration, in the order RA, NGF, and dbcAMP (Fig. 3B). Differences between cellular phenotypes were evident

after 3 days of treatment. The decreases in growth and in [3 H]thymidine uptake were correlated (Fig. 3, C and D). However, the cells were not G1 arrested on cell cycle analysis (data not shown).

Because the cells expressed both c-myc and N-myc mRNA, we tested whether their expression is regulated by differentiation inducers. N-myc and c-myc mRNA expression was down-regulated after NGF, RA, and dbcAMP treatment (Fig. 4A). N-myc and

Figure 3.

Effects of NGF and other biologic response modifiers on JK-GMS cells. A, NGF treatment at a concentration of 100 ng/ml for 5 days induces differentiation of the cells, characterized by the extension of neurites. This differentiation is associated with increased expression of neuronal markers such as bcl-2 and 200-kd neurofilament. Thirty micrograms of proteins was subjected to electrophoresis and immunoblotted with corresponding antibodies. B and C, The fraction of the neurite-positive cells and the number of viable cells after treatment with NGF, RA, and dbcAMP for 5 days are represented. Differentiation and growth inhibition is most prominent after treatment with RA. D, Decrease in [3 H]thymidine uptake shows correlation with growth inhibition. The values of the number of viable cells and [3 H]thymidine uptake are quoted as a percentage of control.

Figure 3.
Continued.

c-myc down-regulations were most prominent after RA treatment, and the decreases were comparable to 19% and 25% of control, respectively. The changes in the expression of EWS-Fli-1 fusion protein, which plays a key role in the tumorigenesis of PNET/ES, were also assessed, but its level did not change after NGF, RA, and dbcAMP treatment (Fig. 4B).

To address whether the effects mediated by NGF are trkA specific, the growth and neuritogenesis of JK-GMS cells were also evaluated after treatment with K-252a, an inhibitor of trkA, and brain-derived neurotrophic factor (BDNF). The treatment with K-252a abrogated the effects of NGF, and BDNF did not significantly alter the biologic properties of the cells (Fig. 5).

Discussion

Although there has been debate on the histogenesis of PNET/ES cells, the current understanding of its histogenesis is that PNET/ES are tumors of neural crest origin, which show both structural and biochemical evidence of primitive neural differentiation (Ambros et al, 1991; Cavazzana et al, 1987, 1992; Llombart-

Figure 4.

Regulation of N-myc, c-myc, and EWS-Fli-1 in JK-GMS cells. A, Concomitant down-regulation of N-myc and c-myc in JK-GMS cells after treatment with NGF, RA, and dbcAMP. The cells have been treated for 5 days with NGF (100 ng/ml), RA (5 μ M), and dbcAMP (100 nM). B, Immunoblotting for EWS-Fli-1 fusion protein with anti-Fli-1 antibody. The protein levels of 68-kD molecular weight EWS-Fli-1 are not significantly altered after the treatments with NGF, RA, and dbcAMP.

Bosch et al, 1996). Askin tumor is a small round cell tumor arising in the thoracopulmonary region, which belongs to the PNET/ES family of tumors (Askin et al, 1979; Linnoila et al, 1986). The primary tumor and the tumor formed in a mouse were histologically identical, with the same karyotype and characteristic chromosomal translocation t(11;22)(q24;q12), and both strongly expressed CD99. Thus the JK-GMS cell line is a relevant model for studying the biologic characteristics of PNET/ES.

NTs and their receptors play a pivotal role in the development of both the central and the peripheral nervous system. TrkA mediates critical effects during human nervous system development, including that of the neural crest (Snider, 1994). In the present study, we demonstrated that the activation of trkA significantly affects the biology of neural crest-derived PNET/ES cells. TrkA activation inhibited growth and induced neuronal differentiation. The effects of trkA activation were extensively studied in another neural crest-derived childhood tumor neuroblastoma because the expression level of trkA is an important prognostic factor in this tumor (Kim et al, 1999; Matsushima and Bogenmann, 1993; Poluha et al, 1995). The tumors expressing high levels of trkA showed good prognosis, whereas those expressing low level of trkA with frequent amplification of N-myc showed poor prognosis (Nakagawara et al, 1993). Multiple defects in the trkA-mediated signaling pathway have been implicated as one of the major mechanisms of tumorigenesis and progression, and trans-

Figure 5.

Assessment of trkA specificity of NGF-mediated effects in JK-GMS cells. A, Significant morphologic changes are not found after NGF treatment in the presence of 100 nM K-252a, and BDNF treatment also does not induce morphologic changes of the cells. B, The cells were treated with NGF at a concentration of 100 ng/ml for 5 days in the presence of 100 nM K-252a and with BDNF at a concentration of 100 ng/ml for 5 days. The number of viable cells and percentage of neurite-positive cells are quoted as a percentage of control.

fection of trkA cDNA is usually associated with the inhibition of growth, the induction of differentiation, and the down-regulation of N-myc in neuroblastoma cells (Azar et al, 1990; Matsushima and Bogenmann, 1993). The biologic effects of trkA activation in JK-GMS cells were quite similar to those of neuroblastoma cells. Besides trkA activation, RA and dbcAMP also induced the neuronal differentiation of JK-GMS cells, showing that JK-GMS cells have the potential for differentiation by various stimuli. The effects were exaggerated by decreasing serum concentration as reported in other PNET/ES cell lines (Noguera et al, 1992), and neuritogenesis was associated with an increased expression of neuronal markers such as bcl-2 and 200-kd neurofilament.

The proto-oncogene expression profiles of PNET/ES and neuroblastoma are different. N-myc expression is dominating in neuroblastomas, and N-myc is believed to play a major role in determining

the biologic characteristics of neuroblastoma (Chan et al, 1997; Rosen et al, 1986). The comparison of proto-oncogene expression in PNET and ES revealed that both c-myc and N-myc are expressed, although c-myc predominates (McKeon et al, 1988). N-myc expression dramatically decreases during neuroblastoma cell differentiation by various differentiation inducers, especially RA (Thiele et al, 1985). Because JK-GMS expressed both N-myc and c-myc, we tested whether their expressions are regulated during differentiation. It is interesting that the expressions of N-myc and c-myc were down-regulated in a similar fashion by NGF and RA treatment, whereas the levels were minimally altered by dbcAMP. The down-regulation of N-myc and c-myc expressions paralleled the degree of growth inhibition and differentiation. Considering the role of myc oncogenes in human cancer, the results seem to be relevant in terms of its effects on growth and differentiation (Nesbit et al, 1999). EWS-Fli-1 fusion protein is the principal factor involved in the tumorigenesis of PNET/ES, and its expression is known to be down-regulated in growth-arrested ES cells (Wang et al, 1999). However, the finding that none of the reagents tested in our study altered the protein level of EWS-Fli-1 raises the possibility that the down-regulation of EWS-Fli-1 characterizes only growth-arrested PNET/ES cells.

Because JK-GMS cells did not express detectable levels of p75 LNGFR on Northern blot analysis (data not shown), the biologic effects after NGF treatment seem to be the consequences of trkA activation by NGF. Furthermore the results of the treatment of the cells with K-252a and BDNF clearly indicated that the biologic effects of NGF on JK-GMS cells are trkA specific. Moreover NGF did not induce significant changes in RD-ES and CADO-ES1 cells, which did not express readily detectable levels of trkA but express p75 LNGFR. All these findings suggest that trkA can potentially play a significant role in determining the biologic characteristics of PNET/ES cells. JK-GMS cells and the other two PNET/ES cell lines, RD-ES and CADO-ES1, expressed NGF mRNA on RT-PCR analysis (data not shown). This raised the possibility that NGF and trkA can establish an autocrine or paracrine loop in PNET/ES, and the significance of this autocrine/paracrine loop should be further investigated.

The newly established cell line JK-GMS should provide a valuable experimental system for the investigation of trkA-mediated effects and the biologic properties of PNET/ES. In the present study, we report for the first time the *in vitro* evidence that activation of trkA can induce favorable biologic phenotype in terms of the oncologic aspects. The expression level of trkA correlated with the biologic changes of cells caused by NGF treatment. The biologic changes after NGF treatment such as growth inhibition and differentiation strongly resembled those of neuroblastoma cells, which suggests that trkA is a key molecule that determines the biologic characteristics of neural crest-derived pediatric tumors.

Materials and Methods

Establishment of Cell Line JK-GMS

A 15-year-old Korean boy died of disseminated Askin tumor despite intensive chemotherapy. The autopsy was performed an hour after death. Tumor tissue was taken from a daughter nodule of a right chest wall mass and minced finely. The cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (GIBCO, Grand Island, New York) and antibiotics. Aliquots of 10^7 cells were injected into the backs of three nude mice subcutaneously, and the mice were kept in a laminar flow cabinet under specific pathogen-free conditions. Tumefaction was found in one mouse, and subsequent culture was performed.

Chromosome Preparation and Karyotype Analysis

The chromosome analysis was performed according to standard methods (Park et al, 1996).

Rainbow-FISH Analysis

The probe (Rx-FISH kit; Applied Imaging, Santa Clara, California) was a mixture of PCR-amplified DNA obtained from flow-sorted Gibbon chromosomes that had been differentially labeled with FITC, Cy3, or Cy5. Slides were denatured in 70% formamide/2× SSC (pH 7.5) at 72° C for 1.5 minutes, quenched in ice-cold 70% ethanol, dehydrated in an ethanol series, and air-dried. The Rx-FISH probe was denatured at 65° C for 10 minutes and placed onto the denatured slides, which were then sealed under coverslips with rubber cement. Hybridization was done overnight at 37° C in a humidified chamber. The next day, posthybridization washing was performed, and counterstaining was done with DAPI II (Vysis, Inc., Downers Grove, Illinois). Image acquisition was performed with a Rx-FISH CytoVision System (Applied Imaging).

FISH with Chromosome Painting Probes

Chromosome-painting experiments were performed on the basis of the G-banding and Rx-FISH results. The human chromosome-specific painting probes were constructed from somatic hybrid mapping panel DNAs (Coriell Cell Repositories, Camden, New Jersey). Human chromosome-specific DNAs were amplified with CL1 primer (5'-TCCCAAAGTGCTGGGATTACAG-3') and CL2 primer (5'-CTGCACTCCAGCCTGGG-3') (Nelson et al, 1989). To prepare painting probes, PCR products were labeled with biotin-dUTP by nick translation.

Briefly, slides were denatured in 70% formamide/2× SSC (pH 7.0) for 2 minutes at 72° C and dipped in a cold ethanol series for 2 minutes each. Probes were denatured at 75° C for 5 minutes, preannealed at 37° C, and applied to the slides. After overnight hybridization, the slides were washed for 10 minutes with 50% formamide/2× SSC (pH 7.0) at 42° C and placed for 5 minutes in 4× SSC, 0.1% Nonidet P-40 (NP-40). Hybridized probes were detected with FITC avidin. Chromosomes were counter-

stained with propidium iodide and DAPI and analyzed by CytoVision.

Northern Blot Analysis

Total cellular RNA was extracted using an RNeasy kit (Qiagen Inc., Santa Clarita, California), and 10 µg of RNA was subjected to electrophoresis in 1.2% formaldehyde agarose gel and was subsequently transferred to a nitrocellulose membrane. The membrane was hybridized overnight at 42° C with [α - 32 P]dCTP-labeled purified inserts of human trkA cDNA, N-myc cDNA, and c-myc cDNA in a solution containing 50% formamide, 1 M NaCl, 1% SDS, 10% dextran sulfate, and 100 µg/ml of salmon sperm DNA. The membrane was washed two times with 2× SSC, 1.0% SDS solution at room temperature for 15 minutes, and additional washing was performed with 0.1× SSC, 1% SDS for 5 minutes at 55° C. Membranes were exposed to Kodak XAR film at -70° C with an intensifying screen.

Assessment of TrkA-Mediated Signaling

For the analysis of trkA autophosphorylation, the cells were grown in 100-mm dishes for 3 days and stimulated with 2.5s mouse NGF (Upstate Biotechnology, Lake Placid, New York) for 5 minutes. They were harvested in tris-buffered saline and lysed with NP-40 lysis buffer (1% NP-40, 10% glycerol, 1 mM PMSF, 10 µg/ml of aprotinin, 1 µg/ml of leupeptin, and 500 µM of sodium orthovanadate in tris-buffered saline). Fifty micrograms of protein from each lysate were subjected to electrophoresis in 8% SDS-polyacrylamide gel, electrotransferred, and subsequently probed with murine monoclonal anti-trkA antibody (E6; Santa Cruz Biotechnology, Santa Cruz, California), which specifically detects the phosphorylated form of trkA. Signals were detected using enhanced chemiluminescence reagents (Amersham Life Science Inc., Arlington Heights, Illinois). Induction of c-fos mRNA after NGF treatment and the expression of p75 LNGFR and NGF were also assessed by RT-PCR according to previously described methods (Furukawa et al, 1997; Labouyrie et al, 1999).

Morphologic Observation and Viable Cell Counting

To assess the change in the number of cells after NGF treatment, 2×10^5 cells were split in a 6-well plate, and 100 ng/ml of NGF was applied on the next day (day 0). The effects of serum concentration on the differentiation were first analyzed, and these experiments were done in RPMI 1640 supplemented with 1% fetal bovine serum. Morphologic changes were monitored throughout the experiment, cells were harvested on day 5, and viable cells were counted. To compare the effects of RA and dbcAMP (Sigma-Aldrich Corporation, St. Louis, Missouri) with those of NGF, the cells were also treated with 5 µM *all-trans* RA (Sigma-Aldrich Corporation) and 0.1 mM of dbcAMP for 5 days. Neurites were defined as a cytoplasmic elongation exceeding two times the cell body diameter, and

the fraction of the neurite-positive cells was calculated. Cell counting was performed after trypan blue staining. To assess the trkA specificity of the biologic effects that followed NGF treatment, the NGF experiments were repeated in the presence of 100 nM of K-252a (Kamiya Biochemicals, Thousand Oaks, California), which was applied 1 hour before NGF treatment. The cells were also treated with 100 ng/ml of BDNF, and its effects were analyzed.

Immunoblotting

The changes in the levels of neuronal markers and EWS-Fli-1 protein were measured by immunoblotting of 30 μ g of total protein lysates obtained from their respective experiments. The proteins were probed with rabbit polyclonal antibody to bcl-2 (N-19; Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-neurofilament antibody (2F11; NeoMarkers, Fremont, California), and rabbit polyclonal anti-Fli-1 antibody (C-19; Santa Cruz Biotechnology, Inc.). The antibody to neurofilament used recognizes the phosphorylated form of the 200-kd and 68-kd subunits of neurofilaments.

[³H]dthymidine Uptake Analysis

In a 96-well plate, 5×10^3 cells were split in quadruplicate. On the next day (day 0), NGF was added to the individual wells at a concentration of 100 ng/ml. Cells were cultured for 3 days (day 3) and 1 μ Ci of [³H]dthymidine (ICN, Costa Mesa, California) was applied to the individual well. Cells were harvested 20 hours after the [³H]dthymidine application, and radioactivity was measured using a liquid scintillation counter. Every experiment was repeated at least three times.

References

- Ambros IM, Ambros PF, Strehl S, Kovar H, Gadner H, Salzer-Kuntschik M (1991). MIC2 is a specific marker for Ewing's sarcoma and peripheral primitive neuroectodermal tumors: evidence for a common histogenesis of Ewing's sarcoma and peripheral primitive neuroectodermal tumors from MIC2 expression and specific chromosome aberration. *Cancer* 67:1886-1893.
- Askin FB, Rosai J, Sibley RK, Dehner LP, McCalister WH (1979). Malignant small round cell tumor of the thoracopulmonary region in childhood: a distinctive clinicopathologic entity of uncertain histogenesis. *Cancer* 43:2438-2451.
- Azar CG, Scavarda NJ, Reynolds CP, Brodeur GM (1990). Multiple defects of the nerve growth factor receptor in human neuroblastomas. *Cell Growth Differ* 1:418-421.
- Bhagirath T, Abe S, Nojima T, Yoshida MC (1995). Molecular analysis of a t(11;22) translocation junction in a case of Ewing's sarcoma. *Genes Chromosomes Cancer* 13:126-132.
- Brodeur GM, Nakagawara A, Yamashiro DJ, Ikegaki N, Liu XG, Azar CG, Lee CP, Evans AE (1997). Expression of trkA, trkB and trkC in human neuroblastomas. *J Neurooncol* 31:49-55.
- Cavazzana AO, Miser JS, Jefferson J, Triche TJ (1987). Experimental evidence for a neural origin of Ewing's sarcoma of bone. *Am J Pathol* 127:507-518.
- Cavazzana AO, Ninfo V, Roberts V, Triche TJ (1992). Peripheral neuroepithelioma: a light microscopic, immunocytochemical, and ultrastructural study. *Mod Pathol* 5:71-78.
- Chan HSL, Gallie BL, DeBoer G, Haddad G, Ikegaki N, Dimitroulakos J, Yeger H, Ling V (1997). MYCN protein expression as a predictor of neuroblastoma prognosis. *Clin Cancer Res* 3:1699-1706.
- Furukawa K, Estus S, Fu W, Mark RJ, Mattson MP (1997). Neuroprotective action of cycloheximide involves induction of bcl-2 and antioxidant pathways. *J Cell Biol* 136:1137-1149.
- Kim CJ, Matsuo T, Lee K-H, Thiele CJ (1999). Up-regulation of insulin-like growth factor-II expression is a feature of trkA but not trkB activation in SH-SY5Y neuroblastoma cells. *Am J Pathol* 155:1661-1670.
- Klein R, Jing S, Nanduri V, O'Rourke E, Barbacid M (1991). The trk proto-oncogene encodes a receptor for nerve growth factor. *Cell* 65:189-197.
- Labouyrie E, Dubus P, Groppi A, Mahon FX, Ferrer J, Parrens M, Reiffers J, de Mascarel A, Merlio JP (1999). Expression of neurotrophins and their receptors in human bone marrow. *Am J Pathol* 154:405-415.
- Ladanyi M, Heinemann FS, Huvos AG, Rao HP (1990). Neural differentiation in small round cell tumors of bone and soft tissue with the translocation t(11;22)(q24;q12). *Hum Pathol* 21:1245-1251.
- Lamballe F, Klein R, Barbacid M (1991). TrkC, a new member of the trk family of tyrosine kinases, is a receptor for neurotrophin-3. *Cell* 66:967-979.
- Linnoila RI, Tsokos M, Triche TJ, Marangos PJ, Chandra RS (1986). Evidence for neural origin and PAS-positive variants of the malignant small cell tumor of thoracopulmonary region ("Askin tumor"). *Am J Surg Pathol* 10:124-133.
- Lipinski M, Braham K, Philip I, Wiels J, Philip T (1987). Neuroectoderm-associated antigens of Ewing's sarcoma cell lines. *Cancer Res* 47:183-137.
- Llombart-Bosch A, Contesso G, Peydro-Olaya A (1996). Histology, immuno-histochemistry, and electron microscopy of small round cell tumors of bone. *Semin Diagn Pathol* 13:153-170.
- Matsushima H, Bogenmann E (1993). Expression of trkA cDNA in neuroblastomas mediates differentiation in vitro and in vivo. *Mol Cell Biol* 13:7447-7456.
- McGregor LM, McCune BK, Graff JR, McDowell PR, Romans KE, Yancopoulos GD, Ball DW, Baylin SB, Nelkin BD (1999). Roles of trk family neurotrophin receptors in medullary thyroid carcinoma development and progression. *Proc Natl Acad Sci USA* 96:4540-4545.
- McKeon C, Thiele CJ, Ross RA, Kwan M, Triche TJ, Miser JS, Israel MA (1988). Indistinguishable patterns of protooncogene expression in two distinct but closely related tumors: Ewing's sarcoma and neuroepithelioma. *Cancer Res* 48:4307-4311.
- Miknyoczki SJ, Land D, Huang L, Klein-Szanto AJ, Dionne CA, Ruggeri BA (1999). Neurotrophins and trk receptors in human pancreatic ductal adenocarcinoma: expression patterns and effects on in vitro invasive behavior. *Int J Cancer* 81:413-427.

- Nakagawara A, Arima-Nakagawara M, Scavarda NJ, Azar CG, Cantor AB, Brodeur GM (1993). Association of high levels of expression of TRK gene and favorable outcome in neuroblastoma. *N Engl J Med* 52:1364–1368.
- Nelson DL, Ledbetter SA, Corbo L, Victoria MF, Ramirez-Solis R, Webster TD, Ledbetter DH, Caskey CT (1989). Alu polymerase chain reaction: a method for rapid isolation of human-specific sequences from complex DNA sources. *Proc Natl Acad Sci USA* 86:6686–6690.
- Nesbit CE, Tersak JM, Prochownik EV (1999). MYC oncogenes and human neoplastic disease. *Oncogene* 18:3004–3016.
- Nogueira E, Navarro S, Pellín A, Llombart-Bosch A (1997). Activation of TRK genes in Ewing's sarcoma: TrkA receptor linked to neural differentiation. *Diagn Mol Pathol* 6:10–16.
- Noguera R, Triche TJ, Navarro S, Tsokos M, Llombart-Bosch A (1992). Dynamic model of differentiation in Ewing's sarcoma cells: Comparative analysis of morphologic, immunocytochemical, and oncogene expression parameters. *Lab Invest* 62:143–151.
- Oelmann E, Sreter L, Schuller I, Serve H, Koenigsmann M, Wiedenmann B, Oberberg D, Reufi B, Thiel E, Berdel WE (1995). Nerve growth factor stimulates clonal growth of human lung cancer cell lines and a human glioblastoma cell line expressing high-affinity nerve growth factor binding sites involving tyrosine kinase signaling. *Cancer Res* 55:2212–2219.
- Park SH, Kim H, Kim HS, Chun YH (1996). Construction of band-specific painting probes from a single microdissected chromosome. *Mol Cells* 6:51–55.
- Poluha W, Poluha DK, Ross AH (1995). TrkA neurogenic receptor regulates differentiation of neuroblastoma cells. *Oncogene* 10:185–189.
- Rosen N, Reynolds CP, Thiele CJ, Israel MA (1986). Increased N-myc expression following progressive growth of human neuroblastoma. *Cancer Res* 46:4139–4142.
- Scotlandi K, Serra M, Manara MC, Benini S, Sarti M, Maurici D, Lollini PL, Picci P, Bertoni F, Baldini N (1996). Immunostaining of the p30/32^{MIC2} antigen and molecular detection of EWS rearrangement for the diagnosis of Ewing's sarcoma and peripheral neuroectodermal tumor. *Hum Pathol* 27:408–416.
- Snider WD (1994). Functions of the neurotrophins during nervous system development: What the knockouts are teaching us. *Cell* 77:627–638.
- Soppet D, Escandon E, Maragos J, Middlemas DS, Reid SW, Blair J, Burton LE, Stanton BR, Kaplan DR, Hunter T (1991). The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptors. *Cell* 65:895–903.
- Stephenson CF, Bridge JA, Sandberg AA (1992). Cytogenetic and pathologic aspects of Ewing's sarcoma and neuroectodermal tumors. *Hum Pathol* 23:1270–1277.
- Thiele CJ, Reynolds CP, Israel MA (1985). Decreased expression of N-myc precedes retinoic acid-induced morphological differentiation of human neuroblastoma. *Nature* 313:404–406.
- Thomson TM, Pellicer A, Greene LA (1989). Functional receptors for nerve growth factors on Ewing's sarcoma and Wilms' tumor cells. *J Cell Physiol* 141:60–64.
- Wang M, Xie Y, Girnita L, Nilsson G, Dricu A, Wejde J, Larsson O (1999). Regulatory role of mevalonate and N-linked glycosylation in proliferation and expression of the EWS-Fli-1 fusion protein in Ewing's sarcoma cells. *Exp Cell Res* 246:38–46.