

Identification of the Neurotrophin Receptors p75 and trk in a Series of Wilms' Tumors

Michael J. Donovan,* B. Hempstead,^{†‡}
L. Julie Huber,[‡] D. Kaplan,[§] Pantelis Tsoulfas,[§]
M. Chao,[‡] Luis Parada,[§] and
Deborah Schofield*

From the Department of Pathology,* Children's Hospital, Boston, Massachusetts; Departments of Hematology/Oncology[†] and Cell Biology,[‡] Cornell University Medical Center, New York, New York; and Molecular Embryology Section,[§] NCI-Frederick Cancer Research and Development Center, ABL-Basic Research Program, Frederick, Maryland

The molecular mechanisms underlying the pathogenesis of Wilms' tumor (WT) are poorly understood, although a variety of growth factors including platelet-derived growth factor and insulin-like growth factor are expressed and are thought to contribute to tumor development. In earlier studies, WT cells in culture were found to express the low affinity nerve growth factor receptor, p75. These WT cells were capable of responding to the neurotrophin (NT) NGF, suggesting that NT may be involved in WT pathogenesis. We have examined a group of WT immunohistochemically with antibodies recognizing known trk receptor proteins, the p75 receptor, and the NTs, NGF and NT-3. Confirmatory immunoprecipitation and Western blots were then performed on representative WT samples from the study group. The p75 receptor was found predominantly in the epithelial and blastemal components where high levels of NT were also identified. The trk A and B receptors were primarily within stromal components, whereas the trk C and C' receptors were present within epithelial structures. Western blot analyses confirmed the presence of the respective receptor proteins with variations correlating in some cases with histological type. The selective presence of NT receptors and growth factors in this series of WT implies autocrine/paracrine mechanisms for tumor development. (Am J Pathol 1994, 145:792-801)

There is convincing evidence for the implication of growth factors as regulators of cell growth and dif-

ferentiation in specific tumors. A variety of peptide growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factors (TGF- α and - β) and insulin-like growth factors (IGF-1 and -2) are reportedly mitogenic *in vitro*^{1,2} and under some conditions promote expression of phenotypic and molecular features of transformed cells.³

A tumor that has been investigated rather extensively in this regard is Wilms' tumor (WT), a pediatric kidney neoplasm that arises from multipotential stem cells of the metanephric blastema.⁴ Because WT recapitulates some aspects of normal kidney development, the expression of a number of fetal growth factors has been examined, specifically PDGF and IGF.^{5,6} Early studies have identified the expression of both PDGF and IGF-2 transcripts in a small series of WT and implicated these fetal growth factors in the etiology of this tumor. More recently, experiments with WT tissue samples reported increased expression of IGF-2 mRNA, increased IGF-2 protein, and increased IGF-1 receptor binding activity.^{7,8} It has been suggested that IGF production may at least be partially responsible for increased proliferation and inhibition of terminal differentiation of this neoplasm. In addition, a role of the WT-1 gene product in the development of these tumors has been postulated given the proteins ability to regulate the expression of the PDGF α -chain and IGF-2 genes.⁹

We would like to suggest that an additional set of growth factors, the neurotrophins (NT) and their receptors, are also involved in the pathogenesis of some WT. We are aware of only one published study that identified the low affinity nerve growth factor (LANGF) receptor, p75, on epithelial WT cells in culture.¹⁰ The authors demonstrated that these cells re-

BLH was supported by grants from the National Institutes of Health, ACS, and the March of Dimes. MVC was supported by grants from the National Institutes of Health, Zenith Award from Alzheimer's Association, and the Dorothy Radbell Cohen Foundation. DK, PL, and LP are supported by the National Institutes of Health.

Accepted for publication July 7, 1994.

Address reprint requests to Dr. Michael J. Donovan, Boston Children's Hospital, Department of Pathology, 300 Longwood Avenue, Boston, MA 02115.

sponded to the NT NGF with an induction in *c-fos* RNA expression. These results, although limited, suggest that WT cells express one class of intact NT receptors capable of responding to a specific ligand. In addition, recent studies have implicated the p75 receptor in normal recent kidney development¹¹ and others have found the p75 receptor in mesangial cells of the developing human kidney.^{12,13} The function of this receptor during the development of the kidney is still elusive. The status of another set of receptors, the high affinity NT receptors or trk receptor tyrosine kinases, on WT cells is currently unknown, however, the trk receptors have been identified at distinct sites during the development of the rodent kidney.¹⁴ Their function in kidney development is unknown.

Members of the trk tyrosine kinase family have recently been identified as functional receptors of the NGF family of NTs. The interactions between NTs and their respective receptors stimulates receptor tyrosine kinase activity and elicits biological responses including differentiation and proliferation, depending on the cellular environment in which the receptor is expressed.¹⁵ Recent evidence has suggested that trk A expression is required for differentiation in some pediatric small round blue cell tumors, such as neuroblastoma.^{16,17} Additional studies have shown high levels of trk protein in terminally differentiated ganglion cells of neuroblastomas and in differentiating rhabdomyoblasts in rhabdomyosarcoma.¹⁸ These results expand the known association between NTs, trk receptors, and differentiation.

The trk gene family encodes three receptor tyrosine kinases, trk A, trk B, and trk C, which selectively interact with the NTs, NGF, BDNF, and NT-3, respectively.¹⁵ In addition to the full-length trk A, B, or C transcripts, each trk gene is subject to alternative splicing events, leading to the generation of numerous isoforms for each trk species. The functional significance of these splice variants with regards to cell signaling is not completely clear. The truncated forms of trk B and trk C have been postulated to inhibit signaling when co-expressed with full-length trk B and trk C receptors.^{15,19} Whether this inhibition of NT signaling reflects a sequestration of ligand by the truncated receptors, or direct inhibition of dimerization and autophosphorylation of full-length trk isoforms is not clear. The trk C gene also encodes additional isoforms that contain 14 or 25 amino acid inserts within the tyrosine kinase domain. The isoforms with kinase inserts display an attenuated tyrosine phosphorylation response to ligand and fail to promote cell differentiation when expressed in a neural crest cell line capable of neuronal differentiation. This system represents the first receptor tyrosine kinase system ex-

pressing variable isoforms that retain both ligand binding and ligand-inducible kinase activation, yet differ in biological capabilities.²⁰

The developmental expression of trk B and trk C is significantly more widespread than that described for trk A. Although most investigations of trk B and trk C expression have focused on the central and peripheral nervous systems, it is now known that all the trks are expressed in extraneural tissues, including the mouse and rat kidney.^{15,19} Based on these studies and by analogy with results derived from neuronal cell lines, the interactions between the NTs and their cognate receptors stimulate receptor tyrosine kinase activity thereby eliciting different biological responses for survival and differentiation.^{20,21} However, the specific roles of the NT receptors in the development of nonneuronal organs is not yet known.

Using antibodies that recognize the NT receptors p75 and trk, including trk A, B, and C, we assessed the expression of these receptors in a series of WTs using immunohistochemistry and Western blot analyses. We also evaluated NT expression in these same WT samples. The localization and involvement of these factors in the pathogenesis of WT is discussed.

Materials and Methods

Ten cases of WT diagnosed at the Children's Hospital of Boston between 1980 and 1993 were identified. After examination of a large series of cases, we selected a cross-sectional study group that consisted of six females and four males, ranging in age from 11 weeks to 8 years of age. There were three stage I, five stage II, and two stage IV categorized as follows:⁴ two anaplastic, two blastemal predominant, two epithelial predominant, two classic triphasic, and two rhabdomyoblastic. After review of the hematoxylin and eosin (H&E)-stained slides on the study group of cases, a representative block was selected for analysis and 4 μ -thick sections were cut. All tissues were processed as previously described using an avidin-biotin-based alkaline phosphatase detection kit (BioGenex Labs, San Ramon, CA).¹⁸ Each of the antibodies were individually titrated first with selective cell lines overexpressing the particular receptor protein.¹⁸ These antibody results served as negative and positive controls and for optimal use on paraffin sections with dilutions ranging from 1:400 to 1:600. Sections were routinely incubated for 1 to 2 hours at room temperature. Additional rinses and preincubation with goat serum (5%) were incorporated as needed to reduce background. Normal goat serum and non-immune serum were routinely used as negative con-

trols. In addition, the peptide immunogen for the trk C' antibody was used as an additional negative control.

Frozen tissue from each category of WT was analyzed using tissue lysates (100 µg/lane) equally loaded and immunoprecipitations with the pan-trk antibody and subsequent Western immunoblotting with trk A, trk B, trk C, and the anti-trk C' antibodies. Detergent (RIPA) extracts of fibroblasts and insect cells stably expressing trk A, B, C, and C' were normalized for protein content and subjected to immunoprecipitation using the pan-trk antisera, followed by Western analyses using the antisera specific for each trk receptor.

Immunocomplexes to protein were detected by incubation with ¹²⁵I-labeled protein A. As shown in Figure 1, no cross-reactivity could be detected. Lysates from insect (SF9) cells (2 × 10⁶) expressing rat trk B were electrophoresed and Westerns performed with anti-trk B and reprobbed with pan-trk antibody for confirmation. A mouse melanoma cell line overexpressing p75 was also used as a control cell line. The methods for Western blot analysis and cell culture utilization have been previously described.²³ The exposure times for the culture utilization have been previously described.²³ The exposure times for the autoradiographs were 18 and 24 hours, as indicated. For the insect cell blots, the exposure times were 7 seconds (chemiluminescence).

Results

Specificity of Antibodies

Antibody specificity for p75 and trk protein was confirmed by Western blot analyses. All antibodies were evaluated immunohistochemically using selective

cell lines overexpressing the individual receptors serving as positive and negative controls (data not shown). The trk and p75 positivity was seen as red, coarsely granular to globular staining within cell cytoplasm. The anti-p75 rabbit polyclonal antibody was generated against the intracellular rat p75 protein and the specificity of this antibody is demonstrated using a human melanoma cell line A875 expressing only the p75 receptor as a methodological control for the Western blots (Figure 3).^{18,22} The complete characterization and utilization of the polyclonal pan-trk antibody has been previously described.^{18,23} The affinity-purified rabbit polyclonal trk A antibody was generated against a unique extracellular sequence of human trk A and identifies a distinct band at 140 kd by Western blot (Figure 1, A). The affinity-purified rabbit polyclonal trk B antibody was generated against a unique juxtamembrane sequences of rat trk B and identifies the variably glycosylated forms (110 and 145 kd; Figure 1, B). The affinity-purified rabbit polyclonal antisera specific for extracellular rat trk C recognizes all trk C isoforms (140 kd; faint band at 100 kd; Figure 1, C) and affinity-purified antisera specific for the unique amino acids within the cytoplasmic domain of rat truncated trk C (C') lacking the catalytic domain are demonstrated (100 kd; Figure 1, D). The antinerve growth factor antibody is a polyclonal antibody that recognizes both nerve growth factor and Nt-3 (BLH, unpublished observations) and was obtained from Collaborative Research, New York.

The p75 Receptor in WT

Previous studies have used a cultured WT cell line to detect p75 receptor expression.¹⁰ However, no published studies have examined the expression of the

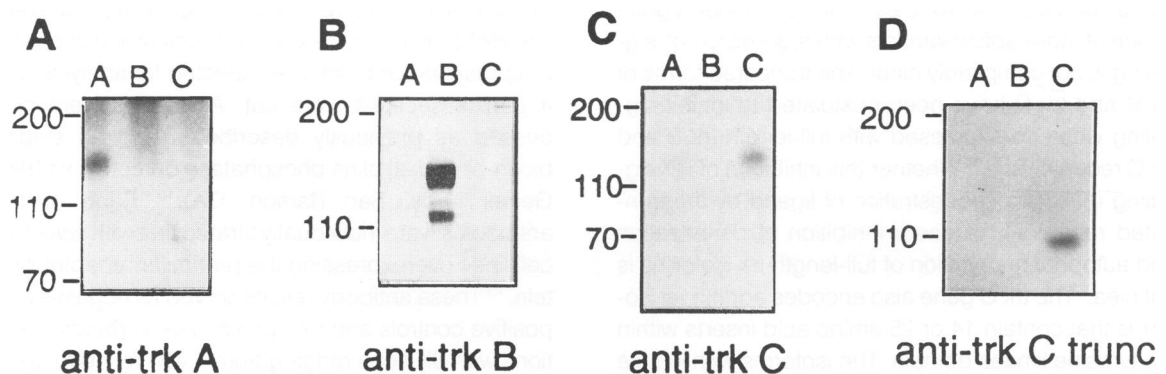


Figure 1. A: Specificity of antisera to trk receptors as detected by one-dimensional Western blot. Cells expressing individual trk family members were extracted in detergent and equivalent amounts of protein were immunoprecipitated with the pan-trk antisera. Immune complexes were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blots were developed using antisera specific for each trk family. A, B, C and D blots probed with anti-trk A, anti-trk B, anti-trk C, and anti-truncated trk C antisera, respectively. A and C: lane a, 3T3-trk A cells; lane b, 3T3-trk B cells; lane c, 3T3-trk C cells. B: lanes a-c, SF9 cells expressing trk A, trk B, or trk C, respectively. D, lanes a-c, 3T3 cells expressing full-length trk C, trk C = 14 amino acid kinase insert, trk C-truncated. A, C, and D used ¹²⁵I-labeled protein A for detection, with 24-hour autoradiography exposure time. B used ECL, 7-second exposure.

low affinity nerve growth factor receptor, p75, in pathological specimens of WT. We report a high level of immunoreactivity for NTs and p75 diffusely in the blastemal component and focally within mature tubules (Figure 2A, 2B, respectively) with a nerve bundle composed of Schwann cells and axons serving as an internal control for p75 (Figure 2, B, inset). The level of NT immunoreactivity was present in all tubules, glomerular structures, and blastemal components identified, independent of the tumor type examined. The level of p75 immunoreactivity was greatest in pure blastemal areas of all tumors and appeared to be somewhat decreased in regions where epithelial differentiation, ie, tubule formation, had occurred. The tubules thought to be the most histologically immature contained the highest levels of p75 antigen. The p75 was not identified in S bodies or glomerular structures. The components of the peripheral nervous system, ie, nerve fibers and perineural cells, as well as Schwann cells and axons have previously been shown by immunohistochemistry to contain the p75 low affinity nerve growth factor receptor.²⁴ The diffusely anaplastic WT exhibited the highest levels of p75 within anaplastic blastemal areas, whereas the nonanaplastic appearing cells were less immunoreactive (data not shown).

We verified that p75 was present in the WT study group by Western blot analysis of individual tumor samples. As shown in Figure 3, the expression of p75 protein varied among individual WT cases examined but correlated for the most part with the immunohistochemistry results. Indeed, the WT sample with the highest level of anti-p75 immunoreactivity (Figure

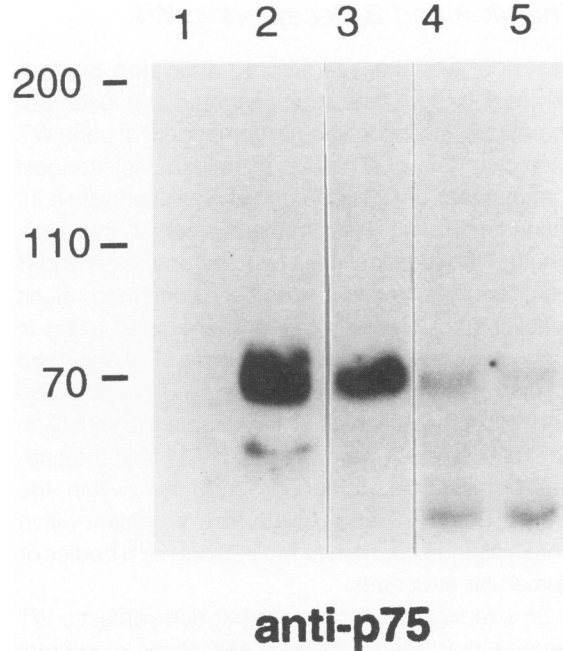


Figure 3. Autoradiograph of 3T3 trk A (lane 1), A875 (lane 2) p75 control cell line, blastemal WT (lane 3), epithelial WT (lane 4), and classical WT (lane 5) after immunoprecipitation and probing with the antibody to p75 (exposure 24 hours).

2, B) contained primarily blastemal components that demonstrated the greatest amount of p75 receptor protein on an equal protein-loaded Western immunoblot (Figure 3, Lane 3). Both of the anaplastic WTs by Western immunoblotting of tissue lysates had high levels of p75 protein identified with no evidence for nonspecific binding (data not shown).

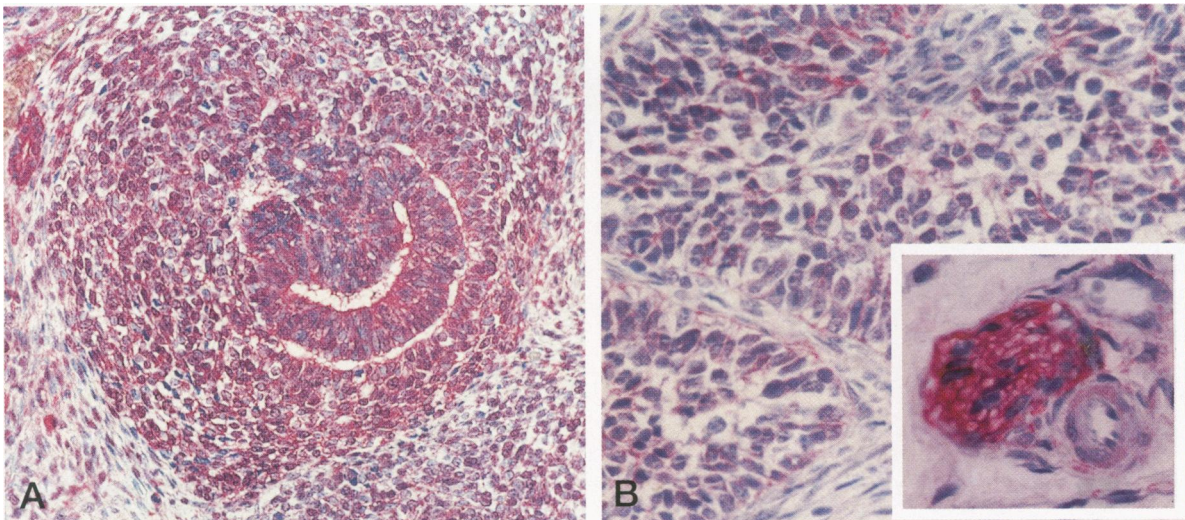


Figure 2. A: Immunostaining of classical WT with polyclonal antineurotrophin growth factor. Note intense epithelial and blastemal staining, (magnification $\times 250$). B: Immunostaining of blastemal component of WT with anti-p75. Note blastemal staining with absence of stromal immunoreactivity. Nerve bundle stains with anti-p75 (inset) (magnification $\times 250$).

The *trk A* and *B* Receptors in WT

Using polyclonal antisera specific for *trk A* or *trk B*, expression could be demonstrated immunohistochemically within the stromal component of each WT. Interestingly, in all WT cases examined, the strongest immunoreactivity for *trk A* and *B* was seen within individual cells at the stromal-blastemal interface. Whether the identical cells express both *trk A* and *B* receptors has yet to be entirely confirmed. Such questions are currently being investigated using *in situ* hybridization with double labeling. The observed staining pattern was true for all the WT types examined with the exception of the anaplastic variety, in which *trk A* and *B* were present only within the anaplastic blastemal component and not within the stroma. Both *trk A* and *B* were focally present within more histologically mature tubules but not S bodies or glomerular structures.

On examination of the two rhabdomyoblastic WT samples that contained focal collections of stromal rhabdomyoblasts, we found additional evidence for differential receptor localization. The *trk B* reaction product was identified within isolated blastemal cells and stromal cells (Figure 4, A). The immunoreactive

cells are distinguished by their more abundant cytoplasm compared with their negative counterparts. Using antibodies that recognize desmin (Biogenex Labs) and smooth muscle actin (Biogenex Labs) on these two WT cases, additional positive cells were found within the blastema and the stroma, suggesting a skeletal muscle phenotype (data not shown). Additional studies are needed to further characterize this observation.

In the anaplastic WT examined, focal *trk A*- and *B*-positive blastemal cells were also noted. These cells were not morphologically similar to those in the rhabdomyoblastic WT in that they were desmin and actin negative. However, special stains for the low molecular weight cytokeratins, ie, CAM 5.2 (Biogenex Labs) antibody, were focally positive for these cells, suggesting epithelial and mesenchymal differentiation (data not shown).

The *trk C* and Truncated *trk C*

We found receptor proteins *trk C* and *trk C'* (truncated) primarily within mature epithelial tubular elements of the classical triphasic WT (Figure 4, B). The

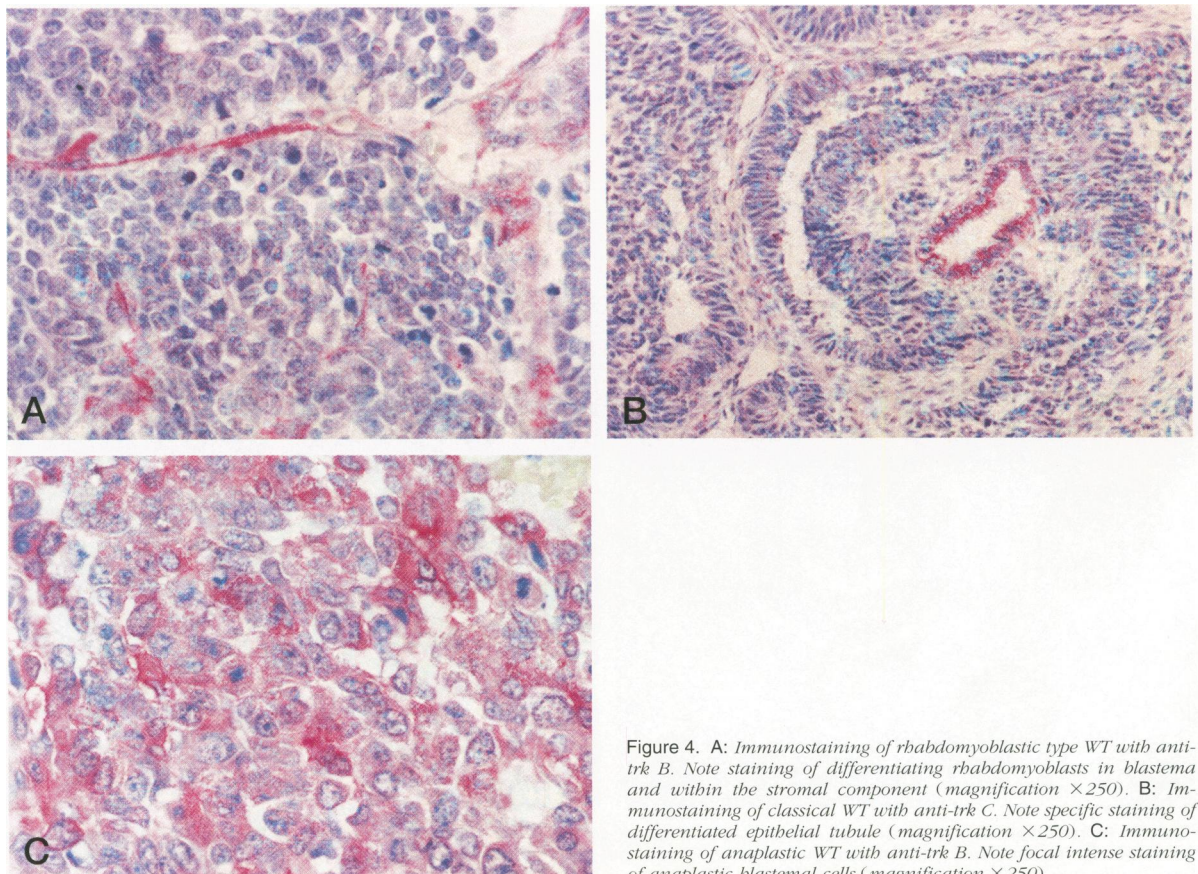


Figure 4. A: Immunostaining of rhabdomyoblastic type WT with anti-*trk B*. Note staining of differentiating rhabdomyoblasts in blastema and within the stromal component (magnification $\times 250$). B: Immunostaining of classical WT with anti-*trk C*. Note specific staining of differentiated epithelial tubule (magnification $\times 250$). C: Immunostaining of anaplastic WT with anti-*trk B*. Note focal intense staining of anaplastic blastemal cells (magnification $\times 250$).

protein was localized to the membrane and cytoplasm of epithelial cells but was not present within the blastema or stromal components. Trk C was also seen focally within the blastema cells of both anaplastic WTs.

We did not find trk receptor protein within the non-rhabdomyoblastic blastemal component of any WT examined except for some focal intense immunoreactivity of trk A, B, and C/C' in the focal and diffusely anaplastic WT samples, as alluded to earlier (Figure 4, C). In these two cases the trk-positive cells were moderately pleomorphic and contained more cytoplasm than the nonanaplastic blastemal cells. They did not have rhabdomyoblastic features in that they were negative for desmin and other skeletal muscle markers.

Western Analysis/Immunohistochemistry Correlation

We confirmed the presence of trk protein by Western blot analysis of individual tumor samples using the pan-trk, trk A, trk B, trk C, and truncated trk C antibodies. It was already established that the pan-trk antibody would identify not only full-length trk proteins but truncated forms as well.²³ Interestingly, with the pan-trk antibody we found several species of trk protein on our Western blot WT samples. By using the specific anti-trk antibodies we were then able to further evaluate trk expression. We did not find evidence of trk A protein within any of the samples that we studied (data not shown). We did find trk B in several samples with a prominent 140-kd band in one sample, probably representing the full-length receptor (Figure 5, lane 1). Interestingly, this sample by immunocytochemistry has prominent trk B reaction product within both isolated blastemal cells and stromal components (Figure 4, A).

In addition, we identified several WT samples that by immunoblot contained distinct bands when probed with either the anti-trk C or truncated trk C' antibodies (Figures 6 and 7, respectively). A 105-kd band (Figure 7, lanes 1, 3, 4) was present in three samples that immunocytochemically contained high amounts of trk C' receptor protein. Furthermore, one sample (Figure 7, lane 3) included a rhabdomyoblastic WT that had trk C' reaction product in blastemal elements and within the stroma. Accompanying trace bands in several of the blots analyzed were thought to represent full-length trk's and variable glycosylated forms. Some of the differences may again be attributable to tumor autolysis and sampling at the

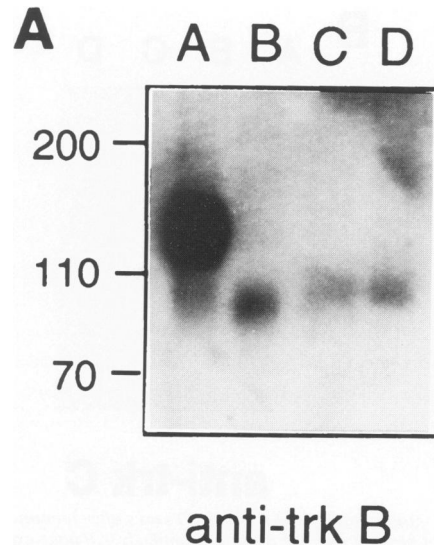


Figure 5. Autoradiograph of selected WT cases after immunoprecipitation and probing with the anti-trk B antibody (24-hour exposure).

Table 1. Immunohistochemical Detection of trk and p75 in a Series of 10 Wilms' Tumors

Wilms' Tumor	TRK A	TRK B	TRK C/C'	p75
Classic				
Blastema	-	-	-	+
Tubules	+	+	+	+
Stroma	+	+	-	-
Rhabdomyoblasts*	-	+	+	+
Anaplastic				
Blastema	(+)	(+)	(+)	+
Tubules	-	+	+	+
Stroma	-	(+)	-	-

(+), focal positivity; +, diffusely positive; -, negative.

* Rhabdomyoblasts in rhabdomyoblastic variant of Wilms' tumor.

time of specimen retrieval. For a summary of immunohistochemical results for WTs in our study group, see Table 1.

Discussion

The p75 and WT

Only one previous study has implicated the NT receptors, specifically p75, in the evolution of WT. In this report, cultured WT cells were found to contain intact low affinity nerve growth factor receptors, p75.¹⁰ By immunohistochemistry we have identified p75 receptors primarily in the blastema and differentiated epithelial tubules of a series of WT. We have confirmed the presence of p75 immunoreactivity by using Western blot analysis on a variety of WT containing varying amounts of blastema and tubular components. The differences observed with the Western immunoblots are thought to reflect differential levels of expression

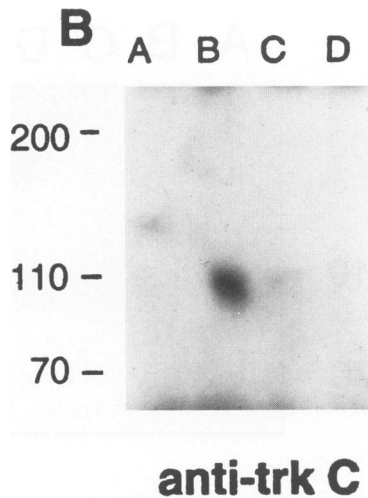


Figure 6. Autoradiograph of selected WT cases after immunoprecipitation and probing with the anti-trk C antibody (18-hour exposure).

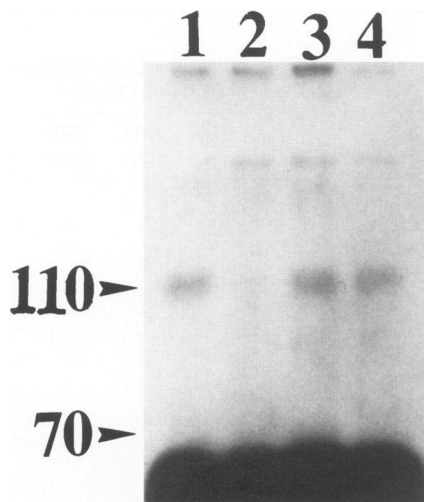


Figure 7. Autoradiograph of selected WT cases after immunoprecipitation and probing with the anti-trk C (truncated trk C) antibody (24-hour exposure).

by different tumor cell types (blastemal predominant versus epithelial), although we cannot rule out secondary effects from tumor autolysis. Indeed, the WT samples that were blastemal predominant contained the greatest amount of p75 protein.

Previous studies have proposed that during tumor progression the blastema retains the capacity to differentiate into the various components of the nephron similar to processes occurring during normal kidney morphogenesis.^{25,26} The differentiative pathway that results in mature kidney is one in which pleuripotential stem cells progressively lose the ability to divide as they become further committed toward their final phenotypes. The factors responsible for the induction and promotion of this differentiative response are likely to reflect the actions of a variety of growth factors and

the regulation in expression of these factors is likely to be a direct consequence of the genetic rearrangements characteristic of WT.

Indeed, the identification of a WT gene at 11p15 in close proximity to the IGF-2 gene^{27,28} and the ability to inhibit WT growth both *in vitro* and *in vivo* with antibodies against the IGF-1 receptor² help support this hypothesis. Previously, it was shown that IGF levels are elevated not only in WT samples examined but also in normal fetal kidney.⁸ *In situ* hybridization of IGF-2 in normal kidney identified expression primarily in blastemal cells, whereas the differentiated glomerular and tubular elements had reduced levels of expression.²⁹ Interestingly, the same authors found elevated IGF-2 expression in differentiated epithelial components of a monomorphous WT. They concluded that IGF-2 is a marker for differentiation and its identification in WT tubules could be a manifestation of unregulated expression.

Recently, both the p75 receptor and the WT-1 gene have been implicated in the early stages of normal kidney development.^{11,30} The p75 has been identified in S bodies of early condensed mesenchyme (blastema) but was lacking on more differentiated tubules.¹¹ Our finding of p75 on mature tubules in WT may in some way reflect a dysregulation of receptor expression as suggested for IGF-2. In the earlier study by Sariola et al,¹¹ the function of p75 in epithelial differentiation and tubular branching was analyzed in organotypic cultures of embryonic rat kidneys. The utilization of antisense oligonucleotides to the p75 receptor (thus inhibiting p75 expression) in kidney cultures resulted in the inhibition of tubule formation with only rudimentary branching of the ureteric bud. The authors concluded that depletion of p75 specifically perturbed epithelial differentiation of the nephrons, although this experimental result has not been duplicated using similar conditions.¹⁴ Additional work³⁰ implicated the WT-1 gene in the promotion of blastemal differentiation because WT-1 transcripts can be found in fetal kidney metanephric blastemal cells and levels of WT-1 increase as cells divide and differentiate. The most recent findings indicate that the WT-1 gene is required for the survival and early differentiation of the metanephric blastema.³¹

Because the WT-1 protein has been implicated in the regulation of the IGF-1 receptor by binding to its promoter,³² so may the WT-1 protein bind to the promoter for p75 and quite possibly the promoter regions for the NT and trk receptors as well. Our immunohistochemical and Western blot results suggest high expression and misregulation of the p75 receptor. By comparison, immunoblots of normal human fetal kidney demonstrate only low levels of p75 expression¹²

(L. Julie Huber, personal communication). We hypothesize that the functional WT-1 protein may serve in a similar regulatory capacity for the p75 receptor and thus a functionally inactive protein would allow for up-regulation of p75. If our preliminary NT studies are accurate, then an autocrine loop involving both p75 and NT may account for some aspects of tumor development. We have recently begun studies to verify the expression of the individual NTs in WT by *in situ* hybridization. Furthermore, the presence of the p75 receptor (and trk receptors) on differentiated tubules allows for competent receptor response to growth factors in a dysregulated setting. We are currently in the process of testing the association between the WT-1 protein and various NT and receptor promoters.

The mechanism by which the low affinity nerve growth factor p75 receptor induces cellular changes remains uncertain. There is evidence that p75 contributes to the formation of high affinity nerve growth factor binding sites³³ and enhances the specificity of the trk family of NT receptors.³⁴ Recent experiments have suggested that this receptor retains some functional similarities to other members of the super family of receptors that include tumor necrosis factor, Fas (Apo-1), and CD40. In this regard p75 may be involved as a constitutive cell death-promoting molecule that is inhibited by nerve growth factor binding when p75 is expressed in a central nervous system-derived cell line.³⁵ These results have been interpreted as implying that some cells become dependent for their survival on the binding of nerve growth factor and this response coincides with an increased expression of p75. Our observations of high expression levels for both p75 and NT in WT blastemal cells and differentiating tubules suggest a similar mechanism for tumor progression.

The trk A and B Receptors

By immunohistochemistry we have identified both trk A and B receptor protein localized to cells that make up the stroma of all WT examined (except the anaplastic variety). In many cases it appeared that the highest level of immunoreactivity was present at the stromal-blastemal interface. The functional significance of this immunoreactivity is unclear. One hypothesis is that these focally positive cells within the stroma may be involved in some inductive pathway involving the proliferating/differentiating blastemal cells and the adjacent stroma. Recent studies using Northern analyses on embryonic rodent kidney identified only the truncated form of the trk B receptor.¹⁴ In addition, by *in situ* hybridization, the authors dem-

onstrated that the transcripts for the trk B receptors were localized to the more uninduced mesenchyme in the upper cortex, which is destined to become the true stromal component of the adult kidney. Immunohistochemical results may again be supporting the theory that during the development of a WT there is continual recapitulation of normal kidney morphology. Furthermore, our Western immunoblots also demonstrate that of the four samples analyzed, three contained truncated trk B receptors and one contained the full-length receptor for trk B. Again, the sample with the full-length receptor was a WT that contained both stromal and isolated blastemal positivity. The full-length receptors are those that contain the tyrosine kinase moiety and are thought capable of responding to signals eliciting proliferation and/or differentiation.

Evidence suggests that the stromal portion of the WT is derived from mesenchymal stem cells that have the capacity to differentiate into a variety of cell and tissue types including skeletal muscle, cartilage, and bone.³⁶ The identification of the trk receptors in this multipotential stroma implies a possible role for these receptors in the subsequent differentiation of this tissue. Indeed, it has already been shown that trk receptors identified with a pan-trk antibody are present in differentiating rhabdomyoblasts of rhabdomyosarcomas.¹⁸ Furthermore, NT mRNA expression has been identified in muscle cells in the major elastic arteries of the developing rat cardiovascular system.³⁷ Intriguing to our study, the only examples of blastemal trk expression are with the anaplastic WT and the two rhabdomyoblastic variants (diagnosed as such because of the large amounts of stromal muscle differentiation).

Skeletal muscle differentiation within a WT is a well-documented histopathological entity. The skeletal muscle is believed to arise from the differentiation of the mesenchymal or stromal component of the tumor.³⁸ On examination of the two rhabdomyoblastic WT samples we found preliminary evidence for differential receptor localization. The trks B, C, and truncated trk C' were found within isolated blastemal cells and stromal cells (Figure 4, A). These preliminary results suggest that these immunoreactive cells (possibly primitive skeletal muscle cells) may arise from the mesenchymally derived blastemal cells. Interestingly, we found trk B receptor expression in several muscle types including vascular tissues (unpublished data) and the p75 receptor was found in fetal skeletal muscle.²⁴ Further studies using *in situ* hybridization and double labeling experiments are required to elucidate the origin of muscle in WT. These studies are currently underway. The anaplastic WT

also contained trk B receptor protein, and this will be discussed later.

The trk C and Truncated trk C (C')

Our identification of trk C and C' receptors in mature tubular structures implies a potential role for these receptors in epithelial differentiation. Previous experiments have already demonstrated preferentially high levels of trk C and C' mRNA in more differentiated structures.^{14,15} It may be that trk C is necessary for early differentiation to occur and trk C' may serve to exert a dominant negative effect on the signal transduction mechanism. This would be similar to the inhibition of the wild-type PDGF receptor by the co-expression of a truncated receptor.³⁹ The trk C gene was already shown to be highly expressed in adult structures of both the brain and extraneural tissues.¹⁵ Recently, full-length trk C transcripts were identified over regions of fully formed collecting ducts in fetal rodent kidney.¹⁴ Our Western immunoblots identified both full-length and truncated receptor proteins on selected WT, including a rhabdomyoblastic type.

We identified trk A, B, and C/C' receptor protein within the nonrhabdomyoblastic blastemal components in the focal and diffusely anaplastic WT samples. We also found high levels of p75 receptor protein present in similar anaplastic blastemal cells. By exhibiting a multireceptor phenotype, we hypothesize that these cells are capable of responding to a variety of NT growth factors, giving these cells a distinct advantage in terms of tumor growth. In addition, by containing so many different receptor types, there is evidence for receptor dysregulation in that the cells will not respond appropriately to differentiative signals, thus allowing for continued tumor progression. We did find evidence of p75 receptor protein on Western immunoblots and trk B protein, further supporting our immunohistochemical evidence. Interestingly, many of the anaplastic blastemal cells also contain epithelial antigens, suggesting a commitment of these cells toward more epithelial structures (tubular components) as opposed to mesenchymal tissues.

In these studies, we have attempted to add the NT and their receptors to the growing list of factors associated with inappropriate cell regulation, differentiation, and tumor formation. The immunoreactive patterns for trk and p75 imply involvement of these factors in the pathobiology of the WT. Furthermore, the presence of the truncated trk C' species in several of the WT examined may have relevance in the overall favorable histology and behavior of this tumor. The evidence of high levels of both p75 and NT in undif-

ferentiated blastemal components lends further support for a misguided autocrine/paracrine signal receptor mechanism in the progression of this tumor. Further studies are underway to characterize both NT and trk receptor function in the pathogenesis of WT.

Acknowledgments

We thank Steve Borack for photographic reproduction and Louis Whitney for illustration layout.

References

1. Deuel TF: Polypeptide growth factors: roles in normal and abnormal cell growth. *Ann Rev Cell Biol* 1987, 3:443-492
2. Gansler T, Furlanetto R, Gramling T, Robinson K, Blocker N, Buse M, Sens, Garvin J: Antibody to type insulin-like growth factor receptor inhibits growth of Wilms' tumor in culture and athymic mice. *Am J Pathol* 1989, 135:961-966
3. Goustain AS, Leof EB, Shipley GD, Moses HL: Growth factors and cancer. *Cancer Res* 1986, 46:1015-1029
4. Beckwith JB, Palmer NF: Histopathology and prognosis of Wilms tumor. *Cancer* 1978, 41:1937-1948
5. Frazier G, Bowen-Pope D, Vogel A: Production of platelet-derived growth factor by cultured Wilms' tumor cells and fetal kidney cells. *J Cell Physiol* 1987, 133:169-174
6. Reeve AE, Eccles MR, Wilins RJ, Bell GI, Millow LJ: Expression of insulin-like growth factor-II transcripts in Wilms' tumor. *Nature* 1985, 317:258-260
7. Haselbacher GK, Irminger J-C, Aapf J, Ziegler WH, Humbel RE: Insulin-like growth factor-II in human adrenal pheochromocytomas and Wilms tumors: expression at the mRNA and protein level. *Proc Nat Acad Sci USA* 1987, 84:1104-1106
8. Werner H, Re G, Drummond I, Sukhatme V, Rauscher F, Sens D, Garvin J, LeRoith D, Roberts C: Increased expression of the insulin-like growth factor I receptor gene, IGF1R, in Wilms' tumor is correlated with modulation of IGF1R promoter activity by the WT1 Wilms tumor gene product. *Proc Nat Acad Sci USA* 1993, 90:5828-5832
9. Wang Z, Madden S, Deuel T, Rauscher F: The Wilms' tumor gene product, WT-1, represses transcription of the platelet-derived growth factor A-chain gene. *J Biol Chem*, 1992, 267:21999-22002
10. Thomson T, Pellicer A, Greene L: Functional receptors for nerve growth factor on Ewing's sarcoma and Wilms tumor cells. *J Cell Physiol* 1989, 141:60-64
11. Sariola H, Saarma M, Saino K, Arumae U, Palgi J, Vaahokari A, Thesleff I, Karavanov A: Dependence of kidney morphogenesis on the expression of nerve growth factor receptor. *Science* 1992, 254:571-573

12. Alpers C, Hudkins K, Ferguson M, Johnson R, Schatteman G, Bothwell M: Nerve growth factor receptor expression in fetal mature and diseased human kidneys. *Lab Invest* 1993, 69:703-713
13. Ernfors P, Wetmore C, Erkiidsdotter-Nilsson M, Bygdemann M, Stromberg I, Olson L, Persson H.: The nerve growth factor receptor gene is expressed in both neuronal and non-neuronal tissues in the human fetus. *Int J Dev Neurosci* 1991, 9:57-66
14. Durbeej M, Soderstrom S, Ebendal T, Birchmeier, Ekblom P: Differential expression of neurotrophin receptors during renal development. *Development* 1993, 119:977-989
15. Tessarollo L, Tsoulfas P, Martin-Zanca D, Gilbert D, Jenkins N, Copeland N, Parada L: *trk C*, a receptor for neurotrophin-3, is widely expressed in the developing nervous system and in non-neuronal tissues. *Development* 1993, 118:463-475
16. Nakagawara A, Arima M, Azar C, Scavarda NJ, Brodeur GM: Inverse relationship between *trk* expression and *n-myc* amplification in human neuroblastomas. *Cancer Res* 1992, 52:1394-1368
17. Nakagawara A, Arima-Nakagawara M, Scavarda NJ, Azar C, Cantor A, Brodeur GM: Association between high levels of expression of the *trk* gene and favorable outcome in human neuroblastoma. *N Engl J Med* 1993, 328:847-854
18. Donovan M, Hempstead B, Horvath C, Chao M, Schofield D: Immunohistochemical localization of *trk* receptor protein in pediatric small round blue cell tumors. *Am J Pathol* 1993, 143:1560-1567
19. Klein R, Conway D, Parada LF, Barbacid M: The *trk B* tyrosine kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. *Cell* 1990, 61:647-656
20. Tsoulfas P, Soppet D, Escandon E, Tessarollo L, Mendoza-Ramirez J-L, Rosenthal A, Nikolics K, Parada L: The rat *trk C* locus encodes multiple neurogenic receptors that exhibit differential response to neurotrophin-3 in PC12 cells. *Neuron* 1993, 10:975-990
21. Ibanez CF, Ebendal T, Barbany G, Murray-Rust J, Blundell TL, Persson H: Disruption of the low affinity receptor binding site in NGF allows neuronal survival and differentiation by binding to the *trk* gene product. *Cell* 1992, 69:329-341
22. Huber LJ, Chao MV: Mesenchymal and neuronal cell expression of the p75 neurotrophin receptor are distinguished during morphogenesis of transgenic animals. *Dev Biol* (in press)
23. Horvath C, Wolfen A, Machadeo D, Huber J, Boter L, Benedetti M, Hempstead B, Chao M: Analysis of the *trk* NGF receptor tyrosine kinase using recombinant fusion proteins. *J Cell Sci* 1993, S17:223-228
24. Garin-Chesa P, Rettig W, Thomson T, Old L, Melamed M: Immunohistochemical analysis of nerve growth factor receptor expression normal and malignant human tissues. *J Histochem Cytochem* 1988, 36:383-389
25. Albeda FW, Molenaar WM, de Leij L, Thijs-Ipema AH: Heterogeneity of Wilms' tumour blastema: an immunohistological study. *Virchows Arch* 1989, 414:263-271
26. Van Heyningen V, Hastie N: Wilms' tumour: reconciling genetics and biology. *Trends Genet* 1992, 8:16-21
27. Brissenden JE, Ullrich A, Francke U: Human chromosomal mapping of genes for insulin-like growth factors I and II and epidermal growth factor. *Nature* 1984, 310:781-784
28. Tricoli J, Rall LB, Scott J, Bell GI, Shows TB: Localization of insulin-like growth factor genes to human chromosomes 11 and 12. *Nature* 1984, 310:784-786
29. Paik S, Rosen N, Jung W, You J, Lippman M, Perdue J, Yee D: Expression of insulin-like growth factor II mRNA in fetal kidney and Wilms' tumor. *Lab Invest* 1989, 61:522-526
30. Prichard-Jones K, Fleming S, Davidson D, Bickmore W, Porteous D, Gosden C, Bard J, Houseman D, van Heyningen V, Hastie N: The candidate Wilms' tumor gene is involved in genitourinary development. *Nature* 1990, 346:194-197
31. Kreidberg J, Sariola H, Loring J, Maeda M, Pelletier J, Housman D, Jaenisch R: WT-1 is required for early kidney development. *Cell* 1993, 74:679-691
32. Werner H, Re G, Drummond I, Sukhatme V, Rauscher F, Sens D, Garvin J, LeRoith D, Roberts C: Increased expression of the insulin-like growth factor I receptor gene, IGF1R, in Wilms tumor is correlated with modulation of IGF1R promoter activity by the WT1 Wilms tumor gene product. *Proc Natl Acad Sci USA* 1993, 90:5828-5832
33. Hempstead BL, Martin-Aanca D, Kaplan D, Parada L, Chao M: High-affinity NGF binding requires coexpression of the *trk* proto-oncogene and the low-affinity NGF receptor. *Nature* 1991, 350:678-683
34. Ip NY, Stitt TN, Tapley P, Klein R, Glass DJ, Fandl J, Greene LA: Similarities and differences in the way neurotrophins interact with the *trk* receptors in neuronal and nonneuronal cells. *Neuron* 1993, 10:137-149
35. Rabizadeh S, Oh J, Li-Tao Z, Yang J, Bitler C, Butcher L, Bredesen D: Induction of apoptosis by the low-affinity NGF receptor. *Science* 1993, 261:345-348
36. Hazen-Martin D, Garvin J, Gansler T, Tarnowski B, Sens D: Morphology and growth characteristics of epithelial cells from classic Wilms' tumors. *Am J Pathol* 1993, 142:893-905
37. Scarisbrick IA, Jones EG, Isackson P: Coexpression of mRNAs for NGF, BDNF, and NT-3 in the cardiovascular system of the pre- and postnatal rat. *J Neurosci* 1993, 13:875-893
38. Garvin J, Surette F, Hintz D, Rudisill M, Sens MA, Sens D: The in vitro growth and characterization of the skeletal muscle component of wilms' tumor. *Am J Pathol* 1985, 121:298-310
39. Ueno H, Colbert H, Escobedo J, Williams L: Inhibition of PDGF B signal transduction by coexpression of a truncated receptor. *Science* 1991, 252:844-848