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bFGF Induces Differentiation and Death of Olfactory Neuroblastoma Cells

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Olfactory neuroblastoma (ONB) is a highly vascularized and malignant tumor arising in olfactory neuronal precursors from the paranasal sinuses. Previously, we showed that treatment of JFEN cells with transforming growth factor (TGF)-α caused them to differentiate and respond to chemical odorants, whereas basic fibroblast growth factor (bFGF) treated cells differentiated and died. In the present study we show that established ONB tumors treated with bFGF upregulate the bFGF receptor (FGFR1) prior to differentiation. This cellular differentiation was evidenced by bFGF-induced expression of the human runt homologue *AML1* (*PEBP2αB, CBFA-2*) that is highly expressed in developing olfactory neuroepithelium and *Trk*A, a preferred nerve growth factor receptor. Since *Trk*A is expressed in supporting cells, but not in mature olfactory neurons, we hypothesize that the expression of *AML1* and *TrkA* in bFGF-treated JFEN cells induced supporting cell differentiation. Collectively, these results have implications for the treatment of patients afflicted with ONB. © 2000 Academic Press

***Key Words: AML1;* basic fibroblast growth factor; *BDNF;* esthesioneuroblastoma; Ewing's sarcoma; *EWS/Fli-1; FGFR-1; NGFR;* athymic mice; olfactory neuroblastoma; pPNET; *Trk*.**

Olfactory neuroblastoma (ONB), or Esthesioneuroblastoma, is an unusual malignant tumor originating in the nasal cavity and often extending into the paranasal sinuses, orbital fossa or anterior skull base (1). ONB is also a highly vascularized tumor and invasive tumor metastasizing in as many as 50% of patients diagnosed (1–3). Patients afflicted with this tumor are treated either singly or in combination with radiotherapy, chemotherapy, and/or surgery (1, 4). Although recent advances in craniofacial surgery have provided improved methods for removal of this tumor (2, 4, 5), little progress has been made in the improvements in noninvasive treatments. Due to the anatomical isolation of the nasal sinuses and the nonspecific signs of disease (nasal congestion, headache), extensive tumor growth may occur prior to detection. Consequently, the difficulty and delay of diagnosis, the advanced stage of tumors at presentation and the high frequency of metastatic spread (1, 6) contribute to the poor long-term prognosis of patients with advanced ONB.

ONB is thought to arise from olfactory mucosa (7), and recent data support its origin from olfactory neuroepithelial precursors within the basal layer of the olfactory epithelium (3, 8). Presently, diagnosis is based on morphological characteristics, anatomical location and the presence of dense core secretory granules and/or Homer-Wright rosettes (3, 7–11). In addition, immunohistochemical-staining profiles that include positivity for neuron specific enolase (NSE), S-100, keratin, synaptophysin and/or chromogranin have also been used for diagnostic determination (3, 7). Unfortunately, none of these markers either alone or in combination are considered definitive for ONB. Furthermore, the primitive nature of its morphology, and the paucity of olfactory-specific markers, has made it difficult to determine the true cellular origin of ONB. Additional diagnostic markers would greatly facilitate the distinction of ONB from other related tumors.

Recent studies have reported the use of markers that provide a more precise classification of ONB tumors (3, 8, 11, 12). For example, the t(11;22)(q24;q12) or t(21;22)(q22;q12) chromosomal translocations associated with Ewing's sarcoma are also found in 100% of the ONB specimens analyzed (11). Similar to murine studies, which indicated a requisite role of the *acheate-scute* 1 gene in olfactory neuronal development (13), we dem-

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onstrated expression of mRNA for the human *achaete-scute* 1 gene (*HASH* 1) in ONB (8). Together, the use of these new genetic probes has helped improve the specificity of ONB tumor diagnosis. These data support the conclusion that ONB is a primitive peripheral neuroectodermal tumor (pPNET) of olfactory neuronal precursors (3, 10).

Little is known about the processes regulating olfactory cell growth and differentiation. In experiments with organotropic cultures from the rat olfactory epithelium, transforming growth factor α (TGF α) has been shown to induce cell proliferation (14), while basic fibroblast growth factors (bFGF) have been shown to be important in the regulation of progenitor cell divisions in the mouse olfactory epithelium (15). In addition, several studies have shown that receptors for various growth factors are expressed in cells of the olfactory epithelium (14). Thus, TGF and bFGF appear to be at least two of the requisite regulators of cell growth and differentiation in the olfactory neuroepithelium.

To study the role of soluble mediators in the abnormal growth/differentiation of olfactory neuroepithelial stem cells we developed a model of tumor progression using the human ONB tumor cell line JFEN (11). JFEN was derived from a metastatic ONB tumor identified using conventional diagnostic methods and confirmed to be ONB with molecular markers (7, 8, 10–12). It also represents the only uncloned well-characterized ONB cell line representing olfactory neuronal tumors. Using this cell line we have demonstrated that transforming growth factor (TGF)-α treated, but not untreated, JFEN cells differentiated into neuron-like cells while basic fibroblast growth factor (bFGF) and insulin-like growth factor (IGF)-I treated cells differentiated and died (12). From these results, we hypothesize that various growth factors could induce the differentiation of ONB tumor cells into different cell types. We predict that each lineage would express distinct genes characteristic of cells derived from olfactory neuroepithelium. To address this hypothesis we examined the expression of genes highly expressed in embryonic olfactory neuroepithelium and/or those whose topological expression patterns have been well characterized in adult olfactory epithelium (16–18). Furthermore, as an *in vivo* assay for tumor differentiation, we evaluated the effects of bFGF on ONB tumors grown in athymic mice. Thus, studying the effects of bFGF therapy *in vivo* has implications for the treatment of patients afflicted with this invasive, and often metastatic, olfactory malignancy.

METHODS

Cell lines and growth factors. The ONB cell line used for these studies, JFEN (19), was kindly provided by Dr. T. Triche, (Department of Pathology, Children's Hospital of Los Angeles). Materials for the culture medium were obtained from Gibco/BRL. Cells were maintained in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum (heat inactivated), 1% MEM nonessential amino acids, 2 mM L-glutamine, and 1% penicillin/streptomycin. All purified recombinant growth factors in this study were obtained from Collaborative Biomedical Products. Lyophilized Growth Factors were diluted into culture medium before use at an optimal concentration of 25 ng/ml (12). The factors used in this study were nerve growth factor (NGF), epidermal growth factor (EGF), insulin-like growth factor-II (IGF-II), basic fibroblast growth factor (bFGF), and transforming growth factor-α (TGF-α). Growth factors were dissolved in sterile distilled, deionized water prior to use. Cultured JFEN cells were treated with specified growth factors for 2 days at 37°C. Each experiment was repeated at least two times.

ONB tumors and growth factor treatment. ONB tumor cells were freshly harvested from tissue culture flasks by trypsinization, washed and resuspended in phosphate buffered saline (PBS). One $\times 10^6$ JFEN cells in 0.1 ml of PBS were injected subcutaneously into 8-week-old *BALB/cBy-nu/nu* female mice. Tumors were grown for 3 weeks or until tumor sizes reached approximately 1.5 cm^3. Endotoxin-free, purified recombinant bFGF (gift of Scios Nova Corp.) was intratumorally injected into mice for 7 days at a dose of 8 mg/kg for a 25 g mouse/day (~200 μg/day) in a total volume of 0.2 ml. This dose was not toxic as assessed by daily observations of malaise or anorexia in treated mice. In addition, previous studies concluded that bFGF is not toxic at the dosage used in this study (20). Control (untreated) mice were injected with 0.2 ml of PBS. Following treatment, mice were sacrificed and tumor tissue was resected for histopathological evaluation. Tumors were fixed in 10% formalin, embedded in paraffin, sliced into 5 μl sections, mounted on slides, and either immunostained using protein-specific antibodies or stained directly with hematoxylin-eosin (H&E) using standard protocols (21).

Human olfactory epithelium. Whole human olfactory organ was obtained within 24 h post-mortem from unrestricted autopsy of patients who died of a disease unrelated to olfaction. The specimens were extracted via a combined intracranial and intranasal approach as reported (5, 21–24). Specimens were immediately placed into Zamboni's fixative solution containing 4% paraformaldehyde for 48 h at room temperature (25–27). Specimens were decalcified in a hydrochloric/formic acid solution for 24–48 h, dehydrated through graded alcohol/xylene series, embedded in paraffin, sectioned, and stained as described (21).

Total RNA extraction. Conditions for nucleic acid purification and RT-PCR have been previously reported (8). Briefly, cell lines were lysed in extraction buffer (7 M guanidinium hydrochloride, 20 mM KOAc, 5 mM EDTA) followed by phenol/chloroform extraction and ethanol precipitation. Each sample was with 10 units of RNase-free DNase I (Boehringer) and incubated at 37°C for 30 min. Following DNAse treatment, reactions were phenol/chloroform extracted and ethanol precipitated. RNA pellets were washed with 70% ethanol, resuspended into 50 μl of RNase-free water, and stored at −70°C.

RT-PCR. The amount of RNA was quantified spectrophotometrically, 5 μg was used in a standard cDNA synthesis reaction (28). Briefly, first strand cDNA was synthesized from total RNA in a 50 μl reaction containing 1× reverse transcriptase (RT) buffer (Gibco/BRL), 0.5 μg oligo dT_{18}, 0.1 μg random hexamers (Gibco/BRL), 200 units Superscript II RNase H^- Reverse Transcriptase (Gibco/BRL), and 1 mM dNTPs. The reaction was incubated at 37°C for 1.5 h and terminated at 95°C for 5 min. For PCR analysis 5 μl of first strand product (approximately 100 ng of cDNA) was added to 45 μl of a 1× PCR mix (20 pmole of each primer, 50 mM KCl, 10 mM Tris–HCl pH 9.0, 0.1% Triton X-100, 1.5 mM $MgCl_2$) containing 2.5 units of *Taq* DNA Polymerase (Promega), and cycled 30 times at 94°C for 30 s, 55–65°C for 30 s, and 72°C for 1 min. Reaction products were resolved on a 2.5% agarose gel and photographed. Annealing temperatures were determined for each primer set and varied from 55–65°C. In all RT-PCR reactions, β-actin primers were used as

controls to ensure the integrity of RNA. Oligonucleotide primers for the *EWS/FLi*-1 fusion gene were designed to flank the breakpoint as described (29–36). Oligonucleotide primers for *AML*1 flank exon 4 and exon 6 containing the runt domain (16, 37, 38). Sequences for PCR primers for genes used in this study were determined from published data (Genebank/EMBL nucleotide sequences) and are summarized in Table 1. Semi-quantitative RT-PCR of *AML*1 was performed by adding 0.2 μl of the primary *AML*1 PCR products and reamplified using the nested oligonucleotide primers *AML*1-2 (Table 1). Templates not treated with RT prior to PCR (RT−), were used as negative controls. All PCR reactions were repeated at least 3 times. Oligonucleotide synthesis and PCR product sequencing was performed with the assistance of the Kimmel Cancer Institute Nucleic Acid Facility.

Immunohistochemical staining. Five μm-thick serial sections were cut from formalin-fixed paraffin-embedded tumor harvested from athymic mice and mounted on Superfrost/Plus slides (Fisher Scientific). Each section was deparaffinized and rehydrated through graded xylene and alcohol series. Slides were placed in citrate-buffered solution (pH 6.0) and heated at 100°C by microwave oven for 20 min for antigen retrieval as previously described (39–42). Endogenous peroxidase was blocked with 3% hydrogen peroxide and nonspecific binding was blocked with 10% normal goat serum. Affinity-purified polyclonal rabbit anti-mouse antibodies specific for *Trk*A (*Trk* 763), *Trk*B (*Trk*B 794), *Trk*C (*Trk*C 798), and FGFR1 (Flg C-15) (Santa Cruz Biotechnology, Inc.) were used at a 1:100 dilution. Secondary goat anti-rabbit antibodies were used at a 1:100 dilution. Ten percent normal goat serum was substituted for the primary antibody in negative controls. Substrate development was performed using Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Diaminobenzidine (DAB) was used as chromogen, and nuclei were lightly counterstained with Hematoxylin.

RESULTS

Olfactory neuroblastoma cells expresses FGFR1, a receptor for bFGF. In earlier studies we reported the cytotoxic effects of bFGF on cultured ONB tumor cells (12). Although cells died 4 days after treatment with 10 ng/ml FGF, the presence of specific growth factor receptors was not previously assessed. Thus, to better understand the mechanism of bFGF effects on JFEN cells, we evaluated the expression of FGF receptors by a RT-PCR assay. To date, four structurally related genes encoding high-affinity FGF receptors (FGFR 1–4) have been identified (43–47). Isoforms of these FGFRs generated by alternative mRNA splicing manifest distinct ligand-binding specificities and affinities which dramatically alter the structure and function of the growth factor receptor (48). For example, FGFR1, a high affinity receptor for both acidic FGF and bFGF, has 3 isoforms which differ in the number of immunoglobulin (Ig)-like domains in the extracellular portion of the molecule (48). In the JFEN cell line each of the three isoforms of FGFR1 are expressed (Fig. 1). To verify the authenticity of the PCR products, three bands, which corresponded to each of the predicted isoforms, were extracted from the gel and sequenced. The sequence confirmed that all three isoform PCR products were representative of the reported FGFR1 extracellular domains. No expression was detected by RT-PCR using the primers for FGFR 2 or FGFR 3 (data not shown).

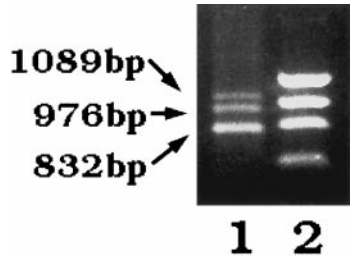

FIG. 1. Expression of FGFR1 in cultured JFEN cells. Shown are the FGFR1 PCR products derived from JFEN cDNA. Three bands corresponding to the three major isoforms of the FGFR1 mRNA, 1098, 976, and 832 bp, are indicated. Molecular weight marker (*Hae* III digested ϕx174) is shown in lane M. PCR product sequences were determined and found to be 100% identical to the cDNA sequence of the FGFR1 isoforms (69). Primer sequences span an intron and did not amplify bands from human genomic DNA (data not shown). Data are representative of two independent experiments.

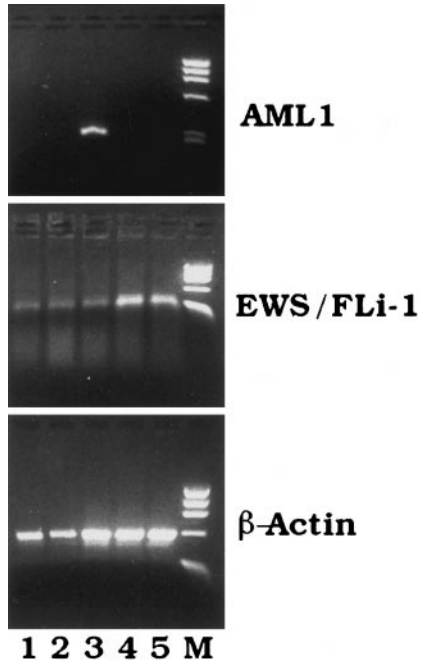

FIG. 2. Expression of *AML*1 and *EWS/Fli*-1 genes in growth factor treated JFEN cells. *AML*1 mRNA was detected exclusively in bFGF-treated JFEN cells in a nested PCR assay (top). RT-PCR products shown represent PCR amplified cDNA from JFEN cells treated with 10 ng/ml of epidermal growth factor (lane 1), TGFα and nerve growth factor (lane 2), bFGF (lane 3), TGF α (lane 4), or saline (lane 5). The *AML*1 nested PCR product size is 284 bp. All factor-treated ONB specimens demonstrated expression of the *EWS/Fli*-1 fusion mRNA characteristic of ONB tumors (middle). JFEN cDNA amplified using β-actin specific primers (bottom) ensured RNA integrity and provided a reference for starting mRNA amounts (33% RNA to cDNA conversion) as previously described (70). Molecular weight marker (*Hae* III digested ϕx174) is shown in lane M. *AML*1 and *EWS/Fli*-1 primer sets span an intron and did not amplify PCR products from human genomic DNA (not shown). Data are representative of three independent experiments.

TABLE 1

Primers Used for RT-PCR Analysis

mRNA	5′ primer	3′ primer	Product size
EWS/Fli-1	TGTTGGGCTTGCTTTTCCGCTC	CCCACTAGTTACCCACCCCAAA	420
FGFR 1	CGCTCTAGAGCAGAACTGGGATGTGGGGCTG	CTCGGATCCAGGGCTTCCAGAACGGTC	1098,976,832
FGFR 2	AACGGCAGTAAATACGGC	GTGAAAGGATATCCCAATAGAATTACC	319
FGFR 3	TTCGACACCTGCAAGCCG	AGCAGGTCGTGGGCAAAC	647
AML1-1	CCAACTTCCTCTGCTCCGTGC	CATCTGACTCTGAGGCTGAGG	518
AML1-2	CCAACTTCCTCTGCTCCGTGC	CTTCGAGGTTCTCGGGGCCCA	284
NGF	CACACTGACGTGCATAGCGT	TGATGACCGCTTGCTCCTGT	389
BDNF	TTCCACCAGGTGAGAGT	ACTAATACTGTCACACACGC	474
NT-3	ATCTTACAGGTGAACAAGGT	TCGGTGACTCTTATGCTCCG	458
GDNF	TGAGCAGTGACTCAAATATG	GTAAACCAGGTTATCATCTA	370
$p75^{NGFR}$	TGGACAGCGTGACGTTCTCC	GATCTCCTCGCACTCGGCGT	370
*Trk*A	GCATCTGGAGCTCCGTGATC	CTCTGCCCAGCACGTCAAGT	564
*Trk*B ECD	AGACACTCAGGATTTGTACTGCC	CCGTGTGATTGGTAACATGTATT	515
*Trk*B TK	AAGACCCTGAAGGATGCCAG	AGTAGTCAGTGCTGTACACG	405
*Trk*C ECD	CCCCCATTTGCGTTATATAAACC	CACACGTGGGGGATAGTAGACA	543
*Trk*C TK	CTACAACCTCAGCCCGACCA	GCTGTAGACATCTCTGGACA	447
β actin	CCCATGCCATCCTGCGTCTG	CGTCATACTCCTGCTTGCTG	320

Note. ECD, extra cellular domain specific; *TK*, tyrosine kinase domain specific.

Expression of EWS/Fli-1 fusion and the human runt (AML1) transcripts in cultured bFGF-treated JFEN cells. ONB tumors are thought to derive from epithelial cells of neuroectodermal origin (3, 8, 11, 12). Thus, we hypothesized that JFEN cells may function like neuroectodermal stem cells and would be capable of differentiating into distinct olfactory precursor cell types like those found in developing olfactory mucosa (49). A marker for such cells is the human runt homologue *AML*1 (50) that is highly expressed in olfactory neuroepithelium of developing mouse embryos (18). To test this hypothesis we treated JFEN cells with factors known to effect neuronal growth/differentiation including EGF, bFGF, TGFα, and NGF and evaluated the expression of *AML*1. To confirm the neuroectodermal nature of this tumor cell line we also evaluated the expression of the *EWS/Fli-1* fusion gene derived from the unique t(11;22)(q24;q12) chromosomal translocation expressed in ONB and Ewings sarcoma (11). Our results show that the ONB cell line expresses the pPNET specific *EWS/Fli-1* fusion mRNA (Fig. 2). Expression of the fusion gene was present in all ONB specimens (Fig. 2, top). Although the amount of the *EWS/Fli*-1 PCR product appeared higher in the TGF α and untreated lanes, this was not consistently observed (data not shown). In contrast, expression of *AML*1 was consistently and exclusively detected in bFGF-treated ONB (Fig. 2, top), in nested PCR assays using the external (*AML1*-1) and internal *AML1* primers (*AML1*-2) listed (Table 1). The PCR products derived from bFGF-treated ONB specimens were sequenced and were 100% identical to the human *AML1* (50) and murine *PEPB2β* (51) runt-domain containing genes. (Four independent experiments, data not shown.)

*Regulated expression of the Trk*A neurotropin receptors but not neurotropins in cultured JFEN cells. Recently, expression of the neurotropins nerve growth factor (NGF), brain-derived neurotropic factor (BDNF), neurotropin 3 (NT-3), glial cell line-derived neurotrophic factor (GDNF), as well as their respective recep-

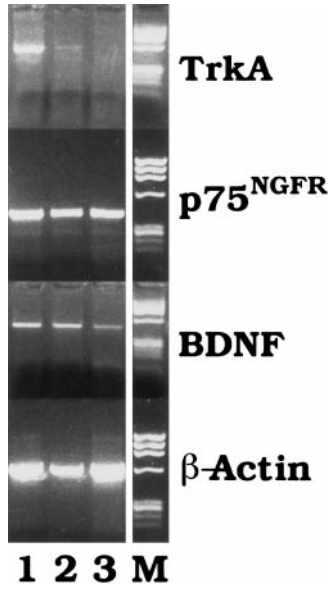

FIG. 3. Expression of neurotropins and neurotropin receptors in ONB. Expression of p75NGFR, BDNF, and *Trk*A mRNAs in JFEN cells were measured by RT-PCR. *Trk*A was highly increased in bFGF-treated cells (lane 1) compared to TGFα (lane 2) or untreated (lane 3) cells. Molecular weight marker (*Hae* III digested ϕx174) is shown in lane M. PCR product sequences were found to be 100% identical to the corresponding genes for each primer set used. Data are representative of three independent experiments.

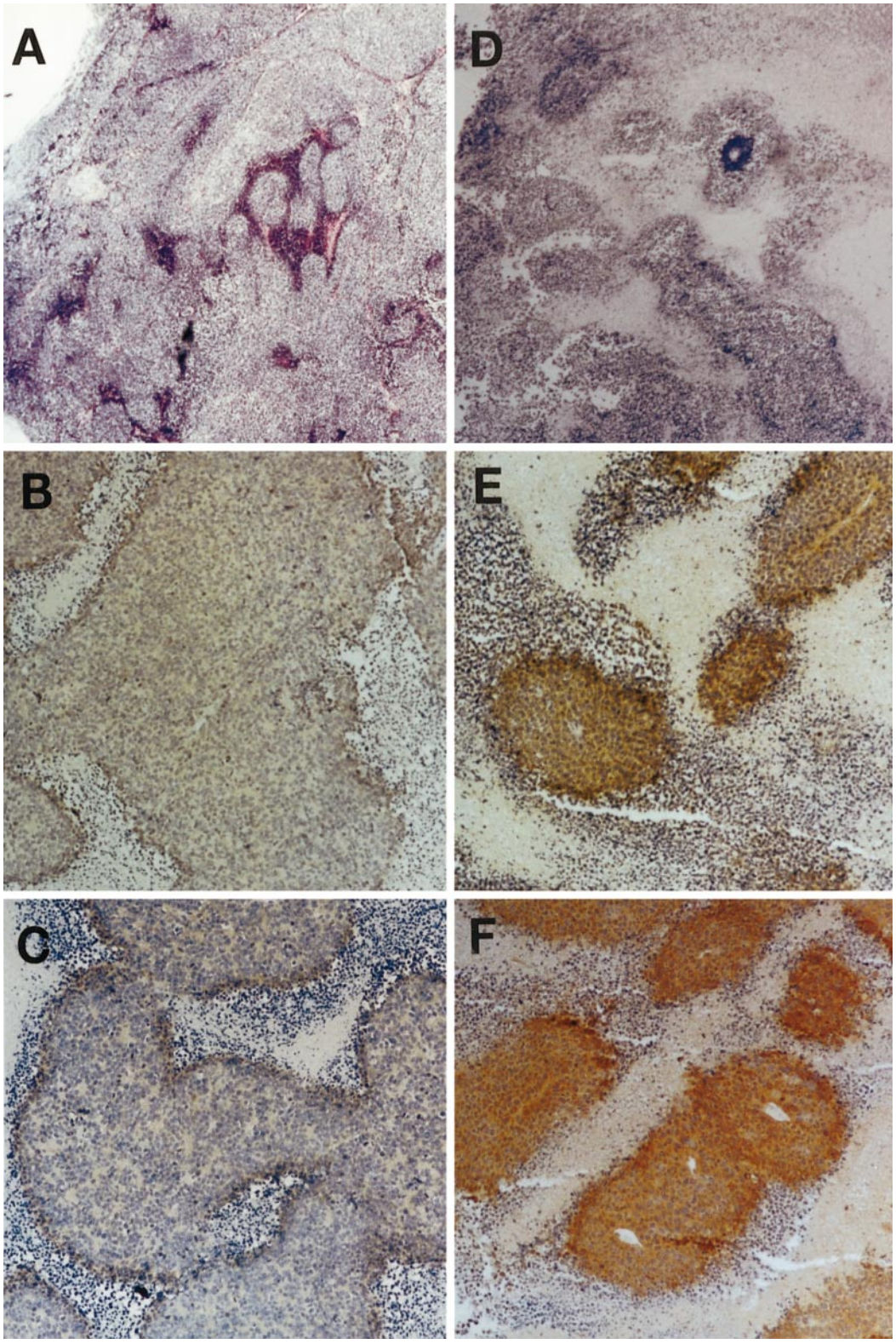

FIG. 4. Treatment of ONB tumors with bFGF. Athymic mice were inoculated with 10^6 JFEN cells as described under Material and Methods. Three weeks following inoculation, tumor-bearing mice were treated with saline (A, B, and C) or with 200 μg bFGF/day for 1 week (D, E, and F). Following treatment, tumors were resected, fixed, and stained with hematoxylin and eosin (A and D) or with antibodies specific for FGFR1 (B and E) or *Trk*A (C and F). Expression of *Trk*A is evident by dark staining in sections derived from treated tumors (F) compared to the light staining observed in untreated tumors (C). Negative control sections (secondary antibody alone) did not show staining in any region examined (not shown). Four tumor-bearing mice were used per treatment group. Data are representative of three independent experiments.

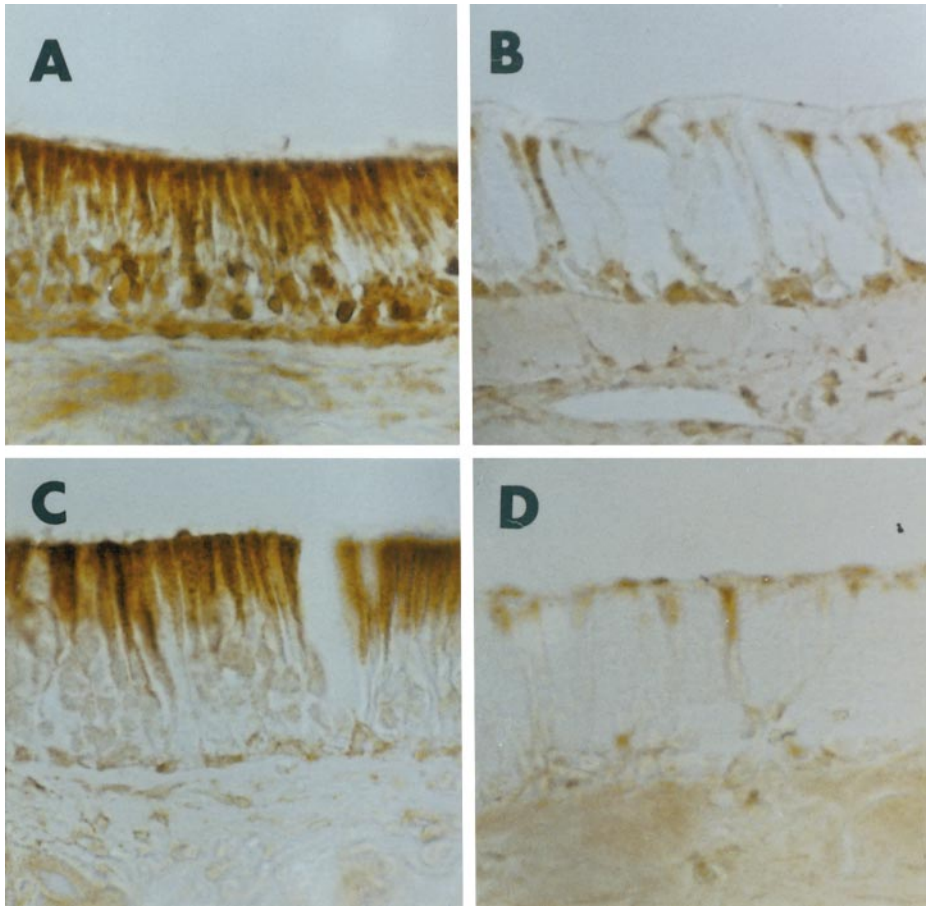

FIG. 5. FGFR1 and *Trk*A expression in olfactory and respiratory epithelium of the human nasal mucosa. Human nasal mucosa stained with FGFR1 (A and B) or *Trk*A specific antibodies (C and D). FGFR1 expression was identified in supporting cells and olfactory neurons in olfactory (A) but not in respiratory (B) epithelium. *Trk*A expression was evident in supporting cells of the olfactory epithelium (C) but not in similar regions of the mucosa devoid of olfactory neurons (D). Controls (secondary antibody alone) showed background staining identical to levels observed in panels B and D (not shown). Data are representative of two independent experiments.

tors $p75^{NGFR}$, *TrkA*, *TrkB*, and *TrkC*, have been studied in rat olfactory epithelium (17) and sensory epithelium of horse vomeronasal organs (52). These studies demonstrated that neurotropin-expressing cells may represent olfactory neuronal precursors at a stem cell stage of development (17). Thus, to evaluate the olfactory stem cell nature of the ONB tumor cell line JFEN following bFGF treatment, we examined the expression of several neurotropins. The JFEN line was found to express *TrkA* after bFGF treatment suggesting a role of this gene product in the differentiation of JFEN cells (Fig. 3, top). Although not modulated by factor treatment, expression of $p75^{NGFR}$ and BDNF was detected in both treated and untreated JFEN cells (Fig. 3). No expression of NGF, NT-3, GDNF, *TrkB*, or *TrkC* gene was detected in any of the JFEN cells by RT-PCR assay (data not shown).

TrkA expression is upregulated in ONB tumors by bFGF treatment in vivo. To evaluate the *in vivo* effects of growth factor treatment, a mouse model of ONB tumors was established by subcutaneous injection of 10^6 JFEN cells into 8-week-old athymic mice. Within one week, tiny nodules appeared under the skin at the injected site. Three weeks after implantation, tumor volumes reached an average of 3.0 ± 1.0 cm^3. At this tumor size, bFGF or normal saline was injected intratumorally. Approximately one week after bFGF therapy, tumors of the treated group stopped growing and were soft when palpated. Tumors of untreated mice continued to grow and invade the peritoneal cavity leading to a large tumor burden and/or moribund state prior to sacrifice (>10 cm^3). Histopathological analysis of the untreated tumors (Fig. 4A) showed lobular and undifferentiated growth patterns characteristic of human ONB (7). In contrast, bFGF-treated tumors showed massive cell damage, leukocyte infiltrations and only scattered viable tumor cell nests were visible in the representative sections (Fig. 4D). Consistent with the *in vitro* experiments, *TrkA* expression was upregulated in the bFGF-treated tumors

(Figs. 4C and 4F). Interestingly, expression of FGFR1 also was upregulated by the bFGF treatment (Figs. 4B and 4E) suggesting that a positive feedback loop may exist between the bFGF ligand and receptor in treated tumors. Unlike *Trk*A, neither *Trk*B nor *Trk*C expression was detected in the treated or untreated ONB tumors (data not shown), consistent with the RT-PCR data of these cells in culture.

Expression of TrkA and FGFR1 in ONB tumors compared to normal human olfactory neuroepithelium. The mammalian olfactory neuroepithelium displays a hierarchical pattern of neuronal maturity, which can be identified by the laminar position of cells within the epithelium (53). The olfactory epithelium is separated from the underlying stroma by a basal membrane, to which a single layer of horizontal basal cells adheres. Superior to this layer are the globose basal cells, (a precursor population of olfactory receptor neurons) situated in a thin band of cells (54). The center of the epithelium is filled with multiple cell layers of mature olfactory receptor neurons, which are ciliated and have extensions nearly reaching the epithelium surface. The top layer of the epithelium contains supporting cells, which are considered non-neuronal (14). To identify cell types within the human olfactory neuroepithelium that correspond to those of the bFGF-treated JFEN cells we examined the expression of *TrkA* and FGFR1 by immunocytochemical analysis of whole human olfactory organs (21). High level of FGFR1 expression was observed in the olfactory epithelium but not in the respiratory epithelium of the nasal cavity (Fig. 5). FGFR1 is expressed in the horizontal basal cells, globose basal cells, olfactory neurons and olfactory supporting cells (Figs. 5A and 5B). *Trk*A was also strongly expressed in the olfactory epithelium but only weakly in the respiratory epithelium. In contrast to FGFR1, *TrkA* is expressed exclusively in the supporting cells, but not in the olfactory receptor neurons, globose basal cells or horizontal basal cells (Figs. 5C and 5D). These data support notion that bFGF-treated JFEN cells differentiate into cells similar to olfactory supporting cells rather than olfactory neurons.

DISCUSSION

We report the successful growth of JFEN cells as subcutaneous solid tumors in athymic mice. Furthermore, we demonstrated that the cytotoxicity of bFGF previously observed *in vitro* (12) was also evident *in vivo* since the treatment of athymic mice bearing advanced, invasive ONB tumors caused tumor differentiation. Only one study has reported the growth behavior of human ONB tissue in athymic mice (9). In that study, a freshly resected uncloned specimen was used for implantation and no continuous tumor passage are treatments were performed. Consequently, neither these tumors nor cells derived from them are available. In the tumor model described herein, however, we have demonstrated the effects of bFGF on tumor cells both *in vitro* and *in vivo* and investigated the mechanism of its antiproliferative effects. We find that bFGF-treated JFEN cells upregulate the FGFR1 prior to differentiation into olfactory supporting cells as measured by the expression of *AML*1 and *Trk*A. Furthermore, the antineoplastic effects of bFGF indicate a new biological property for this family of pleiotropic factors. Consequently, additional studies will be necessary to evaluate the effects of bFGF on additional ONB tumors.

Basic FGF belongs to a family of structurally related heparin-binding proteins, which display a broad range of activities in a diverse spectrum of tissues (46, 55, 56). For example in mouse olfactory epithelium bFGF supports the growth and differentiation of the olfactory bulb (57), but has been shown to limit the growth of olfactory neuronal precursors (58) even though it may support their differentiation (15). Biological responses to bFGF are mediated through specific high affinity transmembrane receptors (FGFR) (46, 55, 56). Four such FGFRs have been identified, FGFR1-4. The most common and widely expressed of these are FGFR1 and FGFR2, while FGFR3 and FGFR4 show a more restriction pattern in the cells/tissues analyzed (44). Our results demonstrate that bFGF-treated JFEN cells express FGFR1 *in vitro* and *in vivo*, which is unregulated after treatment. Furthermore, FGFR1 is expressed strongly in normal olfactory epithelium but not respiratory epithelium, olfactory nerve bundles or other connective tissues of the human nasal cavity. These data suggest that the bFGF-treated JFEN cells represent olfactory supporting cells confirming the olfactory neuroepithelial origin of the ONB (3, 8, 12).

Another bFGF-induced gene identified after ONB differentiation was the human runt homologue *AML*1 (50). The runt gene of *Drosophila* acts in multiple developmental pathways during fly embryogenesis including segmentation, sex determination, and neurogenesis (53, 59, 60). *AML*1 was originally identified because of its association with the t(8;21) translocation found in a variant acute myeloid leukemia (50). Although the physiological function of *AML*1 remains to be determined, recent studies in blood cells suggest that it may function in the control of stem cell differentiation (51). Its role in stem cell differentiation is also supported by the finding that murine runt homologues are expressed following the differentiation of the F9 embryonic stem cell tumor (61). Likewise, studies of the murine runt homologue (*PEPB2α*) have demonstrated expression in the developing olfactory neuroepithelium during olfactory stem cell differentiation (18). In ONB the expression of *AML*1 following bFGF-treatment may thus indicate a comparative stage of differentiation similar to other *AML*1-expressing stem cells. Indeed, the bFGF-treated JFEN cells resemble

*AML*1 expressing precursors within the olfactory epithelium (18).

Other evidence of cellular differentiation induced by bFGF in JFEN cells comes from the expression of *Trk*A and the absence of *Trk* B and C expression. In the rat olfactory epithelium, *Trk*B and *Trk*C are expressed in the olfactory neurons but not in the supporting cells or basal cells, while *Trk*A is expressed in supporting cells but not in olfactory neurons (17). *Trk*A expression was also reported in supporting cells within the sensory epithelium of the horse vomeronasal organ, a tissue closely related to the mammalian neuroepithelium (52). Consistent with these studies, we find *Trk*A expression exclusively in the supporting cells within the human neuroepithelium, but not in the olfactory neurons. Thus, the human JFEN cells expressing *Trk*A after bFGF treatment may represent these supporting cells within the human olfactory neuroepithelium.

Interestingly, JFEN cells expressed mRNA for BDNF in both the bFGF-treated and untreated groups. Although we could not detect the expression of *Trk*B, the preferred receptor for BDNF (62–64), JFEN cells did express $p75^{NGFR}$. As a low affinity receptor, $p75^{NGFR}$ has been reported to bind BDNF and other neurotropins (65). Furthermore, since BDNF has been reported to promote the survival and proliferation of neuronal precursor cells (63), these results suggest an autocrine or paracrine loop in the control of ONB cell growth. Thus, one possibility is that the binding of bFGF to its receptor causes differentiation and the cessation of cell growth through the dysregulation of the BDNF-$p75^{NGFR}$ loop. Future studies will be necessary to address this notion.

The athymic mouse model of human ONB described in this study provide the first system for analyzing factors effecting the growth and progression of this potentially underdiagnosed pPNET (3, 11). In addition, by using this model to measure the effectiveness of anticancer treatments, new applications and/or methods may be developed for improving patient survival. Moreover, the availability of large amounts of purified recombinant bFGF has enabled researchers to test its therapeutic use in the treatment of wounds without detectable side effects (66, 67). Consistent with these previous findings, the intratumoral injections used in the treatment of ONB tumor-bearing mice were nontoxic. Thus combining new drug delivery systems which enable targeted drug treatments (68), along with the treatment of bFGF on other ONB tumors in this mouse model will provide a more comprehensive analysis of the potential of bFGF as a treatment for ONB in afflicted individuals.

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