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SHORT REPORT

The low-affinity neurotrophin receptor, p75, is upregulated in ganglioneuroblastoma/ganglioneuroma and reduces tumorigenicity of neuroblastoma cells *in vivo*

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Neuroblastoma, the most common extracranial tumor of childhood, is derived from neural crest progenitor cells that fail to differentiate along their predefined route to sympathetic neurons or sympatho-adrenergic adrenal cells. Although expression of the high-affinity neurotrophin receptors, TrkA and TrkB, is of major importance in neuroblastoma, the significance of the expression of the low-affinity neurotrophin receptor, p75, is unclear. Here, we analyzed immunohistochemically expression of p75 on a tissue microarray of 93 primary neuroblastic tumors and assessed the functional consequences of p75 expression in neuroblastoma cell lines. We found the p75 receptor protein to be expressed in neuroblastic cells of ganglioneuromas/ganglioneuroblastomas as well as differentiating neuroblastomas, but not in poorly differentiated neuroblastomas. In an unrelated cohort of 110 neuroblastic tumors, p75 mRNA expression levels correlated with differentiation, and patients with tumors that expressed p75 at high levels had an increased event-free and overall survival. In addition, we did not detect p75 expression in 8 established neuroblastoma cell lines examined with FACS analysis. These cell lines exhibited an undifferentiated morphology, and were all derived from aggressive, high-stage neuroblastomas. Ectopic p75 expression in the SH-SY5Y neuroblastoma cell line significantly reduced proliferation, increased the fraction of apoptotic cells *in vitro* and resulted in a loss of tumorigenicity in nude mice. Taken together, our data suggest that expression of the p75 low-affinity neurotrophin receptor is correlated with a reduced level of tumorigenicity, and that induction of p75 expression may be an option to revert features of an aggressive tumor phenotype.

Key words: neuroblastoma; p75; TMA; low-affinity neurotrophin receptor; CD271

Neuroblastoma is the most common extracranial tumor of childhood and accounts for ~ 15% of all childhood cancer deaths.[1,2] This embryonal tumor originates from neural crest progenitor cells that fail to differentiate along their predefined route to sympathetic neurons or sympatho-adrenergic adrenal cells.[3] The exact developmental stage at which cells undergo malignant transformation remains elusive. Neurotrophins mediate survival, differentiation, growth and apoptosis of neurons and neuronal progenitor cells by binding to the tyrosine kinases TrkA, TrkB and TrkC, or the p75 low-affinity neurotrophin receptor.[4] In analogy to their nonmalignant cells of origin, neuroblastoma cells do, indeed, differentially express the TrkA and TrkB neurotophin receptors.[5,6] Expression of TrkA and TrkB is mutually exclusive in neuroblastoma cells, and is a major determinant of outcome in neuroblastic tumors. TrkA-expressing neuroblastomas rarely metastasize, have low angiogenic properties, are sensitive to chemotherapy, are prone to undergo regression or spontaneous maturation, and patients are cured with little or no therapy. In contrast, TrkB-expressing neuroblastomas are highly invasive, metastasize early, have high angiogenic properties, are resistant to therapy and patients often experience rapid disease progression. Despite highly aggressive therapy, fatal outcome frequently occurs. Most features of tumor biology that correlate with TrkA or TrkB expression *in vivo* have also been functionally linked to these receptor tyrosine kinases (reviewed in Ref. 7). Although TrkA and TrkB expression and the functional implications thereof have been studied extensively in neuroblastoma, the expression and function of the p75 low-affinity neurotophin receptor in neuroblastoma was rarely addressed.[8–13] Although initially described as a low-affinity receptor, p75 has the same affinity for NGF as TrkA, but lacks the intrinsic tyrosine kinase activity of other Trks and is able to bind all neurotrophins and proneurotrophins.[14] P75NTR is a member of the tumor necrosis factor receptor superfamily of transmembrane proteins that share significant homology in their extracellular domains. Members of the TNF receptor family, including p75, also have a cytoplasmic death domain.[14] Under physiological conditions, p75 is differentially expressed in subsets of developing neurons, and here the function ranges from controlling trophism to programmed cell death.[15,16] In addition, p75 has broad regulatory functions in regulating death and survival upon neuronal injury, but also in many other nonneuronal cell types.[15,16] Expression of p75 has been reported in several cancer cell populations, including thyroid carcinoma,[17] prostate carcinoma,[18,19] melanoma,[20,21] gastric carcinoma[22] and retinoblastoma.[23–25] In most contexts, p75 acts as a tumor suppressor gene, inducing apoptosis and suppressing invasion and metastasis. Nevertheless, p75 induces invasion and metastasis in some other maligancies, including glioma[26] and melanoma.[20] Ectopic p75 expression in the neuroblastoma cell lines, BE2c and SY5Y, induced apoptosis *in vitro*, which was, at least in part, rescued by TrkA coexpression.[8–10,12] Expression of p75 and its function *in vivo* have not yet been sufficiently addressed.[11] With the introduction of the cancer stem cell concept, the search for a stem cell compartment within neuroblastoma has also pointed some interest toward neural crest stem cells, which are most likely the nonmalignant counterparts of neuroblastoma stem cells.[3] Neural crest stem cells are characterized by coexpression of p75 and Sox10,[27] but to date, no p75-positive, putative neuroblastoma stem cell has been isolated from primary neuroblastomas. Nevertheless, Hansford *et al*.[28] reported p75 positivity of neuroblastoma cells from a bone marrow metastasis, cultured as spheroids.

Additional Supporting Information may be found in the online version of this article.

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TABLE I – SUMMARY OF CLINICAL AND MOLECULAR DATA

No. tumors TMA	93
NB pd	57
NB diff	15
GN/GNB	21
No. tumors/w follow up	64
EFS	48
Event	16
Stage ½	34
Stage 3	10
Stage 4	17
Stage 4s	3

NB, neuroblastoma; GN, ganlioneuroma; GNB, ganglioneuroblastoma; pd, poorly differentiated; diff, differentiating; EFS, Event free survival; tumors/w follow up, tumors for which follow up information is available.

Considering the major importance of TrkA and TrkB in neuroblastoma biology, as well as the functional importance of p75 in regulating apoptosis in the developing neural crest, we asked if p75 plays a role in neuroblastoma pathogenesis *in vivo*. Here, we report that expression levels of p75 correlate with neuroblastoma differentiation *in vivo*. We ectopically expressed p75 in SY5Y neuroblastoma cells, and observed induction of apoptosis as well as decreased proliferation. Furthermore, p75-transfected SY5Y cells lost tumorigenicity in nude mice, suggesting that p75 acts as a tumor supressor in neuroblastoma *in vivo*.

Material and methods

Tissue microarrays

A tissue microarray (TMA) was prepared from formalin-fixed, paraffin-embedded tissue specimens of 93 primary neuroblastic tumors (72 neuroblastomas, 21 ganglioneuromas/ganglio-neuroblastomas) selected from the archival files from the Institute of Pathology, University of Bonn. All tumors were surgically obtained from patients at the time of diagnosis before treatment initiation. Three different tissue cores representative of the respective tumor were arrayed from formalin-fixed, paraffin-embedded tissue blocks using a manual device (Beecher Instruments, Sun Prairie, WI). Four micrometer paraffin sections were cut from every TMA, and used for subsequent immunohistochemical analysis within 1 week. The German Neuroblastoma Study Center provided complete clinical and diagnostically important molecular data for neuroblastoma samples (Table I). Clinical follow-up was available for only 5 of 21 ganglioneuromas/ganglioneuroblastomas, which all were treated by initial surgery alone, and not included into a clinical study. For 13 of 72 neuroblastomas, no follow-up $>$ 5 years was available. Informed consent was obtained from all patients or parents within the German Neuroblastoma Trial for the use of neuroblastoma tumor samples for research purposes.

Immunohistochemistry

After antigen retrieval immunohistochemical stainings were performed on 4 μm sections with a Techmate 500 immunostainer (DAKO, Glostrup, Denmark). NGFR antibody (Novocastra, Newcastle upon Tyne, UK) was used at a dilution of 1:500. The antigen-antibody binding was visualized by means of the avidin-biotin complex (ABC-method) using AEC (3-amino-9-ethylcarbazol) as chromogen. Immunoreactions were visualized with the ABC-complex diluted 1:50 in PBS (Vectastain, Vector, Burlingame, CA). The bona fide positive controls for p75 expression are schwann cells. As schwann cells are present in ganglioneuroma and ganglioneuroblastoma, this stromal cell component serves as an internal positive control in our analysis. In addition, we used stainings with isotype primary antibody as negative control to ensure specificity of the staining reaction. P75 immunostaining was evaluated using a semi-quantitative scoring system. Briefly, the p75 staining intensity of neuroblastoma cells (in neuroblastoma) and ganglionic cells (in ganglioneuromas) was assessed and scaled 0–3 (0 = no expression, 1 = weak expression, 2 = moderate expression, 3 = strong expression). All slides were reviewed independently by 2 pathologists in a blinded fashion.

Affymetrix microarray analysis

RNA was isolated from 110 primary, untreated tumors. Reverse transcription, labeling of total RNA and subsequent hybridization to Affymetrix U133v2 chips were performed according to the manufacturer's protocols, and as previously described.[29] Data processing and normalization were performed according to standard procedures using the MAS5 algorithm.

Statistics

Statistical analysis was performed using R statistical language (www.r-project.org). A Kruskal-Wallis test was used to compare different groups [differentiation, MYCN status, event-free survival (EFS) *vs*. event, tumor stage]. The survival package of R was used for Kaplan-Meier analysis. Event was defined as either local or distant relapse or as disease progression. Cut-off for Kaplan-Meier analysis was 100 relative expression units, as expression levels below 100 were considered as absent expression.

Cell culture

All neuroblastoma cell lines were grown in RPMI medium supplemented with 10% FCS, L-glutamine, penicillin, streptomycin and amphotericin B as described. Neither BDNF nor NGF was detectable in the FCS by ELISA (data not shown). SH-SY5Y (referred to in the text as SY5Y) is a subclone from the human SK-N-SH neuroblastoma cell line described previously. The full-length p75 cDNA was cloned into the pTet retroviral expression vector (Clontech, Palo Alto, CA).[10] Transfected cells were selected with 200 μg/ml hygromycin (Sigma, Munich, Germany), and subcloned by limited dilution to obtain single-cell clonal lines. As negative controls, SY5Y cells were infected with a retrovirus bearing the empty retroviral vector (SY5Y(c)). Sequencing confirmed the identity of all transfectants.

mRNA expression analysis by RT-PCR

The mRNA expression of p75 was analyzed by RT-PCR using specific biotinylated primers (forward primer: 5′-tcgtggagagtctgtgcagt-3′, reverse primer: 5′-tggacaggaagtgtggtcag-3′). Total RNA was extracted with the RNeasy Kit (Qiagen, Valencia, CA), reverse transcribed and amplified for 20 cycles on a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Watertown, MA) using the Superscript amplification system (Life Technologies, Inc.). The PCR products were run on a 6% polyacrylamide gel and transferred to a nylon membrane (Hybond N, Amersham, IL). Biotinylated signals were detected using the Southern Light Detection system (Tropix) and exposed to X-ray film. GAPD was co-amplified as a housekeeping gene to normalize p75 expression levels.

FACS analysis of p75 expression

Expression of p75 protein was monitored by FACS analysis. Cells were trypsinized, washed with PBS and incubated with the anti-p75 Ab3125 mouse monoclonal antibody (Abcam, Cambridge, UK) or isotype control. After 20 min incubation, cells were washed with PBS, incubated with the FITC-conjugated secondary antibody (SoutherBiotech, Birmingham, AL) for 15 min and analyzed in a Beckman Coulter EpicsXL4 using EXPO32 software (Beckman Coulter, Krefeld, Germany).

MTT cell viability assay

Cells were seeded at a density of 2500 cells per well, and cultured in standard medium, replaced daily. An MTT assay was performed according to the manufacturer's protocol (Roche, Mannheim, Germany).

BrdU proliferation assay

Cells were cultured with BrdU-labeling solution for 24 hr, followed by washing with PBS. They were fixed with 70% ethanol in hydrochloric acid for 30 min, and incubated with monoclonal antibody against BrdU for 90 min, followed by peroxidase substrate. The BrdU absorbance in each well was measured directly in a spectrophotometric microplate reader at 430 nm.

Annexin V apoptosis assay

Apoptotic cell death was confirmed by staining the cells with annexin V (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. Apoptosis was then monitored by flow cytometric analysis with a FACScan using CELLQuest software (Becton-Dickinson Immunocytometry Systems, Mountain View, CA).[10] Assays were performed twice with similar results.

Growth of xenograft tumors in nude mice

Neuroblastoma cells were cultured to 80% confluency, harvested, and suspended in Matrigel (BD Bioscience, Germany). Eight week-old female athymic NCR (*nu*/*nu*) mice were inoculated s.c. in the flank with 2.5×10^7 SY5Y-p75#1 cells, SY5Y-p75#2 cells or 2.5×10^7 control cells in 200 μl Matrigel (sample sizes: 8 mice inoculated with SY5Y-p75, *i.e.*, 4 mice inoculated with SY5Y-p75#1 and 4 mice SY5Y-p75#2, 10 mice inoculated with control cells). In addition to the before mentioned cohorts, 2 mice each were injected with 1×10^4, 1×10^5, 1×10^6 and 1×10^7 cells. Tumor size was measured with a digital calliper to calculate tumor volume. Mice were sacrificed at day 180, or if tumor volume was >1000 mm^3.

Results

P75 expression correlates with differentiation and survival

To assess the potential role of p75 in neuroblastoma, we first analyzed p75 protein expression in a cohort of primary neuroblastic tumors. These included neuroblastomas as well as their more benign and differentiated derivates, ganglioneuroblastomas and ganglioneuromas. A TMA was established incorporating 93 primary, untreated neuroblastic tumors, including 21 ganglioneuromas/ganglioneuroblastomas and 72 neuroblastomas (Table I). No p75 expression was detected in poorly differentiated neuroblastoma cells by immunohistochemistry (0/57 tumors, Supp. Info. Table 1), whereas significantly higher p75 expression levels were detected in differentiating neuroblastoma (4/15 tumors, Supp. Info. Table 1) (*t*-test, $p = 8.4 \times 10^{-05}$, Fig. 1*b*), and in the neuroblastic tumor cells of ganglioneuroblastomas or ganglioneuromas (13/21 tumors, Supp. Info. Table 1) (*t*-test, $p = 1.7 \times 10^{-10}$, Fig. 1*b*). Because of the low numbers of ganglioneuroblastomas (all of the intermixed type) and ganglioneuromas, and due to the similar clinical and biological behavior of ganglioneuroblastomas and ganglioneuromas, both subtypes were grouped together and no further subgroup analysis was performed. In contrast to its absence in poorly differentiated neuroblastoma cells, p75 was expressed at different levels in nonmalignant cells of all types of neuroblastic tumors, such as perivascular cells, septal cells, and especially, Schwannian stromal cells (Fig. 1*a*, Supp. Info. Table 1). Similar results were obtained in an independent cohort of 110 neuroblastic tumors previously analyzed on Affymetrix microarrays (Fig. 1*c*). Kaplan-Meier analysis revealed that high p75 mRNA expression was associated with event-free and overall survival (EFS/OS) in the latter cohort ($p = 0.011$ and 0.027, respectively, Fig. 1*d* and Supp. Info. Fig. 1). The cut-off for Kaplan-Meier analysis was 100, *i.e.*, background level expression of p75 mRNA *vs.* significant p75 mRNA expression. This cut-off turned out to be more valid than a cut-off separating neuroblastic tumors with low and intermediate *vs.* high p75 mRNA expression (data not shown). P75 expression did not correlate with stage, clinical course or outcome in the first cohort (Kruskal Wallis Test, Supp. Info. Table II) or with stage in the second cohort (data not shown).

P75 is absent in neuroblastoma cell lines derived from high-stage tumors

P75 expression was analyzed in 8 neuroblastoma cell lines, all derived from aggressive, high-stage primary neuroblastomas and demonstrating an undifferentiated morphology in cell culture. Flow cytometric analysis did not detect expression in any cell line analyzed (Fig. 2*a*).

Ectopic p75 expression reduces viability and induces apoptosis in vitro

No p75 protein was detectable at the cell surface of human SY5Y neuroblastoma cells by flow cytometric analysis (Fig. 2*a*). SY5Y cells were retrovirally transfected with a cDNA encoding p75 or with an empty retroviral vector. After obtaining single-cell clones, stable expression of p75 was confirmed using flow cytometry (Fig. 2*a*) in clones harboring p75 cDNA, whereas no expression was detected in control cells. Consistent with this observation, the single cell clones SY5Y-p75#1 and SY5Y-p75#2 expressed high levels of p75 mRNA, whereas p75 mRNA was hardly detectable in the control cells (Fig. 2*b*). Cell growth was assessed in the presence and absence of the neurotrophin, NGF, using the MTT cell viability assay. The increase in viable cells over a time-course of 12 days was significantly lower in p75-expressing SY5Y cells than in control cells (Fig. 2*c*). The presence of NGF did not influence the number of viable cells in p75-expressing SY5Y cells or control cells. To analyze whether decreased proliferation or increased apoptosis resulted in the difference in cell viability, we analyzed proliferation rate and apoptosis in the presence and absence of the neurotrophin NGF. BrdU incorporation over 24 hr was moderately decreased in p75-expressing SY5Y cells compared with p75-negative control cells, as measured by BrdU incorporation ELISA (Fig. 3*a*). In contrast, a clear increase in the number of apoptotic cells was observed in an annexin V assay (Fig. 3*b*). These effects were independent of the presence or absence of exogenous neurotrophin NGF (Figs. 2*d*, 3*a*, and 3*b*). However, we cannot rule out the possibility that the cells may be expressing a neurotrophin and hence activating p75 by an autocrine mechanism.

Ectopic p75 expression inhibits xenograft tumor growth

A xenograft mouse model was used to assess the effect of p75 on neuroblastic tumors *in vivo*. Nude mice (nu/nu) were subcutaneously injected with 1×10^4, 1×10^5, 1×10^6, 1×10^7 (2 mice each) or 2.5×10^7 SY5Y neuroblastoma cells (10 mice) in the flank or with up to 2.5×10^7 SY5Y-p75 cells (8 mice inoculated with 2.5×107 SY5Y-p75, *i.e.*, 4 mice inoculated with 2.5×10^7 SY5Y-p75#1 cells and 4 mice inoculated with 2.5×10^7 SY5Y-p75#2 cells). Tumor growth was observed in all mice injected with SY5Y neuroblastoma cells regardless of the number of cells inoculated (10^4–2.5×10^7 cell/mouse), and the time necessary to reach a tumor volume of 1000 mm^3 inversely correlated with the number of cells injected (data not shown). No tumor growth was observed in mice injected with either SY5Y-p75#1 or SY5Y-p75#2 cells in any cell number during 180 days. Mice were sacrificed on day 180, and necropsy revealed no evidence of tumor cells (Fig. 4). We therefore concluded that ectopic expression of p75 reduces or abrogates tumorigenecity of SY5Y neuroblastoma cells ($p = 2.2 \times 10^{-05}$, Fisher's exact test, comparison of 8 mice injected with 2.5×10^7 SY5Y cells ectopically expressing p75 with 10 mice injected with 2.5×10^7 SY5Y without ectopic p75 expression).

Discussion

Here, we report that p75 expression correlates with differentiation in neuroblastic tumors, and that p75 is not expressed in neuroblasts of primary, poorly differentiated neuroblastomas. We then analyzed the functional consequence of ectopic p75 expression in SY5Y neuroblastoma cells. Although our *in vitro* findings are in

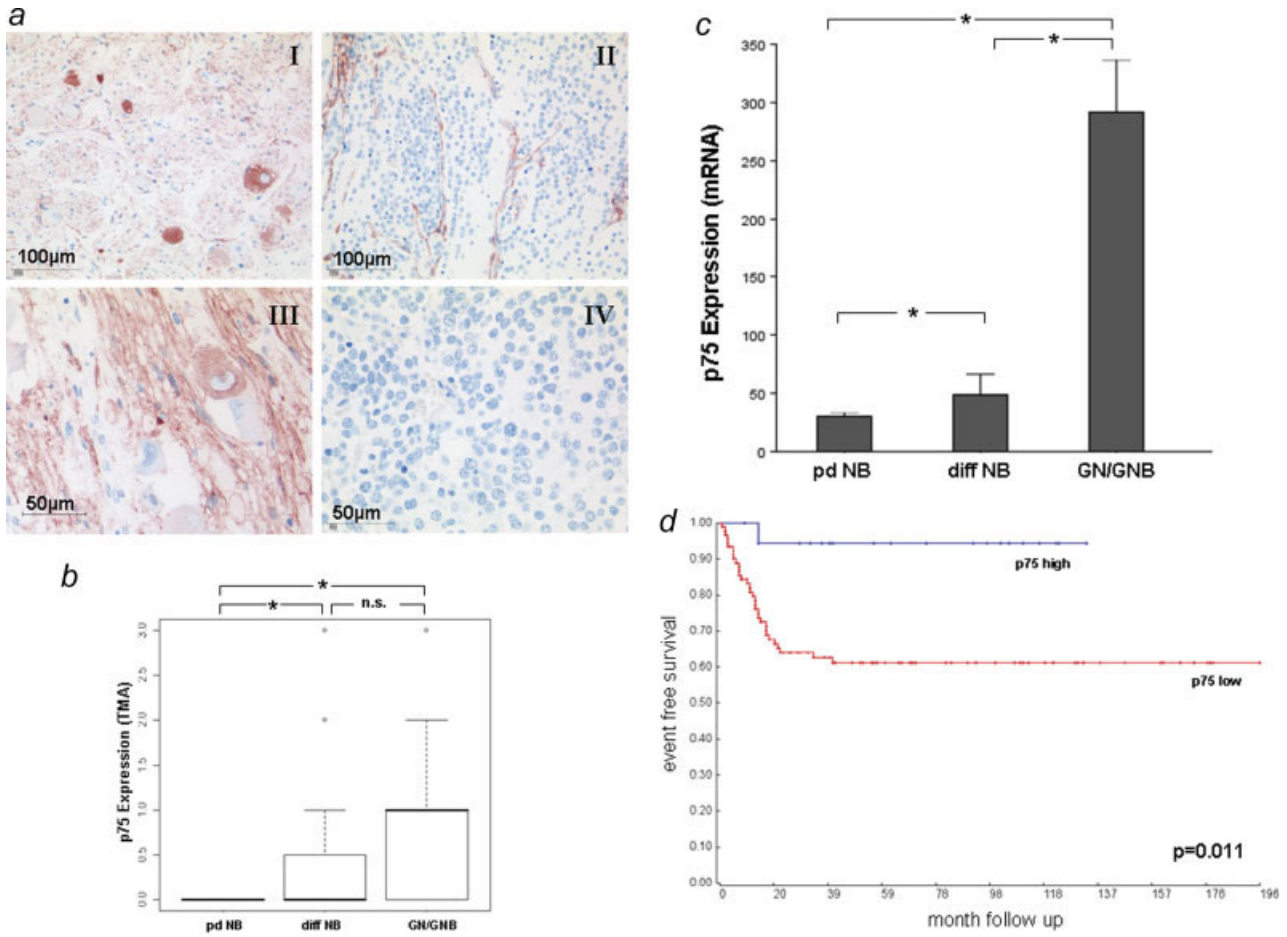

FIGURE 1 – (*a*) Immunohistochemical staining of p75 in neuroblastic tumors. In poorly differentiated neuroblastomas (II and IV), no p75 staining is observed in any tumor cells, although perivascular and septal stromal cells express p75. In contrast, in benign ganglioneuromas (I and III), p75 staining is intense in both tumor and stromal cells. Note that Schwannian stroma does display bright immunoreactivity for p75 (I/III) and thereby serves as an internal positive control. (*b*) A tissue microarray with 93 primary neuroblastic tumors was utilized to analyze p75 expression in neuroblastoma and its benign derivates. Expression was absent in poorly differentiated neuroblastomas (NB pd) and significantly higher in differentiated neuroblastomas (NB diff) or ganglioneuroblastomas/ganglioneuromas (GNB/GN). Differences reaching a statistical significance of $p < 0.01$ are marked with "*." (*c*) p75 mRNA expression in an independent cohort of 110 neuroblastic tumors analyzed with Affymetrix microarrays. Differences reaching a statistical significance of $p < 0.01$ are marked with "*." (*d*) Kaplan-Meier analysis of 110 neuroblastic tumors demonstrates that high p75 mRNA expression levels are associated with event-free survival (EFS). Cut-off low *vs.* high p75 mRNA expression = 100 relative expression units.

line with previous reports, we provide first evidence that ectopic p75 expression abrogates tumorigenicity of SY5Y neuroblastoma cells in nude mice.

The starting point of our study was the analysis of p75 expression in primary neuroblastic tumors, including neuroblastoma, but also in its benign stroma-rich derivates, ganglioneuroblastoma and ganglioneuroma.[30] This allowed analysis of p75 expression in the entire spectrum of neuroblastic differentiation. Immunohistochemistry allowed the attribution of p75 expression to specific cell types found within the tumors. This is of special interest for p75, as p75 is strongly expressed in Schwann cells.[15] Consistently, we detected strong p75 expression in the Schwannian stroma of neuroblastic tumors. As the origin and nature of Schwannian stroma is still a matter of debate,[31–33] we consider it essential to attribute expression of p75 to neuroblasts and (schwannian-) stroma cells separately. This is of utmost importance, as it is known that the functional implication of p75 is highly context dependent, and p75 might induce opposing phenotypes in different cellular backgrounds. It is, therefore, important to stress that with increasing differentiation, p75 was not only expressed in the larger mass of Schwannian stroma, but also in the neuroblastic tumor cells with neuronal differentiation. In contrast, no p75-positive neuroblasts were detected in primary poorly differentiated neuroblastomas. Especially in light of the current efforts to identify neuroblastoma stem cells, this finding is of high importance (see below). Although neuroblasts were negative, perivascular and septal stromal cells in poorly differentiated neuroblastomas did express p75 at different levels. This is consistent with the expression pattern observed in several other tumors (*e.g.*, Ref. [34]), and might also reflect some Schwann cells or Schwannian progenitors residing in the septae of poorly differentiated neuroblastomas. Our findings are supported by a previous report from Fanburg-Smith *et al.*,[11] who analyzed p75 expression in 1150 tumors, including some neuroblastomas and ganglioneuromas. Additionally, we reproduced our results in a second cohort of neuroblastic tumors on mRNA levels, although attribution of p75 expression levels to either neuroblasts or stroma cells was not feasible in this cohort. Although high p75 expression levels were significantly correlated with EFS in the second cohort, correlation did not reach significance in the first cohort. This is most likely because we lacked clinical information for most stroma-rich tumors in the first cohort.

Consistent with the lack of p75 expression in poorly differentiated neuroblastomas, p75 expression was not detected in neuroblastoma cell lines, as neuroblastoma cell lines have only been

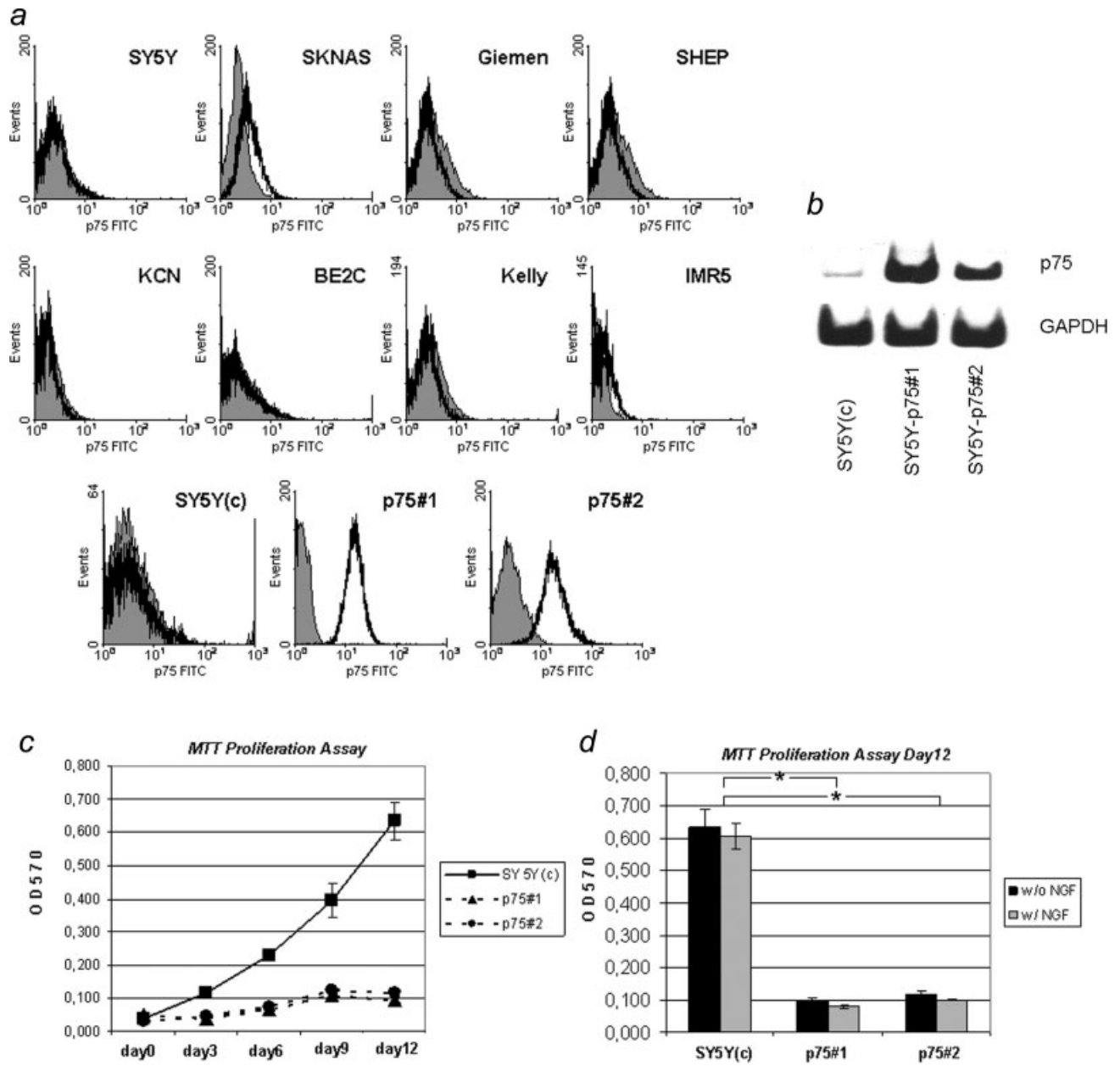

FIGURE 2 – (*a*) Flow cytometric analysis of p75 expression in neuroblastoma cell lines, and in independent single-cell subclones of the SY5Y neuroblastoma cell line stably transfected with a cDNA encoding p75. (*b*) RT-PCR p75 mRNA expression analysis of SY5Y neuroblastoma cells ectopically expressing p75 and control cells. (*c*) MTT cell viability assay of two independent SY5Y subclones ectopically expressing p75 and control cells detected a significant reduction in cell number in p75-positive clones. (*d*) No effect of NGF treatment was detectable in the MTT assay. Differences reaching a statistical significance of $p < 0.01$ are marked with "*."

successfully established from the most aggressive high-grade tumors. To further study the function of p75 in neuroblastoma cells, we established several independent clones of neuroblastoma cell lines ectopically expressing p75. Ectopic expression of p75 led to a decreased cell viability, and induced high levels of apoptosis. This was previously reported by another group using the SK-N-BE neuroblastoma cell line.[8,12] In addition to the clear increase in apoptosis, we observed a moderate reduction in cell proliferation, as detected in an ELISA-based BrdU incorporation assay. Although *in vitro* analysis of cells ectopically expressing p75 revealed some insight into the functional importance of p75 expression in neuroblastoma, analysis of the tumorigenicity of the respective cells *in vivo* is necessary to allow assessment of the effect of p75 on all aspects of tumor biology. Here, we provide the first report that p75 expression abrogates tumorigenicity in neuroblastoma cells *in vivo*. This appears to be a qualitative rather than a quantitative effect, as we were not able to overcome the effect by increasing the number of grafted SY5Y-p75 neuroblastoma tumor cells from 1×10^4 to 1×10^7 and finally to 2.5×10^7 cells, even though as few as 1×10^4 SY5Y control cells resulted in tumors (Fig. 4, data not shown).

With growing interest in isolation and analysis of tumor stem cells, analyzing expression and function of p75 is of high importance. The neural crest stem cell is characterized by expression of p75 and Sox10,[27,35] and thus, an obvious hypothesis is that these may also be markers of neuroblastoma stem cells. Consistent with this hypothesis, Hansford *et al.*[28] found that neuroblastoma cells

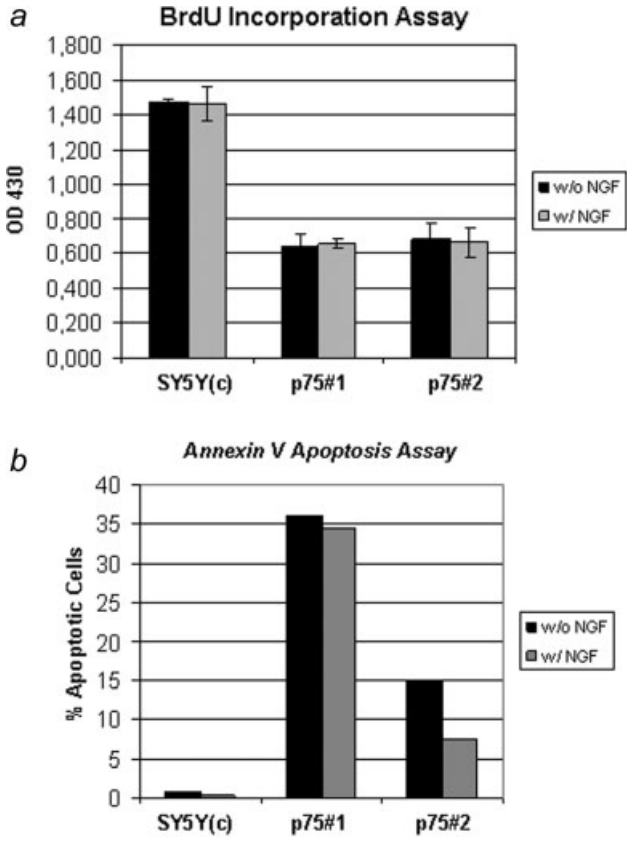

Figure 3 – (*a*) ELISA-based BrdU incorporation assay detected a moderate decrease in proliferation of p75-positive SY5Y cells in comparison with control cells. Differences reaching a statistical significance of $p < 0.01$ are marked with "*." (*b*) Ectopic expression of p75 led to a clear increase in apoptosis in independent single-cell clones compared with p75-negative control cells. Differences reaching a statistical significance of $p < 0.01$ are marked with "*."

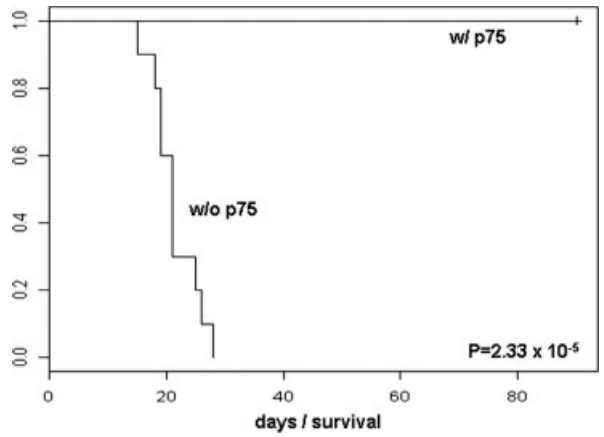

Figure 4 – Kaplan-Meier analysis of survival of nude mice xenografted with 2.5×10^7 SY5Y cells ectopically expressing p75 (8 mice, *i.e.*, 4 mice xenografted with SY5Y-p75#1 and 4 mice xenografted with SY5Y-p75#2) or 2.5×10^7 control cells (10 mice). Although no tumor growth was observed in mice xenografted with p75-positive cells, all mice xenografted with p75-negative control cells developed tumors. Mice were sacrificed when the tumor volume reached 1000 mm^3.

isolated from bone marrow and cultured under special conditions as spheroids were tumorigenic in mice. In addition, p75 was expressed in these cells. We did not detect such cells in the aggressive, poorly differentiated variant of neuroblastoma. However, we cannot exclude the existence of a stem cell compartment that consists of very few p75 positive cells that were not detectable in our analysis.

Taken together, we suggest that p75 expression, at least in primary neuroblastic tumor cells, accompanies differentiation into a benign tumor and is functionally linked to a loss of tumorigenicity in a xenograft mouse model.

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