# Expression of TWEAK/Fn14 in neuroblastoma: Implications in tumorigenesis

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**Abstract.** Tumor necrosis factor-like weak inducer of apoptosis (TWEAK), a member of the tumor necrosis factor (TNF) family of cytokines, acts on responsive cells via binding to a cell surface receptor called Fn14. TWEAK binding to an Fn14 receptor or constitutive Fn14 overexpression has been shown to activate nuclear factor κB signaling which is important in tumorigenesis and cancer therapy resistance. In the present study, we demonstrate that TWEAK and Fn14 are expressed in neuroblastoma cell lines and primary tumors, and both are observed at increased levels in high-stage tumors. The treatment of neuroblastoma cell lines with recombinant TWEAK *in vitro* causes increased survival, and this effect is partially due to the activation of NF-κB signaling. Moreover, TWEAK induces the release of matrix metalloprotease-9 (MMP-9) in neuroblastoma cells, suggesting that TWEAK may play a role in the invasive phase of neuroblastoma tumorigenesis. TWEAK-induced cell survival was significantly reduced by silencing the TWEAK and Fn14 gene functions by siRNA. Thus, the expression of TWEAK and Fn14 in neuroblastoma suggests that TWEAK functions as an important regulator of primary neuroblastoma growth, invasion and survival and that the therapeutic intervention of the TWEAK/Fn14 pathway may be an important clinical strategy in neuroblastoma therapy.

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## Introduction

The tumor necrosis factor (TNF) superfamily consists of numerous cytokine ligands and receptors that regulate many biological processes, including cell proliferation and survival, and their balance is important in maintaining normal cellular functions (1,2).

TNF superfamily ligands are mostly expressed as type II transmembrane proteins that can be processed into smaller soluble proteins exerting a wide variety of biological effects by binding their cognate family of TNF receptors (1,2). Tumor necrosis factor-like weak inducer of apoptosis (TWEAK), a member of the tumor necrosis factor superfamily, was first described as an inducer of apoptosis in transformed cell lines (3). It has later become apparent that TWEAK is a multifunctional cytokine that regulates survival (4), proliferation (5-7), migration (6,8,9), differentiation (10,11), and induces apoptosis in certain tumor cell lines (12,13). TWEAK acts on responsive cells via binding to an inducible high affinity cell surface receptor known as fibroblast growth factor-inducible 14 (Fn14) (14). It has been demonstrated that TWEAK binding to Fn14, or constitutive Fn14 overexpression, activates nuclear factor κB (NF-κB) signaling pathway, which is known to play a pivotal role in immune- and inflammatory processes, oncogenesis and cancer therapy resistance (15,16).

The aberrant expression of both TWEAK and Fn14 has been detected in human tumor samples (7,17), and the possibility that TWEAK and Fn14 could contribute to tumor growth has recently been reviewed (16,18,19). TWEAK activates cellular processes attributed to tumor growth such as matrix metalloprotease (MMP) secretion (20-22), migration (6,8,9), proliferation (5-7), and apoptotic resistance (4). TWEAK is also mitogenic for endothelial cells and stimulates angiogenesis (5,23).

Neuroblastoma, an embryonic tumor of the sympathetic nervous system, is the most common and deadly extracranial tumor associated with childhood. Neuroblastoma

exhibits heterogeneous biological and clinical features ranging from spontaneous regression to highly malignant disease with metastatic spread. More than 40% of children with neuroblastoma are diagnosed as high-risk patients and despite intensive treatment modalities; the cure rate for these patients is less than 50% (24). Hence, there is a great need for new therapies based on a biological understanding of this disease.

The aim of this study was to assess the expression of TWEAK and Fn14 in childhood neuroblastoma, particularly how it relates to the functional importance in neuroblastoma cell growth and survival.

## Materials and methods

*Reagents and antibodies*. Phospho NF-κB p65 (Ser536; cat. 3031), 65 kDa, NF-κB p65 (cat. 3034) 65 kDa and Fn14 polyclonal antibody were purchased from Cell Signaling (Beverly, MA, USA). NF-κB p65 (sc-109), TWEAK (FL-249), and TWEAK (S-20) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary anti-goat Alexa 594 and anti-rabbit Alexa 488 antibodies were purchased from Invitrogen (Carlsbad, CA, USA). Human recombinant IL-1β, TNF-α and TWEAK were purchased from R&D Systems (Abingdon, UK), and human TWEAK ELISA was purchased from PeproTech (London, UK).

*Human tissue samples*. Primary neuroblastoma samples from tumors and non-malignant adrenals were obtained during surgery, snap-frozen in liquid nitrogen, and transferred to -80˚C for future analysis. Twenty six neuroblastoma samples derived from children of different ages and all clinical stages, including different biological subsets (MYCN amplification, 7 of 27; 1p deletion, 9 of 27; Table I) were analyzed. Three childhood ganglioneuromas and three samples of non-malignant adrenals from children aged 12-25 months were also included. Ethical approval was obtained from the Karolinska University Hospital Research Ethics Committee.

*Neuroblastoma cell lines*. Human neuroblastoma cell lines [SK-N-BE(2), SK-N-DZ, SH-SY5Y, SK-N-SH, SK-N-FI, IMR-32, SK-N-AS and SHEP-1] were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and antibiotics. The cultures were kept at 37˚C in a humified 5% $CO_2$ atmosphere.

*RNA isolation and RT-PCR*. Total RNA was extracted from cultured neuroblastoma cells using RNeasy Mini kit (cat. no. 74104, Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. cDNA was synthesized using 2.0 $\mu$g total RNA that was reverse transcribed in a final volume of 50 $\mu$l using the SuperScript preamplification kit (Life Technologies, Inc., Gaithersburg, MD, USA).

Gene specific PCR was performed in 50 $\mu$l of reaction mixture containing 2-10 $\mu$l cDNA (from isolated RNA), 2.5 U of Taq DNA polymerase (Promega, Madison, WI, USA), 10 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1% Triton® X-100, 2.0 mM $MgCl_2$, 1 mM deoxynucleotide triphosphate mix, and 1 $\mu$M of each primer.

PCR for TWEAK and Fn14 was performed as follows: 94˚C for 5 min (first denaturation/hot start) and then at 94˚C for 1 (denaturation), 52˚C for 1.5 min (annealing), and 72˚C for 1 min (extension) for 35 cycles with a 10 min final extension at 72˚C. PCR conditions for β-actin were identical except for a 1 min annealing at 55˚C, and a total of 27 cycles, with a 10 min final extension.

PCR amplifications were performed in a PTC-200 Peltier Thermal Cycler (MJ Research Inc. Waltham, MA, USA). PCR products were analyzed by agarose gel (1.5%) electrophoresis and photographed under UV light. Nucleotide sequences of PCR primers used were as follows: Fn14, 5'-GAC CTG GAC AAG TGC ATG GAC-3' (sense) and 5'-AGC TGT TTT GTG TGA GCC AGC- 3' (antisense); TWEAK 5'-ATC GCA GCC CAT TAT GAA GTT C-3' (sense) and 5'-GAT GGA AAA CAC GTG AAC AGG C-3' (antisense); β-actin, 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3' (sense) and 5'-ACT CGT CAT ACT CCT GCT TGC TGA TCC A-3' (antisense); PCR fragments of 500 (Fn14), 607 (TWEAK), and 625 bp (β-actin) were expected.

*Microarray expression analysis*. Raw data files from four European expression microarray studies generated from two Affymetrix platforms (HU133A and HU133plus2) were obtained from ArrayExpress (www.ebi.ac.uk/microarray-as/ae/) and the r2 data base (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi). The three studies run on the HU133A platform (25-27), were reanalyzed using gcRMA by Bioconducter for R 2.9.2 (library BioC 2.4) in two separate groups: i) De Preter data set comprising preamplified primary neuroblastoma samples (n=17, stages 1-4), ii) McArdle (n=16) and Wilzén (n=8) data sets comprising non-preamplified primary neuroblastoma samples. Also, expression values (log2) from 76 MAS5.0 normalized neuroblastoma samples (stages 1-4) run on the Affymetrix HU133plus2 platform were obtained from the r2 database (28) and referred to as the Versteeg data set.

Neuroblastoma samples from all three data sets (25-27) were divided into two groups based on their clinical stage (INSS stage) (29) and investigated for differential expression of TNFSF12 (TWEAK) and TNFRSF12A (Fn14) between groups. The significance was tested by Welch's t-test (2-tailed, 2 sample comparison, unequal variance).

*Treatment of cells with TWEAK*. To prevent influence of endogenously produced TWEAK, neuroblastoma cells were serum starved in an RPMI-1640 medium containing 0.1% FCS for 24 h prior to the incubation of recombinant human TWEAK for the indicated concentrations and time-points.

*Immunohistochemistry*. Formalin-fixed and paraffin-embedded tissue sections were deparaffinized in xylene and graded alcohols, hydrated and washed in PBS. After antigen retrieval in sodium citrate buffer (pH 6.0) in a microwave oven, the endogenous peroxidase was blocked by 0.3% $H_2O_2$ for 15 min. Sections were incubated overnight at 4˚C with primary antibody (TWEAK; FL-249). As a secondary antibody, the anti-rabbit horseradish peroxidase (HRP) SuperPicTure Polymer detection kit was used (Zymed-Invitrogen, San Francisco, CA,

Table I. Immunohistochemical assessment of TWEAK, Fn14 and pNF-κB expression in neuroblastoma tissue samples.

| Sample | DIA[a] | Age months | Gender | Stage INSS[b] | MYCN ampl | 1p del | DNA ploidy | High-risk[c] | Outcome | TWEAK | TWEAK-R/ Fn-14 | pNF-κB |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | NB[d] | 21 | M | 1 | No | No | 4n | No | NED[h] | +++ | ++ | +++ |
| 2 | NB | 123 | F | 1 | No | No | 3n | No | NED | ++(+) | + | +++ |
| 3 | NB | 7 | F | 1 | Yes | Yes | 2n | No | DOD[i] | ++ | ++ | ++ |
| 4 | NB | 13 | M | 1 | No | No | | No | NED | ++ | ++ | ++ |
| 5 | NB | 18 | F | 1 | No | No | | No | NED | ++ | + | +++ |
| 6 | NB | 31 | M | 2B | No | No | 3n | No | NED | +++ | ++ | +++ |
| 7 | NB | 33 | F | 2A | No | No | 3n | No | NED | ++ | + | +++ |
| 8 | NB | 8 | F | 2 | No | No | 3n | No | NED | ++ | ++ | +++ |
| 9 | NB | 110 | M | 2 | No | No | 2n | No | NED | +++ | ++ | +++ |
| 10 | NB | 5 | F | 2 | No | No | 3n | No | AWD[j] | ++ | + | ++ |
| 11 | NB | 103 | F | 2B | No | No | 2n | No | NED | +++ | ++ | +++ |
| 12 | NB | 6 | M | 3 | No | ND | 3n | No | NED | +++ | +++ | +++ |
| 13 | NB | 12 | F | 3 | No | No | 5n | No | NED | +++ | ++ | ++ |
| 14 | NB | 0 | M | 3 | No | No | 3n | No | DOC[k] | ++ | ++ | +++ |
| 15 | NB | 79 | M | 3 | Yes | Yes | 3n | Yes | NED | +++ | ++ | +++ |
| 16 | NB | 136 | M | 4 | Yes | Yes | 2n | Yes | DOD | ++ | +++ | +++ |
| 17 | NB | 39 | F | 4 | Yes | Yes | 2n | Yes | DOD | +++ | +++ | +++ |
| 18 | NB | 35 | F | 4 | No | Yes | | Yes | NED | ++ | ++(+) | +++ |
| 19 | NB | 28 | M | 4 | Yes | Yes | 3n | Yes | NED | ++ | ++ | +++ |
| 20a | NB | 8 | M | $4_M$[g] | No | No | 3n | No | NED | +++ | ++ | +++ |
| 20b | NB | 8 | M | $4_M$ | No | No | 4n/5n | No | NED | +++ | +++ | +++ |
| 21 | NB | 22 | M | 4 | Yes | Yes | | Yes | DOD | +++ | ++ | ++ |
| 22 | NB | 50 | F | 4 | Yes | Yes | | Yes | DOD | ++ | ++ | ++ |
| 23 | NB | 0 | M | 4S | No | No | 3n | No | NED | +++ | ++ | ++ |
| 24 | NB | 10 | M | 4S | No | No | 3n | No | NED | +++ | ++ | ++ |
| 25 | NB | 0 | M | 4S | No | ND | 4n | No | NED | +++ | ++ | +++ |

Ampl, amplification. Del, deletion. +, low. ++, median. +++, significantly strong.

USA). A matched isotype control was used as a control for non-specific background staining. Routine standard staining showing a normal histology of neuroblastoma was performed with hematoxylin and eosin.

For immunofluorescence studies, cells were grown on fibronectin-coated chamber slides (Nunc, Roskilde, Denmark) for 24 h. Cultures were then washed and fixed with 2% paraformaldehyde for 15 min and 70% cold methanol for 5 min. After washing with PBS buffer, goat-anti TWEAK (S-20) and rabbit-anti Fn14 antibodies were incubated with cultures overnight at 4˚C. After rinsing in PBS, cultures were incubated with secondary antibodies conjugated with Alexa 488 and Alexa 599, respectively. A matched isotype control was used as a control for non-specific background staining. The cells were examined in a Zeiss axiophot photomicroscope (Carl Zeiss, Oberkochen, Germany). Nuclear translocation of NF-κB upon stimulation with TWEAK was performed by immunofluorescence studies using anti-NF-κB p65 antibody (sc-109) that recognizes both non-phosphorylated and phosphorylated forms of NF-κB p65.

*TWEAK ELISA*. TWEAK ELISA was performed to measure the endogenous production of TWEAK in neuroblastoma cells upon stimulation by cytokines IL-1β and TNF-α. Neuroblastoma cells [SK-N-AS, SK-N-BE(2) and SH-SY5Y] were seeded in a regular growth medium in 96-well plates and allowed to attach. Cells were then treated with two concentrations (10 and 50 ng/ml) of IL-1β and TNF-α, respectively, for 12 h. Supernatants from treated cells were collected and 100 $\mu$l medium per well were analyzed by using the Human TWEAK ELISA Development kit (PeproTech, Rocky Hill, NJ, USA), following the manufacturer's instructions.

*Protein isolation and immunoblotting*. Proteins from TWEAK-treated cells and control cells were extracted in RIPA lysis buffer (cat. no. 20-188, Upstate Biotechnology, USA) containing complete, mini, EDTA-free protease inhibitor (cat. no. 11 836 170 001, Roche). The protein content was measured using Bradford reagents (Bio-Rad Laboratories, CA, USA). Equal amounts of protein were separated by NuPAGE, Novex

and Tris-Acetat Mini Gels (Invitrogen) 4-12% in reduced conditions, and proteins were transferred to a PVDF (Pierce, Rockford, IL, USA) membrane and incubated with primary antibodies at 4˚C overnight. Alkaline-phosphatase conjugated antibodies were used as secondary antibodies. Detection and visualization were performed using Pierce Super Signaling solutions (Pierce), the Fujifilm Luminescent Image Analyzer LAS-3000 and the Fujifilm MultiGauge (Ver. 3.0) analysis software.

*NF-κB luciferase reporter gene assay*. The NF-κB-responsive reporter plasmid κBcon A-LUC was provided by E. Sontag (30) and also described in Johannesen *et al* (31). SK-N-AS and SK-N-BE cells were seeded in 6-well plates in triplicates the day before transfection. Cells were transfected using Lipofectamine 2000 (Invitrogen), 4 $\mu$g/$\mu$l DNA per well and calf thymus DNA (Amersham Pharmacia, Sweden), and incubated for 6 h at 37˚C in a humidified 5% $CO_2$ atmosphere. Cells were then serum starved for 14 h and subsequently incubated for 6 h with TWEAK (100 ng/ml) or TNF-α (30 ng/ml). Cells were washed in 1X PBS and lysed in TROPIX lysis buffer containing 0.5 mM DTT. Luciferase activity was determined using the Dual-Light Luciferase Gene Assay System (Applied Biosystems Inc., Foster City, CA, USA) in a Luminoscan RT (Labsystems, Helsinki, Finland). Luciferase measurements were corrected to protein concentrations in each cell lysate, and protein measurements were performed as described above. The difference between the groups was analyzed on log-transformed values after normalization to total protein content in the cell lysates using a repeated measures one-way ANOVA test, followed by a Bonferroni multiple comparison test.

*Fn14 and TWEAK siRNA*. SK-N-AS and SK-N-BE(2) cells were seeded in 6-well culture plates in RPMI medium at a 30-50% confluence. Cells were transfected with target-specific Fn14 (sc-43764), TWEAK (sc-37522), control (Fluorescein conjugate-A) (sc-36869) or scrambled control (sc-37007) siRNA (Santa Cruz Biotechnology), respectively, at a concentration of 33 nM using Lipofectamine 2000 in OptiMEM. To evaluate cell viability, western blot analysis of protein extracts and trypan blue exclusion assay were performed 72 h after the initial transfection. Transfection efficiency was assessed in SK-N-AS cells transfected with control (Fluorescein conjugate-A) using flow cytometry.

*Detection of MMP-2 and MMP-9 in cell conditioned media using gelatin zymography*. The presence of MMP-2 and MMP-9 in serum-free media from cells treated with or without recombinant TWEAK was determined by SDS-gelatin zymography. Approximately 30,000 cells were seeded in 96-well plates and left to attach in 10% RPMI-1640 overnight. Then, SK-N-AS and SK-N-SH cells were starved in 0.1% RPMI-1640 for 24 h prior to incubation with TWEAK (0-1000 ng/ml) for 48 h. SDS-substrate PAGE was done as previously described (32,33) with gels containing 0.1% (w/v) gelatin. The gelatin zymograms were calibrated with a mixture of conditioned serum-free medium from THP-1 and humans skin fibroblast cells (32). Conditioned medium (10 $\mu$l) was mixed with 2.5 $\mu$l of loading buffer (250 mM Tris-HCl, pH 6.8, 10% SDS, 0.03% bromophenol blue and 50% glycerol). Eight $\mu$l of this non-heated mixture was applied to the gel, which was run at 20 mA/gel at 4˚C. Thereafter, the gel was washed twice in 100 ml of washing buffer [2.5% (v/v) Triton X-100 in water], and then incubated in 100 ml of assay buffer (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 0.2 M NaCl and 0.02% Brij-35) for ~20 h at 37˚C. Gels were stained with 0.2% Coomassie brilliant blue R-250 (30% methanol) and destained in a solution containing 30% methanol and 10% acetic acid. Gelatinase activity was evident as cleared regions.

*Statistical analyses*. All statistical analyses were performed with GraphPad Prism Software (GraphPad Software, San Diego, CA, USA). The t-test was used to determine whether the mean of a single sample differed significantly from control. To compare several treatment groups, one-way ANOVA with Tukey multiple-comparisons tests were used. $P<0.05$ was considered statistically significant.

## Results

*TWEAK and Fn14 are expressed in neuroblastoma primary tumors and cell lines*. We investigated eight different neuroblastoma cell lines for the expression of TWEAK and Fn14. All human neuroblastoma cell lines investigated showed varying degrees of both TWEAK and Fn14 mRNA and protein expression as detected by RT-PCR and western blot analysis, respectively (Fig. 1A and B). The 30-35-kDa band for TWEAK represents the transmembrane form of TWEAK as the soluble form could not be detected by western blot analysis. We selected 3 cell lines SK-N-AS, SK-N-BE(2) and SH-SY5Y for the *in vitro* experiments. The rationale behind choosing these cell lines was based on the genetical and phenotypical differences between the cell lines. SK-N-AS and SH-SY5Y are typical non-*MYCN*-amplified cell lines, whereas the SK-N-BE(2) cell line is *MYCN*-amplified and P53-mutated. Furthermore, while the SK-N-BE(2), SK-N-SH and SK-N-AS show multi-drug resistant (MDR) phenotype, the SH-SY5Y cell line does not. In order to investigate whether neuroblastoma cells were able to produce and secrete TWEAK *in vitro*, we assessed the TWEAK production by ELISA. The TWEAK levels in cell supernatants from SK-N-AS cells showed an elevated secretion upon stimulation with pro-inflammatory cytokines IL-1β and TNF-α (Fig. 1C). Similar results were obtained for SH-SY5Y and SK-N-BE(2) cell lines (data not shown).

Immunofluorescence staining of neuroblastoma SK-N-AS cells stained with antibodies towards TWEAK (red) and Fn14 (green) revealed the cellular distribution of the ligand and receptor, demonstrating a distribution of TWEAK and Fn14 in the cytoplasm but also to a certain degree to the nuclear compartment (Fig. 1D).

The staining of primary neuroblastoma tumor tissue with antibodies against TWEAK and Fn14 revealed significant cytoplasmic and nuclear expression of both TWEAK and Fn14 in all primary tumors that were analyzed (Fig. 1E and Table I). No significant difference in staining intensity between favorable and non-favorable tumors (*MYCN*-amplified vs non-*MYCN*-amplified) could be detected by immunohistochemistry and no staining was observed in sections incubated with isotype

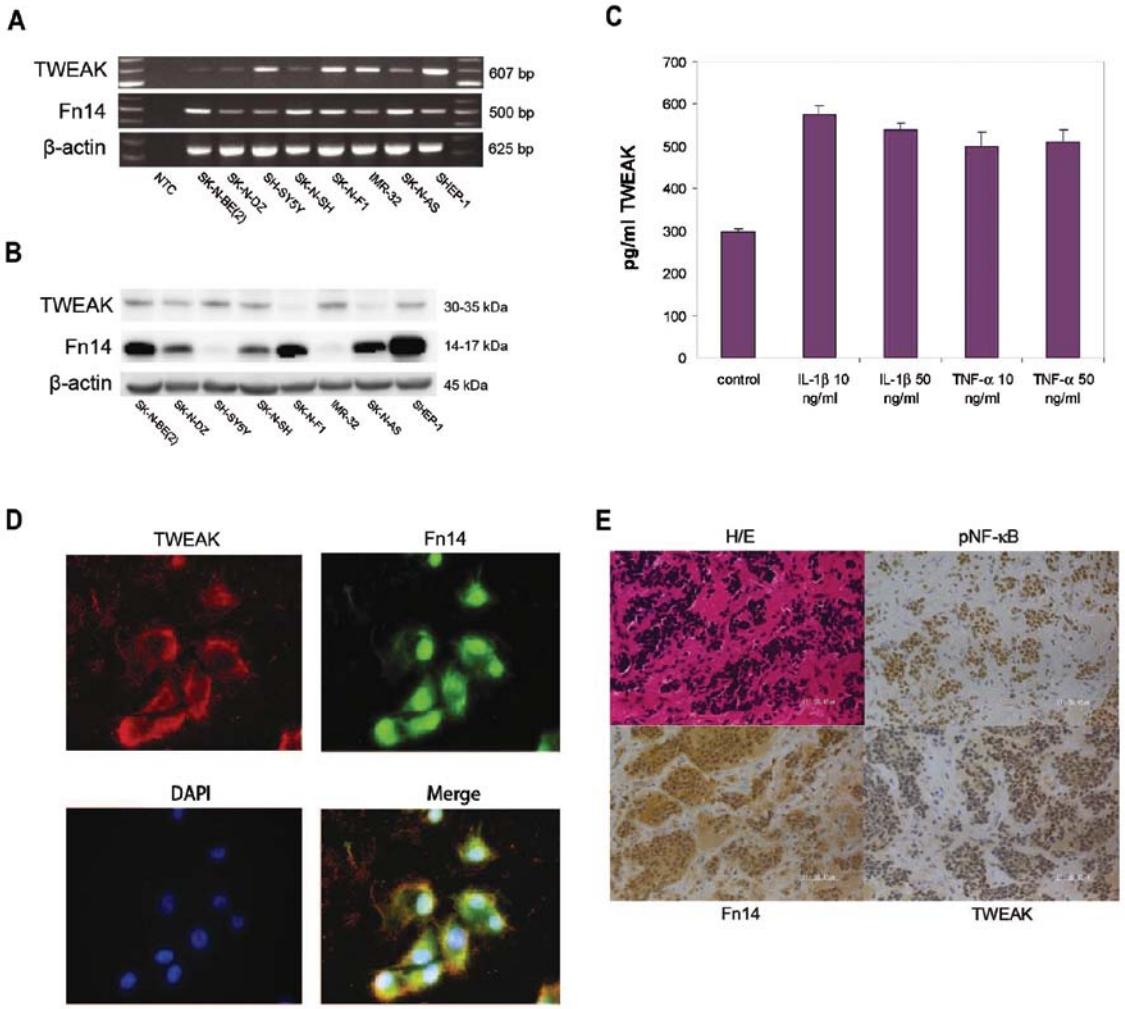


Figure 1. TWEAK and Fn14 expression and secretion in neuroblastoma cell lines and primary tumors. (A) RT-PCR showed expression of TWEAK and Fn14 in all neuroblastoma cell lines investigated. NTC, negative control. (B) Western blot analysis detected protein bands of ~30-35 kDa corresponding to TWEAK and 14-17 kDa corresponding to Fn14 in neuroblastoma cell lines. β-actin was used to ensure equal loading. (C) TWEAK secretion in neuroblastoma cells. SK-N-AS cells were treated with 10 or 50 ng/ml IL-1β and TNF-α and cell supernatants were analyzed for TWEAK secretion measured by ELISA. (D) Immunofluorescence images of neuroblastoma cell line SK-N-AS showing cellular distribution of TWEAK (red) and Fn14 (green). Merge shows colocalization of ligand and receptor. DAPI shows staining of the nucleus. (E) Immunohistochemical detection of TWEAK (x400), Fn14 (x200) and phospho-NF-κB (x200) in primary human neuroblastoma tumor tissue (H/E hematoxylin/eosin; x400).

control antibody. Moreover, mRNA levels of both TWEAK and its receptor Fn14 were found to be generally higher in primary high stage tumors (stage 3-4) compared to low stage tumors (stage 1-2), when investigating three European microarray data sets (Fig. 2). The up-regulation was significant for TWEAK and Fn14 in one out of three data sets respectively, *i.e.* the De Preter and Versteeg data sets ($p<0.05$, Welch's t-test; Fig. 2). Also, the expression variance of both TWEAK and Fn14 was considerably higher in the high-stage group, and the up-regulation seemed to involve a sub-set of tumor cases. Ten out of 117 cases from all three data sets showed up-regulation of TWEAK (fold change >2), 13 showed up-regulation of Fn14 (fold change >2), and 9 showed up-regulation of both genes (fold change >2) compared to the mean expression levels of the low-stage group (Fig. 2).

*TWEAK promotes NF-κB activation and nuclear translocalization in neuroblastoma cells.* TWEAK treatment has previously been shown to stimulate NF-κB activation in different cell types (15,34-36). To determine if TWEAK could induce activation of NF-κB in neuroblastoma cells, we examined the intracellular localization of NF-κB in TWEAK stimulated SK-N-AS cells. No nuclear staining for NF-κB was observed in untreated cells, but after 20 min of stimulation with recombinant TWEAK, nuclear translocation of NF-κB was detected (Fig. 3A). To validate the TWEAK induction of NF-κB in SK-N-AS cells grown in 0.1% FCS, we isolated total protein fractions of cells treated with TWEAK (100 ng/ml) and immunoblotted for both the NF-κB and the pNF-κB subunit. Blots revealed an increased level of pNF-κB (p65) subunit shortly after stimulation by TWEAK at the same time that NF-κB showed a decreased signal (Fig. 3B). This is consistent with the immunostaining results showing activation of pNF-κB (Fig. 3A), and the results of SK-N-AS cells upon stimulation with TWEAK showing increase of NF-κB transcriptional activity upon transient transfection using the NF-κB-responsive reporter plasmid κBcon A-LUC (Fig. 3C).

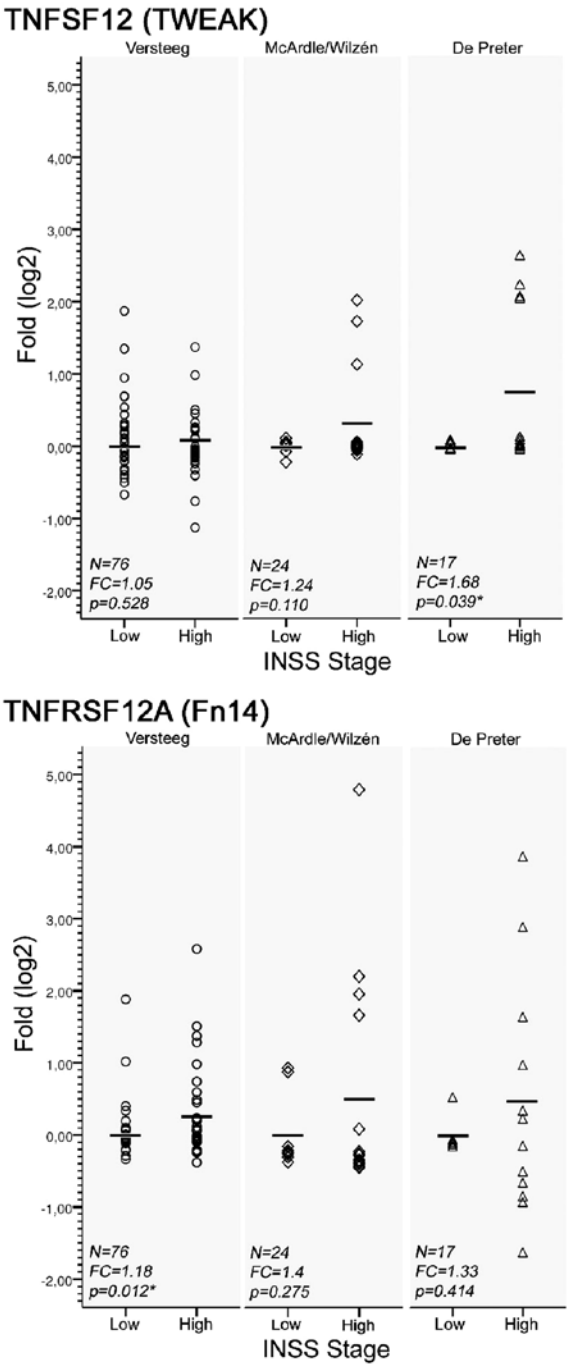


Figure 2. Fold scatter plot of TWEAK (TNFSF12) and Fn14 (TNFRSF12A) mRNA expression in primary tumors. The log2 fold between high stage (INSS stage 3-4) and low stage (INSS stage 1-2) tumors from four Affymetrix microarray studies, presented as three data sets are plotted. The log2 fold mean in each group is marked by horizontal line and related to the mean in low stage tumors (log2 fold, 0). Open circles, Versteeg data set (28) from the r2 database data set (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi. Open squares, McArdle/Wilzén data set (26,27). Open triangles, De Preter data set (25). N, the number of cases. FC, relative fold change between groups. p, significance by Welch's t-test (*p<0.05). See text for more detail.

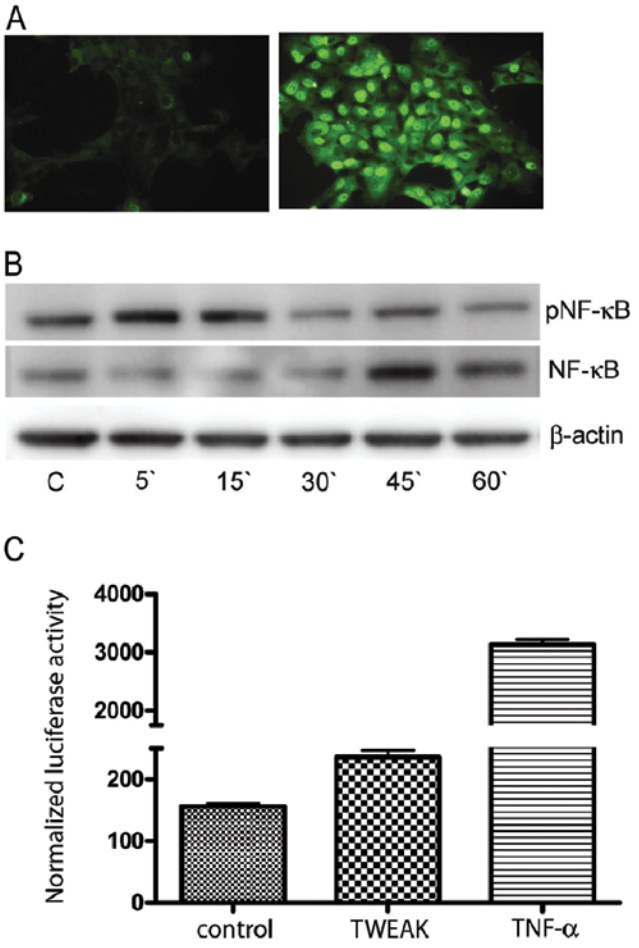


Figure 3. Effect of TWEAK treatment on NF-κB cellular localization in neuroblastoma cells. (A) NF-κB immunostaining of TWEAK-stimulated neuroblastoma cells. SK-N-AS cells were cultured under reduced serum conditions (0.1%) for 16 h prior to stimulation with TWEAK (100 ng/ml). Cells were fixed and immunostained for the p65 subunit of NF-κB. The left panel shows non-treated SK-N-AS cells, whereas the right panel shows cells treated with TWEAK for 20 min. (B) TWEAK stimulates NF-κB p65 phosphorylation in neuroblastoma cells. SK-N-AS cells were cultured under reduced serum conditions (0.1%) for 16 h prior to stimulation with TWEAK (100 ng/ml) for various time periods as indicated or reduced serum for control. Following treatment cells were lysed and total cell extracts were immunoblotted for both the phospho-NF-κB and NF-κB subunit. β-actin was used as a control for equal loading. (C) TWEAK induces NF-κB transcriptional activity in neuroblastoma cells. SK-N-AS cells transiently transfected with a NF-κB plasmid containing luciferase reporter gene were exposed to TWEAK (100 ng/ml), TNF-α (30 ng/ml) or culture medium only (control) for 6 h. Luciferase activity was measured in cell lysates and values were normalized to total protein content. The experiment was repeated three times with similar results. Data presented are mean ± SEM from a representative experiment, n=3 per group.

Furthermore, immunohistochemical analysis of neuroblastoma primary tumors using phospho-specific NF-κB antibody (p65) revealed a significant nuclear staining (Fig. 1E).

*Silencing of TWEAK and Fn14 reduces neuroblastoma cell viability*. To investigate the influence of TWEAK on neuroblastoma cell survival, SK-N-AS cells were transfected with siRNA targeting TWEAK or Fn14. As shown in Fig. 4A, the silencing of both TWEAK and Fn14 resulted in significant decrease in neuroblastoma cell survival compared to cells transfected with a scrambled siRNA construct ($p<0.05$). Addition of recombinant TWEAK partly restored TWEAK expression and cell survival in TWEAK siRNA-treated SK-N-AS cells, underscoring a role for TWEAK in cell survival (Fig. 4A and B). Increased expression of Fn14 in TWEAK siRNA-treated cells compared to scramble- and non-treated cells (Figs. 1B and 4B) is possibly due to the absence

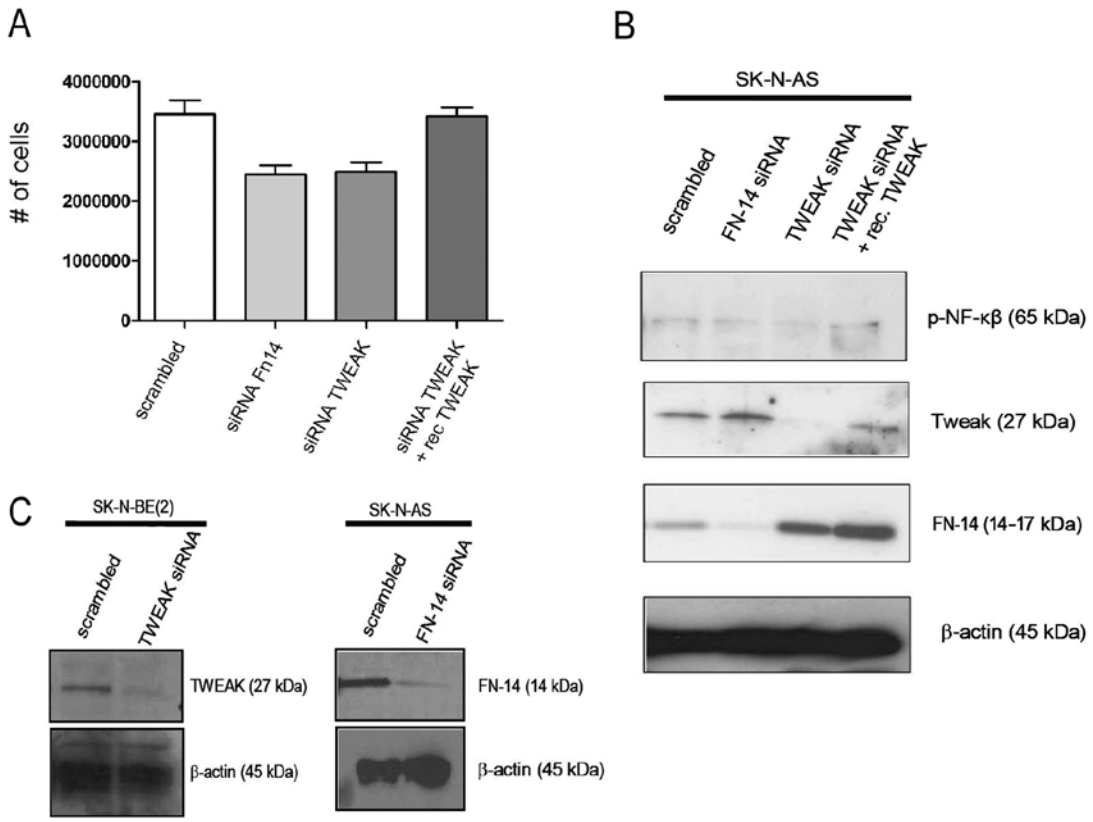


Figure 4. Silencing of TWEAK and Fn14 in neuroblastoma cells. (A) TWEAK and Fn14 expression are important for neuroblastoma growth. Trypan blue exclusion assay performed 72 h after initial transfection showing cell viability upon transfection of SK-N-AS cells with siRNA towards TWEAK or Fn14 in addition to siRNA transfection followed by recombinant TWEAK treatment ($p<0.05$). (B) Expression of TWEAK and Fn14 in SK-N-AS cells after transfection with scramble siRNA and siRNA towards TWEAK or Fn14. (C) Cell viability assay results were verified by western blot analysis showing a decrease in the endogenous protein expression of TWEAK in SK-N-BE(2) cells and Fn14 in SK-N-AS cells upon silencing with siRNA.

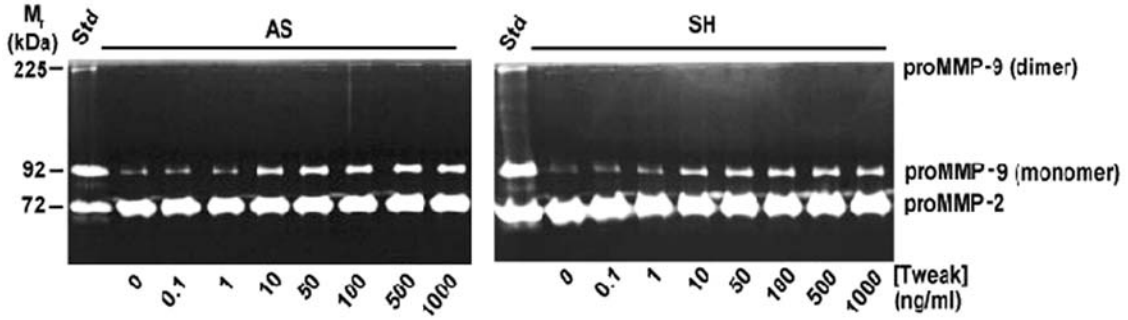


Figure 5. TWEAK induces MMP-9 activity in neuroblastoma cells. Gelatin zymography of TWEAK-treated SK-N-AS and SK-N-SH culture supernatants shows that TWEAK induces pro-MMP-9 (monomer) in a dose-dependent manner. MMP-2 production is constitutive.

of the ligand resulting in lowered receptor internalization and degradation of receptor-ligand complex. Additional western blotting was performed to also confirm the specific down-regulation of TWEAK or Fn14 expression in SK-N-AS cells following siRNA transfection (Fig. 4C).

*TWEAK induces MMP-9 expression in neuroblastoma cells.* Since MMP-9 is a NF-κB responsive gene and TWEAK has been shown to induce MMP-9 protein level in other cell systems (21,22), we investigated the effect of TWEAK on the secretion of MMP-2 and MMP-9 in four different neuroblastoma cell lines. Zymography performed on conditioned medium from neuroblastoma cell lines revealed that MMP-2 was constitutively expressed, whereas TWEAK induced the release of MMP-9 in a dose-dependent manner in SK-N-AS and SK-N-SH cells (Fig. 5). Similar results were obtained for SK-N-BE(2) cells whereas SK-N-SY cells did not release MMP-9 upon stimulation with TWEAK (data not shown), indicating a heterogeneity with respect to TWEAK induction of MMP-9 in neuroblastoma cells.

## Discussion

The TNF superfamily of proteins has been implicated in the regulation of cell survival and proliferation. We have previously studied the role of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) in neuroblastoma (37). In the present study, we describe the expression of TWEAK in neuroblastoma. TWEAK is a member of the TNF family of cytokines that acts on responsive cells via binding to a cell surface receptor called Fn14. TWEAK is a multifunctional cytokine expressed in a variety of normal tissue, but its expression has also been implicated in certain types of cancer [reviewed in refs. 16,18]. Equivalent to TWEAK, Fn14 is expressed in most cells and tissues (16) although the expression level is normally low. However, Fn14 expression can be induced by several cytokines, hormones and inflammatory mediators (16). A high expression of Fn14 has been detected in various adult cancers (7,15,38) and Fn14 mRNA expression correlates with glioma grade and patient outcome (15). In addition, increased Fn14 mRNA and protein have been shown to be associated with disease progression in esophageal adenocarcinoma (17,39) and cancer of the mammary (21).

We analyzed neuroblastoma primary tumors from different biological subsets and clinical stages, and detected TWEAK and Fn14 in all samples investigated (Table I and Fig. 1E). TWEAK and Fn14 mRNA and protein were all detected in various degrees in all neuroblastoma cell lines investigated (Fig. 1A and B), and soluble TWEAK was detected in supernatants from neuroblastoma cells (Fig. 1C). The discrepancy between mRNA and protein levels of TWEAK and Fn14 may have root in several aspects such as mRNA stability and post-translational modification. Furthermore, the mRNA analysis was performed by reversal transcription PCR which is not a quantitative method. Immunofluorescence images of SK-N-AS cells demonstrate that the endogenously produced ligand and its receptor are co-localized to the cytoplasm, and to a certain degree in the nucleus (Fig. 1D). Interestingly, a 'short' variant of endogenously formed TWEAK possessing a nuclear localization sequence has been shown to colocalize with GSK3β in the nucleus of human neuroblastoma cells (40).

The significance of TWEAK and Fn14 expression in neuronal cells is not well understood. TWEAK has been shown to be expressed in primary murine neurons (41) and in the peripheral nervous system. TWEAK and Fn14 have been reported to regulate neurite outgrowth and regeneration (42), and a recent study has also demonstrated the role of TWEAK/Fn14 in neurite extension in neural progenitor cells (11).

We observed an up-regulation of both TWEAK and Fn14 mRNA in several primary tumors from three publicly available European neuroblastoma data sets. It is presently unknown why the Fn14 gene is elevated in certain solid tumors. Fn14 gene amplification may possibly occur during the development and progression of these tumors although this is not frequently detected in neuroblastomas (43). Alternatively, the expression of Fn14 might be driven by a range of cytokines and growth factors produced within the tumor microenvironment. Additionally, TWEAK produced by tumor cells or other cells within the tumor microenvironment may activate Fn14 and NF-κB in cancer cells by use of a positive feedback loop (15). In this study, both TWEAK and Fn14 mRNA were found at significantly higher levels in a sub-set of neuroblastoma samples. Among 10 cases showing up-regulation of TWEAK (fold change >2), 8 also show up-regulation of Fn14 (fold change >2) supporting this hypothesis (Fig. 2).

To examine the effect of TWEAK on the proliferation of neuroblastoma cells, we stimulated serum-starved cells with an increasing concentration of TWEAK (1-1000 ng/ml) for 24 and 48 h. In contrast to other reports using other types of tumor cells (5,7,23), we did not detect any significant increased proliferation of neuroblastoma cells upon stimulation with TWEAK (data not shown). However, this is in line with results from studies on murine postnatal neural progenitor cells (11) and human embryonic kidney (HEK 293) cells (44). TWEAK stimulation through Fn14 activates the NF-κB signaling pathway in various cells and induces the expression of pro-inflammatory molecules (16,45). Inflammatory mediators are critical components of tumor growth and the possibility that TWEAK produced by tumor cells may act on stromal cells within the tumor is supported by studies showing that TWEAK can induce secretion of cytokines from endothelial cells (7), fibroblasts (46) and macrophages (22). In addition, the activation of stromal cells may promote the infiltration of innate immune system cells that in turn may be potential sources for TWEAK and other pro-inflammatory cytokines. In our study, pro-inflammatory cytokines were shown to enhance TWEAK secretion by neuroblastoma cells (Fig. 1C). Therefore, inflammatory mediators within the tumor microenvironment may contribute to the further induction of TWEAK secretion by neuroblastoma cells.

Numerous genes have been described that are regulated by NF-κB and mediate the survival of cancer cells. These include genes such as *MMP-9* (47), *VEGF* (48), and *COX-2* (49), which have been closely associated with invasion and angiogenesis.

In the present study, we detected the nuclear expression of phospho-NF-κB in all primary neuroblastoma tissue samples by the use of immunohistochemistry (Fig. 1E). The phosphorylation of NF-κB (p65) was induced upon TWEAK stimulation of neuroblastoma cells as shown by western blot analysis (Fig. 3B). Furthermore, we demonstrate the translocation of NF-κB into the nucleus of TWEAK-stimulated neuroblastoma cells (Fig. 3A). A recent study demonstrated the the Fn14 expression in gastric cancer was inversely correlated with patient survival. Furthermore, expression level of Fn14 was shown to affect cell growth which in turn was mediated by NF-κB activity (50).

Among proteases involved in tumor invasion and metastasis are the matrix metalloproteases (MMPs), a large family of endopeptidases which together can process all extracellular matrix proteins as well as other non-matrix proteins such as growth factors, cytokines and receptors (51,52). Neuroblastoma cells are able to produce extracellular matrix degrading enzymes such as MMP-2 and MMP-9 (53-55). TWEAK has been shown to upregulate MMP-9 expression in both normal (22) and transformed cells (21,56). Stimulating neuroblastoma cells with TWEAK induced the expression of MMP-9 in a dose-dependent manner as observed in gelatin zymograph. Neither of the cell lines responded to TWEAK stimulation by a change in MMP-2 expression (Fig. 5). These results suggest that TWEAK may have important functions during the metastasis conversion in neuroblastoma.

In conclusion, our data demonstrate that TWEAK and Fn14 are expressed in neuroblastoma, and may play an important role in pro-survival features of the tumor. Given that targeted antibody therapy for Fn14 in several human xenografts results in significant antitumor effects (57,58) a further evaluation of the mechanisms behind TWEAK and Fn14 expression may reveal new therapeutic options for neuroblastoma.

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