Role of neurotrophins on dermal fibroblast survival and differentiation

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NT: neurotrophin; DF: dermal fibroblasts; DM: dermal myofibroblasts

Abstract

Neurotrophins (NTs) belong to a family of growth factors that play a critical role in the control of skin homeostasis. NTs act through the low-affinity receptor p75NTR and the high-affinity receptors TrkA, TrkB and TrkC. Here we show that dermal fibroblasts (DF) and myofibroblasts (DM) synthesize and secrete all NTs and express NT receptors. NTs induce differentiation of DF into DM, as shown by the expression of α-SMA protein. The Trk inhibitor K252a, TrkA/Fc, TrkB/Fc or TrkC/Fc chimera prevents DF and DM proliferation. In addition, p75NTR siRNA inhibits DF proliferation, indicating that both NT receptors mediate DF proliferation induced by endogenous NTs. Autocrine NTs also induce DF migration through p75NTR and Trk, as either silencing of p75NTR or Trk/Fc chimeras prevent this effect, in absence of exogenous NTs. Finally, NGF or BDNF statistically increase the tensile strength in a dose dependent manner, as measured in a collagen gel through the GlaSbox device. Taken together, these results indicate that NTs exert a critical role on fibroblast and could be involved in tissue remodelling and wound healing.

Introduction

Neurotrophin (NT) family comprises a group of functionally and structurally related proteins that play a fundamental role in the survival and differentiation of neuronal cells (Ernsberger et al, 2009). NT family includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). These proteins exert their effects through the binding and activation of two kinds of transmembrane receptors, the low-affinity receptor p75NTR, which interacts with all of the NT with the same affinity, and the high-affinity receptors Trk (TrkA, TrkB and TrkC), which bind a particular NT (Snider, 1994). NTs are synthesized and released by many skin cells that also express NT receptors, thus creating a complex network between the different cellular populations (Botchkarev et al, 2006). Therefore, in skin, NTs act not only as trophic molecules for skin innervation but also as growth/survival factors for skin cells, being involved in different autocrine and paracrine functions (Peters et al, 2007).

Fibroblasts (DF) represent the main cellular component of the dermis, and are implicated in the homeostatic maintenance of skin extracellular matrix (ECM). They are metabolically active cells and their role is associated with the synthesis and secretion of collagens, proteoglycans, fibronectin and metalloproteases. Dermal fibroblasts play a role in different physiopathological processes in the skin, including wound healing and fibrosis (Werner et al, 2007; Krieg et al, 2007). After injury, different factors, in particular TGF-β_1 (Desmoulière et al, 2003), promote DF differentiation into myofibloblasts (DM), characterized by the expression of α-Smooth Muscle Actin (α-SMA) (Hinz, 2007), which is therefore used as DM differentiation marker. DM are the key effectors in injury/repair processes and fibrosis, as they control ECM component deposition, tissue contraction and wound resolution (Hinz, 2007), and their subsequent apoptosis is essential for the tissue re-epithelization (Desmoulière et al, 2005).

While the expression and function of NTs and their receptors in keratinocytes and melanocytes were extensively studied (Marconi et al, 2003; Marconi et al, 2006), little is known on the correlation between NTs and

fibroblasts. In the corneal and muscular systems, NGF promote fibroblast migration and differentiation into DM (Hasan et al, 2000; Micera et al, 2007; Poduslo et al, 1998).

In the present work, we report that DF and DM express all NTs and their receptors. We also show that NTs promote fibroblast survival, differentiation and migration. Finally, NTs can stimulate fibroblast contraction in a 3D collagen system.

Material and methods

Cell cultures

Human dermal fibroblasts were obtained by explant culture from foreskin and grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum S. TGF-β_1 (1 ng/ml, Sigma, St. Louis, MO) was added in fibroblast secondary culture for six days to promote differentiation into myofibroblasts. The myofibroblast phenotype was check by immunostaining of α-SMA.

Immunofluorescence

Human dermal fibroblasts were plated into chamber slides and stimulated or not with 100ng/ml TGF-β_1 or 100ng/ml recombinant NT (NGF, BDNF, NT-3 or NT-4) (Sigma) in medium with BSA 0,1%. After six days, cell were washed in PBS and fixed in situ in 3,6% formaldehyde for 20 minutes. After washing, cells were permeabilized by incubation for 5 minutes with 0.5% Triton X-100, for 15 minutes with 0.5% bovine serum albumin and 5% goat serum, and then for 60 minutes at 37°C with the mouse monoclonal α-SMA antibody (1:400, Sigma, St Louis, MO). After a brief washing, the cells were incubated for 60 minutes with the secondary antibody anti-mouse AlexaFluor 488 (1:130 dilution) (Molecular Probe Inc, Eugene, OR, USA). Fluorescent specimens were analyzed by a confocal scanning laser microscope (Leica TCS SP2; Leica, Heerbrugg, Switzerland).

NT ELISA assay

Dermal fibroblasts and myofibroblasts were plated and cultivated in 60 mm^2 tissue culture petri dishes. The medium was changed with 2 ml of medium with BSA 0,1% 24 hrs after seeding. Cells were lysed in 100 μl of RIPA buffer pH 7.4 (50mM Tris-HCl, 150mM NaCl, 1% deoxycolate, 1% TritonX-100, 0.1% SDS, 0.2% sodium azide) and conditioned medium were collected and storaged with protease inhibitor. Media were store for 48 hrs at –80°C. The NGF, BDNF, NT-3 and NT-4 quantitation was performed by a two-site enzyme immunoassay (Quantikine™, Promega Corporation, Madison, Wisconsin, USA) according to manufacturer instructions. The samples concentration was determined by absorbance at 540 nm against a known standard of recombinant human NT. NT protein levels are given in pg/mg of cell lysate and results are expressed as mean ± SEM of triplicate from three different experiments.

Flow Cytometry Analysis

About 100,000 dermal fibroblasts and myofibroblasts were plated and cultivated in 60 mm^2 tissue culture petri dishes. After 24 hrs from treatment with 200nM K252a, BrdU (10 μM) was added in each plate. Afters 48 hrs from tratment with 200nM K252a, cells were trypsinized and BrdU incorporation and DNA amount were analysed with BrdU Flow Kits (BD Biosciences Pharmigen, San Diego, CA). Results are calculated as the mean ± SD of three different experiments.

Western blotting analysis

Cells were washed with PBS and lysed on ice, for p75NTR, α-SMA, cyclin B and procaspase-3 detection in lysis buffer pH 7.5 (NaCl 150mM, MgCl 15mM, EGTA 1mM, HEPES 50mM, Glicerolo 10%, Triton 1%), for Trks detection in lysis buffer pH 7.4 (50mM Tris-Hcl, 1% NP40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EGTA, 1mM Na_3VO_4, 1mM NaF) containing protease inhibitors, as described previously (Marconi et al, 2003). Total proteins (20 μg) were analyzed under non reducing conditions on 7% polyacrylamide gels for

p75NTR, under reducing condition on 10% polyacrilammide gels for Trk receptors and α-SMA and under reducing condition on 12% polyacrilammide gels for cyclin B and procaspase-3. The proteins were blotted onto nitrocellulose membranes. To verify equal loading of total proteins in all lanes, the membranes were stained with Ponceau Red. The blots were blocked for 2 hrs in blocking buffer (PBS buffer, pH 7.4 with 0.2% Tween 20 and 5% nonfat milk) and incubated with mouse monoclonal anti-human p75NTR antibody (1μg/ml Upstate Biotechnology, Lake Placid, NY), rabbit polyclonal anti-TrkA (1:1000, Upstate Biotechnology, Lake Placid, NY), rabbit polyclonal ant-TrkB (1:1000; Upstate Biotechnology, Lake Placid, NY), goat polyclonal anti-TrkC (1:750; Upstate Biotechnology, Lake Placid, NY), mouse monoclonal anti a-SMA (1:3000; Sigma, St Louis, MO), anti-cyclin B mouse monoclonal (1:1000; BD Biosciences Pharmingen, San Diego, CA, USA), anti-caspase-3 rabbit polyclonal (1:1000; Cell Signaling Technology, Danvers, MA, USA), mouse monoclonal anti-β-actin (1:1000; Sigma, St Louis, MO) and mouse monoclonal anti-vinculin antibody (1:400; Sigma, St Louis, MO), overnight at 4°C. Then membranes were washed in PBS/Tween 20, incubated with peroxidase-conjugated goat anti-mouse or goat anti-rabbit (1:3000; Biorad, Hercules, CA) or donkey anti-goat (1:5000; Santa Cruz Biotechnology Inc, Santa Cruz, CA) antibodies for 45 min at room temperature. Finally, membranes were washed and developed using the ECL chemiluminescent detection system (Amersham Biosciences UK Limited, Little Chalfont Buckinghamshire, England).

Reverse transcription and polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from cultures using TRI Reagent method performed as described by Sigma. One microgram of total cellular RNA extracted was reverse-transcribed and amplified as described (Pincelli et al, 1994). To verify the integrity of the RNA in each sample, the α-actin mRNA was amplified by RT-PCR. Nucleotide sequences of the oligomers used were as follows: p75NTR :

TrkA: 5’-ggctcctcgggactgcgatg-3’, 5’-caggagagagactccagagcg-3’ (nucleotides 214-233 and 459-479, fragment 266 bp); TrkB: 5’-gacactcaggatttgtactgcc-3’, 5’ tccgtgtgattggtaacatgtatt-3’ (nucleotides 990-1011 and 1481-1504, fragment 515 bp); TrkC: 5’-ctctcttccgcatgaacatc-3’, 5’-tcttctggtttgtgggtcac-3’ (nucleotides 748-767 and 1416-1435, fragment 688 bp); α-actin: 5'-tggatgatgatatcg ccgcgctcg-3', 5'-cacataggaatccttctgaccca-3' (nucleotides 75-98 and 213-235, fragment 161 bp). The PCR was carried out at least three times for each sample. No reverse-transcribed mRNA and buffer without template were used as controls**.**

siRNA transfection of fibroblasts

About 100,000 cells/well, 50,000 cells/well, 1500 cells/well were plated respectively on 60 mm^2 petri dishes, 12-well plates and 96-well plates in penicillin/streptomycin free medium. 24 hours later cells were transfected with 100nM p75NTRsiRNA (Dharmacon Inc, Lafayette, CO, USA) or scrambled RNAi as mock control, combined with Lipofectamin 2000 and Opti-MEM (both from Invitrogen Corporation) as datasheet suggests. Fibroblasts were transfected twice and used for MTT, migration assay and to evaluated cyclin B and procaspase-3 expression by Western blotting. p75NTR protein level was also detected by Western blotting, as described above.

MTT assay

Dermal fibroblasts and myofibroblasts were plated in 96-well tissue culture plate (2500 cells/well). 72 hours after seeding, cells were treated with 100ng/ml human recombinant NT (Sigma-Aldrich, St Louis, MO) or 2μg/ml recombinant human TrkA/Fc, 1 μg/ml recombinant human TrkB/Fc and TrkC/Fc chimeras (R&D Systems, Minneapolis, MN, USA), 200nM K252a, or 100nM p75NTR siRNA (Dharmacon Inc, Lafayette, CO, USA), in medium with BSA 0,1%, for 24, 48 and 72 hours. Proliferative cells were detected by incubating with MTT (Sigma-Aldrich, St Louis, MO) solution at 37°C for 4 hours. They were solubilizated with DMSO and the formazan dye formation was evaluated by scanning multiwell spectrophotometer at 540 nm. The results are expressed

as optical density units (OD) or as viability percentage respect of control. Results are calculated as the mean ± SD of three different experiments.

Migration assay

A total of 200,000 dermal fibroblasts were plated on 12-well tissue culture plates and then treated with 5 μg/ml mitomycin C for 1 hour and 30 minutes. Subsequently, the cells were washed three times in serum-free medium and a line for each well were drawn along the cell monolayer with a sterile plastic tip. Plates were washed twice with serum-free medium to remove all detached cells and incubated in medium with BSA 0,1% with 100ng/ml TGF-β_1, 100ng/ml human recombinant NT (Sigma-Aldrich, St Louis, MO), 2 μg/ml human recombinant TrkA/Fc, 1 μg/ml human recombinant TrkB/Fc and TrkC/Fc chimeras (R&D Systems, Minneapolis, MN, USA), 200nM K252a, or 100nM p75NTR siRNA (Dharmacon Inc, Lafayette, CO, USA). Serum-free medium, water (diluent) or water containing Lipofectamin 2000 and Opti-MEM (mock) were used as controls. Cells were monitored at 24 and 48 hours from stimulation. The result of each experiment was expressed as the mean of migrated cells from three different areas. The final results are expressed as the mean±SD of three different experiments.

Tensile strength measurement

Contractile strengths were measured with the GlaSbox® device as already described by Viennet (Viennet et al, 2005). This box contains 8 rectangle plates in which living dermal equivalents (LDE) are placed and maintained by 2 thin flexible silicium blades with a grid in its inferior part on which LDE attach after a few minutes polymerisation. LDE are composed of 6 volumes of 1.76 concentrated DMEM, 3 volumes of 2mg/ml type I collagen and 1 volume of a 8.10^5 fibroblasts/ml suspension. During contraction of the LDE, the gold covering blades get out of shape inducing changes in the electrical resistance measured by a Wheastone bridge. Variations are registered real time for 24 hours by a computer and converted as milliNewton (mN). The effects of neurotrophin concentrations (10–100ng/ml; Sigma-Aldrich, St Louis, MO) were

examined in triplicate and positive (2.5ng/ml TGF-b_1, Sigma-Aldrich, St Louis, MO) and neutral control (DMEM alone) were performed.

Statistical Analysis

Results from each experiments were analysed by Student's T-Test, obtaining a p-values refered to the mean comparison between treated samples and its controls in each triplicate experiment.

Results

DF and DM synthesize and secrete all NTs and express NT receptors

It was previously demonstrated that epidermal cells express NT and their receptors at different levels and that NTs are implicated in different physiopathological processes in the skin (Botchkarev et al, 2006). In order to evaluate the role of these proteins also in DF and DM, we have analyzed their production in fibroblasts with or without treatment with TGF-β_1 (Figure 1a-b).

We have observed that DF and DM synthesize and release NTs, although at different levels, as shown by ELISA Assay (Figure 1c-1f). Overall, the more differentiated DM release higher levels of NTs than DF. In particular, DF and DM synthetize highest levels of NT-3 and NT-4, while they release NGF, NT-3 and NT-4 in similar amounts. BDNF is expressed at low levels in both cell lysates and in the medium.

In order to evaluate whether DF and DM could also be the target of NTs, we have analyzed NT receptor expression at the mRNA and protein levels. Both DF and DM express TrkA, TrkB, TrkC and p75NTR mRNA, as shown by RT-PCR (Figure 1g). NT receptor expression is also confirmed by Western Blotting (Figure 1h). DF express TrkA protein at higher levels than DM, while TrkB and p75NTR are expressed at higher levels in DM than in DF. TrkC protein is slightly more expressed in DM than in DF. These findings suggest that DF and DM could participate in the NT network not only by releasing NTs but also by responding to their action.

NTs maintain DF and DM viability through Trk receptors.

Given that DF and DM express NT receptors and release NTs, we first analyzed the effect of endogenous and exogenous NT on DF and DM proliferation. When NT were added (100ng/ml) to fibroblast and myofibroblast cultures, no effect was observed on their proliferation up to 72 hrs (Figure 2a-b). Likely, autocrine release of NT could suffice for proliferation of DF and DM. To confirm this hypothesis, we treated DF and DM with either K252, an inhibitor of Trk phosphorylation or specific TrkA, TrkB or TrkC/Fc chimeras that act as soluble receptors to prevent binding of NTs to their membrane receptors. Addition of TrkA/Fc or TrkC/Fc, but not of TrkB/Fc statistically reduced DF proliferation from 24 up to 72 hrs, as compared to control (Figure 2c). This is consistent with the negligible amount of BDNF protein released by DF and with the almost absence of TrkB protein in DF (see figure 1f and g). Similarly, K252 statistically diminished DF proliferation at all time points. Moreover, all Trk chimeras statistically reduced DM proliferation from 24 up to 72 hrs, as compared to control (Figure 2d). In these cells, also TrkB/Fc statistically reduced DM proliferation, in agreement with the higher expression of TrkB protein in DM with respect to DF (see figure 1f and g). Similarly, K252 statistically diminished DM proliferation at 48 and 72 hrs.

To better understand DF and DM reduced viability, we treated these cells with K252a and analyzed cell cycle and caspase-3 activation after 48 hrs (Figure 2e-f). Inhibition of Trk receptor signalling induced cell cycle arrest in G0-G1 phase. At the same time, blocking Trk resulted in activation of caspase-3 both in DF and DM.

NTs maintain DF viability through p75NTR.

Because p75NTR can act as a co-receptor that refines Trk affinity and specificity for NT, we investigated its role in DF proliferation. To this purpose, we silenced p75NTR by siRNA (100nM) (Figure 3a) and evaluated DF proliferation. p75NTR siRNA-treated DF proliferated to a significantly lesser extent, as compared to DF treated with scrambled siRNA (Figure 3b). p75NTR

siRNA-treated DF underwent G2-M arrest, as shown by increased cyclin B expression at 48 hrs, while caspase-3 was not activated (Figure 3c).
These findings indicate that p75NTR co-operates with Trk receptors to mediate DF proliferation induced by autocrine NTs.

Neurotrophins promote DF differentiation into DM

It has been shown previously that NTs mediate differentiation in neuronal and non-neuronal cells (Spittau et al, 2010), and NGF can induce a-SMA expression in lung and skin fibroblasts, with an effect comparable to that of TGF-β_1 (Micera et al, 2006). 24 hrs after treatment with NGF, BDNF, NT-3 or NT-4, α-SMA expression was induced to the same extent as after stimulation with TGF-β_1 (Figure 4a). α-SMA protein expression was maintained for six days after addition of all NTs with a pattern similar to that induced by TGF-β, (Figure 4a-b). This finding indicates that NTs induce the differentiation of DF into DM at early and later points, with the same TGFβ_1 kinetics.

Neurotrophins induce DF migration

Fibroblast migration plays an essential role in several physiopathologic processes at the skin level, including wound healing (Walter et al, 2010) and melanoma progression (Wu et al, 2010). Because NTs are involved both in wound healing (Sun et al, 2010) and in melanoma cell migration (Truzzi et al, 2008), we wanted to investigate the migratory capacity of fibroblasts after treatment with NTs (Figure 5a). NGF, BDNF, NT-4 or NT-3 stimulated DF migration in a statistically significant fashion both at 24 and 48 hrs, as compared to diluent alone (Figure 4a). NGF was shown to act as the strongest migratory stimulus, effect being more potent than TGF-β_1, as shown by the number of migrated cells in the scratching assay (Figure 5b). Addition of K252a, TrkA/Fc, TrkB/Fc, TrkC/Fc chimeras statistically prevented DF migration as compared to control, in absence of exogenous NT (Figure 5c). This was shown by the reduction of migrated cells in the scratching assay (Figure 5d). Since p75NTR contributes to the high affinity and cooperates with Trk receptors in several activities, we tested the role of p75NTR in NT-induced DF

migration. Silencing p75NTR mRNA markedly reduced p75NTR protein expression (Figure 6a) and significantly inhibited DF migration, as shown by the number of migrated cells (Figure 6b) in the scratching assay (Figure 6c). This indicates that autocrine and paracrine NTs promote DF migration through the combined activity of NT receptors.

NGF and BDNF stimulate dermal fibroblast contraction *in vitro*

In order to evaluate DF contraction we have used GlaSbox®, a previously described device that measures tensile strength generated by human dermal fibroblasts embedded in a collagen gel to create a Living Dermal Equivalent (LDE) (Viennet et al, 2005). All generated curves exhibit 2 distinct phases: the first 8 hrs phase shows a fast increase of the contractile strength reaching a maximum and then a 8h to 24h phase with a maximal and constant contractile strength.

We analyzed the effect of NTs on the contractile strength in the GlaSbox. NGF significantly stimulated contractile strength in a dose dependent manner, as compared to controls, while only 100ng/ml dose was as effective as TGF-β_1, used as a positive control (Figure 7a). The maximal strength was reached around 8-10 hrs with 100ng/ml and 50ng/ml. BDNF significantly stimulated the contractile strengths at 8 hrs only with 100ng/ml dose, although it was less effective than NGF at the same dose (Figure 7b). Contractile properties of NGF and BDNF were observed in the collagen gel, after extraction of the LDE from the GlaSbox® (Figure 7c and d). By contrast NT-3 and NT-4 failed to exert any effect on the contractile strength (Figure 7e, f, g and h).

Discussion

NTs form an intricate system at the epidermal level where they are released by several cell types which in turn express NT receptors (Raap and Kapp, 2010; Botchkarev et al, 2006). Fibroblasts participate in a number of critical functions in the skin, such as tissue remodeling and fibrosis (Werner et al, 2007; Krieg et al, 2007). Furthermore, fibroblasts play an important role in the skin

immune system, as they promote the migration of dendritic cells and support the expansion of IL-17 producing T cells (Saalbach et al, 2010; Albanesi et al, 2009; Schirmer et al, 2010). Fibroblasts together with the ECM are also essential for tumor invasion and metastasis (Li et al, 2009). In this work, we demonstrate that NTs, by modulating several fibroblast functions, extend their influence also in the dermis, making the skin NT network more and more complex.

We have shown that DF and DM synthesize and release all NTs and express both the high affinity receptors, TrkA, TrkB and TrkC, and the low affinity receptor p75NTR. In particular, NGF and NT-3 are secreted at higher levels than other NTs, and DM produce higher amounts of NT than DF.

It has already been shown that NGF is released by damage tissues (Hattori et al, 1996). In this context, metabolically active DM could release NGF and NT-3 and stimulate tissue remodeling. Interestingly, NGF and NT-3 also induce keratinocyte proliferation (Marconi et al, 2003), suggesting that these NTs act on the two most important cells involved in wound healing. While TrkA is more expressed in DF than in DM, p75NTR is almost exclusively expressed in DM, in agreement with data reported for the corneal system (Micera et al, 2006). It is conceivable that NGF and NT-3 stimulate the differentiation of DF into DM through TrkA and initiate tissue remodeling. On the contrary, in DM, p75NTR could act as death receptor, as demonstrated in other systems, where p75NTR activation contributes to DM elimination in the final phase of the wound healing process (Desmoulière et al, 1995). Consistently, p75NTR-mediated apoptosis of DM is also necessary to prevent hepatic fibrosis (Kendall et al, 2009).

Exogenous NTs *per se* fail to stimulate DF and DM proliferation, possibly because endogenous production of NTs is sufficient to normal cell proliferation and survival. However, blocking their signaling pathway, through inhibition of Trk receptors, in absence of exogenous NTs, strikingly reduces DF and DM proliferation, in agreement with previous reports where the use of chimeric receptor that block Trk activity, inhibit cell proliferation in melanoma cell lines (Truzzi et al, 2008). In particular, inhibiting Trk receptor signalling induces DF and DM G0-G1 arrest, and caspase-3 activation, further confirming the

importance of NT in maintaining DF and DM viability through Trk receptors. On the other hand, blocking p75NTR promotes G2-M arrest, demonstrating that in this context p75NTR only acts as Trk co-receptor.

Interestingly, NTs also promote DF differentiation into DM, by inducing α-SMA expression, indicating that NTs could have a functional role in the fibro-myofibroblasts system. In fact, it has been shown that NGF induce fibroblast-like keratinocyte differentiation into DM (Micera et al, 2001; Micera et al, 2007a), their contraction in 3D collagen matrix (Micera et al, 2001) and the expression of MMP-9 (Metalloprotease-9) in keratoconjunctivitis-derived fibroblasts (Micera et al, 2007b) and different works show the applicative possibility of this NGF capacity (Landi et al, 2003; Aloe et al, 2008; Sun et al, 2010).

DF migration and contractile strength are critical for matrix remodeling (Rhee, 2009). We have demonstrated that all NTs promote DF migration while NGF, and BDNF promote DF contractile activity. Therefore NGF and BDNF, produced by dermal and epidermal cells, could be key regulators of the biomechanical properties in the dermis. Taken together, these results add a further functional element to the complex network created by NTs in the skin. In particular, NTs seem to participate in a cross talk between dermal and epidermal cells, pointing to a fundamental role in both physiologic conditions and diseases.

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Legends

Figure 1. Fibroblasts and myofibroblasts synthetize and secrete all NT and express NT receptors. (a) DF and DM in culture. DM were obtained by maintaining DF in TGF-β_1-additioned medium. **(b)** α-SMA expression was evaluated by direct immunofluorescence in both DF and DM. **(c-f)** NGF, NT-3, NT-4 and BDNF protein level of both DF and DM were evaluated by ELISA assay in cell lysates and conditioned medium, as describe in Materials and Methods. Data are expressed as the mean ± SEM of triplicate from three different experiments. **(g)** TrkA, TrkB, TrkC and p75NTR mRNA were evaluated by RT-PCR and ethidium bromide staining. β-actin mRNA was used as an internal control. Control lanes (1, 2) represent amplification with no template and RNA without reverse transcription, respectively. **(h)** TrkA, TrkB, TrkC and p75NTR protein expression was studied by Western blotting in DF and DM, as described in Materials and Methods. β-actin was used as control.

Figure 2. Neurotrophins support fibroblast viability through Trk receptors. (a-b) DF and DM were plated onto 96-well tissue culture plate and stimulated with NGF, BDNF, NT-3 or NT-4 (100ng/ml). MTT assay was performed at 0, 24, 48 and 72 hours later. **(c-d)** DF and DM were plated onto 96-well tissue culture plate and treated with recombinant human TrkA/Fc (2μg/ml), TrkB/Fc, TrkC/Fc chimeras (1μg/ml), k252a (200nM) or diluent alone. MTT assay was performed 24, 48 and 72 hrs later. **(e)** DF and DM were treated with K252a (200nM) or diluent alone and after 24 hrs BrdU was added in each plate. After 48hrs from stimulus with K252a, control and treated cells were trypsinized and stained with. Cell cycle was analyzed by flow cytometry; one representative experiment is shown. **(h)** DF and DM were treated with K252a (200nM) and lysed after 48 hrs. Cyclin B and Procaspase-3 were evaluated by Western Blotting. β-actin was used as internal control.

Figure 3. Neurotrophins support fibroblast viability through p75NTR. **(a)** About 100,000 DF were plated onto 60 mm^2 petri dishes and transfected with 100nM p75NTRsiRNA or scramble siRNA on the next day. p75NTR expression was controlled 24 and 48 hrs later by Western blotting. (**b**) About 1500 DF were plated onto 96-well tissue culture plate and transfected with 100nM p75NTRsiRNA or scramble siRNA on the next day. MTT assay was performed 24 and 48 hours later. Data are expressed as mean +/- SEM of triplicate from three different experiments. **(c)** About 100,000 DF were plated onto 60 mm^2 petri dishes and transfected with 100nM p75NTRsiRNA or scramble siRNA on the next day. Cyclin B and procaspase-3 expression was evaluated by Western Blotting 24 and 48 hrs later. β-actin was used as internal control.

Figure 4. Neurotrophins induce fibroblast differentiation into myofibroblast. DF were stimulated or not with TGF-β_1 (100ng/ml) or NGF, BDNF, NT-3 and NT-4 (100ng/ml). **(a)** 24, 48 hrs and six-day after treatment, cells were lysed and α-SMA protein expression was evaluated by Western blotting. Vinculin was used as internal control. **(b)** DF were stimulated as previously described and six-day later, cells were fixed with formalin and stained with FITC conjugated antibody anti-α-SMA. Nuclei were stained with DAPI. Cells were analyzed by confocal microscopy. Bar, 15 μm

Figure 5. Neurotrophins stimulate fibroblast migration through Trk receptors. (a) DF were plated onto 12-well tissue culture plate and treated with mitomycin C (5μg/ml) for two hours. One line for each well was drawn along the cell monolayer with a sterile tip. After washing with serum free medium, cells were incubated with TGF-β_1 (100ng/ml) or NGF, BDNF, NT-3, NT-4 (100ng/ml) or diluent alone. After 24 and 48 hours, six areas were counted and expressed as mean of cell migrated/area. Data are expressed as mean +/- SEM of triplicate from three different experiments. **(b)** Cells were observed and photographed at 48 hours after stimuli. **(c)** DF were plated onto 12-well tissue culture plate and treated with mitomycin C (5μg/ml) for two

hours. One line for each well was drawn along the cell monolayer with a sterile tip. After washing with serum free medium, cells were incubated with recombinant human TrkA/Fc (2μg/ml), TrkB/Fc, TrkC/Fc chimeras (1μg/ml) or k252a (200nM) or diluent alone. After 24 and 48 hours, six areas were counted and expressed as mean of cell migrated/area. Data are expressed as mean +/- SEM of triplicate from three different experiments. **(d)** Cells were observed and photographed at 48 hours from stimuli.

Figure 6. Neurotrophins stimulate fibroblast migration through p75NTR. (a) About 100,000 DF were plated onto 60 mm^2 petri dishes and transfected with 100nM p75NTRsiRNA or scramble siRNA on the next day. p75NTR expression was controlled 48 hrs later by Western blotting. **(b)** About 50,000 DF were plated onto 12-well plates and transfected with 100nM p75NTRsiRNA or scramble siRNA on the next day. Mock and p75NTRsiRNA cells were treated with 5μg/ml mitomycin and scratched as previously described. Subsequently, cells were incubated or not with TGF-β_1 (100ng/ml) or NGF, BDNF, NT-3 or NT-4 (100ng/ml). After 48 hours three areas were counted and expressed as the mean of cell migrated per area. Data are expressed as the mean ± SEM of triplicate from three different experiments. **(c)** Cells observed and photographed at 48 hours from stimuli.

Figure 7. Neurotrophins induce fibroblast tensile strength. (a-h) Living dermal equivalent (LDE), formed as described in M&M, were placed into GlaSbox devices and stimulated with TGF-β_1 (100ng/ml) or NGF, BDNF, NT-3 or NT-4 at the concentration of 1ng/ml, 10ng/ml or 100ng/ml for each stimulus or neutral control (DMEM alone). Tensile strenght generated from each LDE was measured as changes in elettrical resistence by a Wheaston bridge and converted in milliNewton (mN) by a computerized system. Data are expressed as the mean of three different experiments. Each GlaSbox device, containing LDE, was observed and photographed after 24 hours.