

## Nuclear localization of TRK-A in liver cells

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**Summary.** The liver represents a site of expression of neurotrophins and their receptors. We have characterized the expression and intracellular localization of the nerve growth factor (NGF) receptor, Trk-A, in liver cells *in vivo* and *in vitro*. In both normal and fibrotic liver tissue, Trk-A immunostaining was present in different cell types, including parenchymal cells and cells of the inflammatory infiltrate. In hepatocytes and activated stellate cells (HSC), Trk-A showed a predominant nuclear localization, both in the presence and absence of injury. In cultured HSC, Trk-A was found to be functional, because exposure of the cells to recombinant NGF resulted in stimulation of cell migration and activation of intracellular signaling pathways, including Ras-ERK and PI3K/Akt. Remarkably, in cultured HSC, Trk-A staining was found constitutively in the nucleus. In these cells, Trk-A could be stained only by antibodies directed against the intracellular domain but not by those recognizing the extracellular portion of Trk-A suggesting that the intracellular portion of the receptor is the major determinant of nuclear Trk-A staining. In contrast to HSC, freshly isolated hepatocytes did not show any nuclear localization of the intracellular portion of Trk-A. In pheochromocytoma cells, nuclear staining for Trk-A was not present in conditions of serum deprivation, but could be induced by exposure to NGF or to a mixture of soluble mediators. We conclude that nuclear localization of the intracellular domain of Trk-A is observed constitutively in liver cells such as HSC, while in other cell types it could be induced in response to soluble factors.

**Key words:** Liver fibrosis, Hepatic stellate cells, PC12, Nerve growth factor

### Introduction

The family of neurotrophins comprises several polypeptides that regulate proliferation, differentiation, survival and development in the central and peripheral nervous system (Huang and Reichardt, 2003). Nerve growth factor (NGF) represents the most widely studied cytokine of this family. NGF signals mainly through two molecules located on the cell surface, namely, a high affinity tyrosine kinase-coupled receptor, Trk-A, and a low-affinity receptor belonging to the TNF-receptor superfamily known as p75<sup>NGF-R</sup> (Kaplan and Miller, 1997). While these molecules have been originally identified in the nervous system, subsequent studies have indicated that the distribution of Trk-A and p75 is widespread in many tissues, where they regulate different biological activities in epithelial and stromal cells (Shibayama and Koizumi, 1996; Descamps et al., 1998; Khan et al., 2002).

The liver represents a site of expression of several neurotrophins and their receptors, including NGF, Trk-A and p75. Interestingly, the relative expression of these molecules has been shown to be modulated during the process of tissue healing (Cassiman et al., 2001). Similar to what is observed in other tissues, the response of the liver to an acute or chronic injury requires a complex cross-talk involving different cell types. In particular, following damage to the hepatocytes, all non-parenchymal cell types participate in a coordinated response aimed at reconstituting the integrity of the tissue. In this context, a pivotal role is played by myofibroblastic cells derived from activation of hepatic

stellate cells (HSC) and from other cellular sources within the liver and possibly from the bone marrow (Forbes et al., 2004; Ramadori and Saile, 2004). HSC may be considered as paradigmatic of the biology of the cells implicated in liver repair. Upon liver injury, HSC undergo a damage-induced activation process that enables them to coordinate the liver "wound-healing" response (Friedman, 2000). In particular, activated HSC acquire a myofibroblast-like phenotype and are major determinants, together with myofibroblasts derived from other populations, of the development of fibrosis in the setting of chronic liver damage. HSC are a source of several neurotrophins in the liver, and they are known to express neurotrophin receptors (Cassiman et al., 2001).

This study was undertaken to investigate the expression and subcellular localization of the NGF receptor, Trk-A, in liver cells, with particular emphasis on HSC. In addition, the biological effects of NGF on cultured human HSC was also analyzed. We show that Trk-A is mainly localized at the nuclear level in both hepatocytes and HSC *in vivo*, and that nuclear localization occurs constitutively in cultured HSC. Moreover, we demonstrate that nuclear localization involves the intracellular domain of Trk-A and that this process is modulated by soluble mediators.

## Materials and methods

### Reagents

Primary Antibodies against Trk-A used in this study are summarized in Table 1. Fluorescent secondary antibodies used in immunofluorescence were from Molecular Probes (Eugene, OR). Mouse monoclonal antibodies against  $\alpha$ -smooth muscle actin (clone 1A4) and against the splicing factor SC-35 were purchased from Sigma Chemical Co. (St. Louis, MO). Polyclonal antibodies against the phosphorylated form of ERK, Raf-1, and Akt (Ser473), were from Cell Signaling Technology (Beverly, MA). Polyclonal anti-ERK antibodies used for Western blotting and antibodies against p75NGF-R were purchased from Santa Cruz

Biotechnology (Santa Cruz, CA). Human recombinant NGF and PDGF-BB were purchased from Peprotech (Rocky Hill, NJ). All other reagents were of analytical grade.

### Tissues

Normal liver tissue (3 samples) used for all experiments was obtained during surgical liver resection for secondary liver cancer. The tissue was obtained at a minimum of 5 cm from the tumor, and normal histology was assessed by routine examination. Samples of patients with chronic hepatitis C and cirrhosis used for immunohistochemistry were obtained from livers explanted for orthotopic transplantation (n=3). No patients had a history of alcohol intake or other known causes of liver damage. The specimens were snap-frozen in liquid nitrogen until analyzed. The procedures followed in the study were in accordance with the ethical standards of the Regional Committee on Human Experimentation.

### Immunohistochemistry

These experiments were conducted on frozen sections as described in detail elsewhere (Bonacchi et al., 2003). Dried sections were sequentially incubated with the primary antibody and, after washing, with mouse monoclonal anti-rabbit antibodies, and finally with affinity-purified rabbit anti-mouse antibodies. At the end of the incubation, sections were washed twice in TBS and then incubated with alkaline phosphatase anti alkaline phosphatase (APAAP) and developed. Negative controls were treated with omission of the primary antibody or its substitution with non-immune rabbit immunoglobulins.

### Immunofluorescence

Six  $\mu$ m-thick sections were cut from frozen tissue, allowed to dry on glass slides and sequentially fixed for 30 minutes in acetone and chloroform. Sections were

**Table 1.** List of the antibodies against Trk-A used in this study.

Antibody	Source	Epitope	Use	Reference(s)
Trk-A	Santa Cruz sc-118	C-terminus (intracellular)	IHC WB IF	Colangelo et al. 2005 Özbas-Gerçeker et al. 2004
Trk-A	Cell Signaling Technology #9142	C-terminus (intracellular)	WB IF	Rossi et al. 2002
Trk-A	Upstate Biotechnology 06-574	N-terminus (extracellular)	IHC IF	Chattopadhyay et al. 2003 Chiarenza et al. 2001
Phospho-Trk-A Tyr 490	Cell Signaling Technology #9141	Intracellular domain (residues around Tyr 490)	WB IF	Binder et al. 1999
Phospho-Trk-A Tyr 674/675	Cell Signaling Technology#9144	Intracellular domain (residues around Tyr 674/675)	WB IF	Wahab et al. 2005

IF: immunofluorescence; IHC: immunohistochemistry; WB: Western blotting.

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then washed three times for 5 minutes in PBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% tritonX-100), incubated for 60 minutes with blocking buffer (5.5% horse serum in PBST) and then with primary antibodies (1:100 dilution in PBS containing 3% BSA) for 20 hours at 4°C. After washing with PBST containing 0.1% BSA for 15 minutes, sections were incubated with secondary antibodies diluted in PBST-3% BSA for 1 hour at room temperature and washed 3 times for 5 minutes in PBST and once for a few seconds in deionized water. Finally, slides were mounted using mounting medium and a coverslip and viewed with a fluorescence microscope (Leica, Wetzlar, Germany).

For confocal microscopy, after extensive washes in TBST, cells or tissue samples were mounted with glycerol plastine and observed under a laser scanning confocal microscope (Bio-Rad MRC 1024 Es; Hercules, CA). A series of optical sections (512x512 pixels) were taken through the depth of the specimen with a thickness of 1 µm at intervals of 0.2 µm. Twenty optical sections were examined and then projected as a single composite image by superimposition (Confocal Assistant version 4.02).

### Cell culture

Human HSC were isolated from wedge sections of liver tissue unsuitable for transplantation by collagenase/pronase digestion and centrifugation on Stractan gradients. Procedures used for cell isolation and characterization have been extensively described elsewhere (Casini et al., 1993). All the experiments were conducted on cells cultured on uncoated plastic dishes, showing an "activated" or myofibroblast-like phenotype.

PC12 were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 10% calf serum, glutamine, and non-essential aminoacids. Before the experiments, cells were deprived of serum for 16 hours.

Primary human hepatocytes were isolated as described elsewhere (Giannini et al. 2003) from the healthy liver tissue of surgical specimens (approximately 20-25 cm<sup>3</sup>) collected after informed consent had been obtained from patients undergoing therapeutic partial hepatectomy for liver metastasis or benign hepatic tumor.

### Cell proliferation

Measurement of thymidine incorporation was performed on serum-starved, confluent HSC incubated with agonists for 24 hours and then pulsed with [<sup>3</sup>H] thymidine. DNA synthesis was measured as described elsewhere (Marra et al., 1999). Measurement of cell numbers was conducted in serum-starved, sub-confluent cells plated on 12-well dishes and exposed to different conditions for 5 days. Cells were then washed with PBS and incubated with 0.5% (w/v) crystal violet in 20% methanol for 5 min at room temperature. After three washes with PBS, the monolayer was incubated with

0.1M sodium citrate, pH 4.2, for 1 hour at room temperature. At the end of incubation, an aliquot of this solution was transferred to a 96-well dish and read at 565 nm. All assays were conducted in triplicate.

### Cell migration

Confluent HSC were serum-starved for 48 h and then washed, trypsinized, and resuspended in serum-free medium containing 1% albumin at a concentration of 3x10<sup>5</sup> cells/mL. Chemotaxis was measured in modified Boyden chambers equipped with 8 mm pore filters (Poretics, Livermore, CA) coated with rat tail collagen (Collaborative Biomedical Products, Bedford, MA), as previously de-scribed (Marra et al., 1999). When inhibitors were used, cultured cells were incubated with the drugs to be tested or with their vehicle for 15min before trypsinization, and equal concentrations were added in the Boyden chamber.

### NGF Elisa

Serum-deprived sub-confluent HSC were maintained in serum-free medium for 24 hours. The medium was then collected and stored at -20°C until assayed. NGF assay was performed as described in detail elsewhere (Rosini et al., 2000), using a commercially available NGF mouse monoclonal antibody (Boehringer Mannheim, Mannheim, Germany). The sensitivity of the test ranged between 8 and 20 pg/ml.

### Reverse-transcriptase-polymerase chain reaction (RT-PCR)

mRNA was extracted from human HSC, reversed to first strand cDNA and amplified by RT-PCR, as previously described (Bonacchi et al. 2003). The primers used were as follows: NGF forward 5'-TAAAAAG CGGCGACTCCGTT-3'; reverse 5'-GTAGAAGGTGTC CCCGCTTA-3' (Labouyrie et al. 1999); β-actin forward 5'-TCGACAACGGCTCCGGCATGTGCAAG-3'; reverse 5'-AGCCACACGCAGCTCATTGTAGAA G-3'.

### Preparation of Cell Lysates and Western Blotting

Confluent, serum- starved HSC were treated with the appropriate conditions, quickly placed on ice, and washed with ice-cold phosphate-buffered saline. The monolayer was lysed in radioimmunoprecipitation buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride, 0.05% (w/v) aprotinin). Insoluble proteins were discarded by high speed centrifugation at 4°C. Protein concentration in the supernatant was measured in triplicate using a commercially available assay (Pierce, Rockford, IL). Equal amounts of total cellular proteins were separated by SDS-PAGE and analyzed by Western blot as previously described (Bonacchi et al., 2003).

### Data presentation and statistical analysis

Autoradiograms and autoluminograms are representative of at least three experiments with comparable results. Data in bar graphs are presented as mean $\pm$ SD, and were analyzed by Student's t-test.

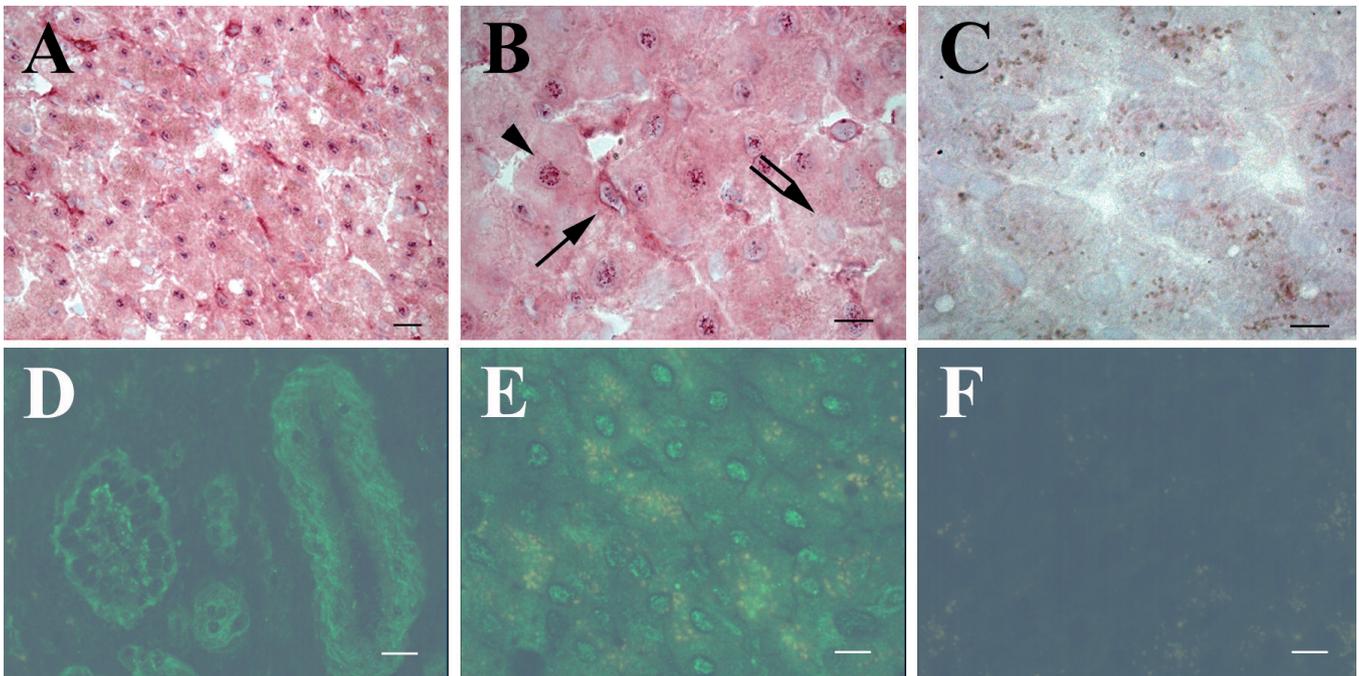
## Results

### Nuclear localization of Trk-A in liver cells *in vivo*

We first analyzed the expression pattern of Trk-A in normal human liver tissue and in condition of chronic liver damage. In normal liver, a diffuse immunostaining for this receptor was observed in both hepatocytes and cells localized in the hepatic sinusoids (Fig. 1A). At higher magnification, nuclear localization of Trk-A immunostaining was detected in some of the hepatocytes and cells of the hepatic sinusoid, although the intensity of staining and the presence of nuclear localization were not uniformly observed in all cells (Fig. 1B). Remarkably, nuclear staining was not a characteristic of all cells, because it was almost absent in some of the hepatocytes and sinusoidal cells. Using immunofluorescence, positive immunostaining for Trk-A was also observed in structures located in the portal tract, including epithelial cells of the bile ducts and cells located in the wall of hepatic artery branches (Fig. 1D). Of note, nuclear localization was rarely observed in cells

belonging to these structures, while it was well evident, with the same technique, in the hepatocytes (Fig. 1E).

In the liver with chronic damage and fibrosis, such as that due to HCV-related cirrhosis, lymphocytes infiltrating the portal tracts and forming inflammatory lymphoid follicles also appeared to express Trk-A, albeit without any evidence of nuclear localization (Fig. 2A), in agreement with previous observations in isolated cells (Torcia et al., 1996). Positive cytoplasmic and nuclear staining of the hepatocytes was observed also during chronic inflammation (Fig. 2B), and the intensity of nuclear staining appeared to be higher than in control liver tissue. In close proximity to the hepatocytes, several spindle-shaped cells within the fibrotic septa also expressed Trk-A, which in several instances was localized at the nuclear level, especially at the interface between septum and nodule (Fig. 2B). The location of these cells is compatible with the one of matrix-producing myofibroblasts derived from activated HSC and/or activated portal myofibroblasts (Cassiman and Roskams, 2000). To confirm that activated HSC express this NGF receptor *in vivo*, serial sections from fibrotic livers were analyzed by immunofluorescence using antibodies directed against Trk-A or  $\alpha$ -smooth muscle actin, as a marker for these cells. Merging the images derived by the two immunostainings revealed a clear colocalization of the specific signals, together with evidence of nuclear localization (Fig. 2F). Taken together, these data indicate that in some, but not all



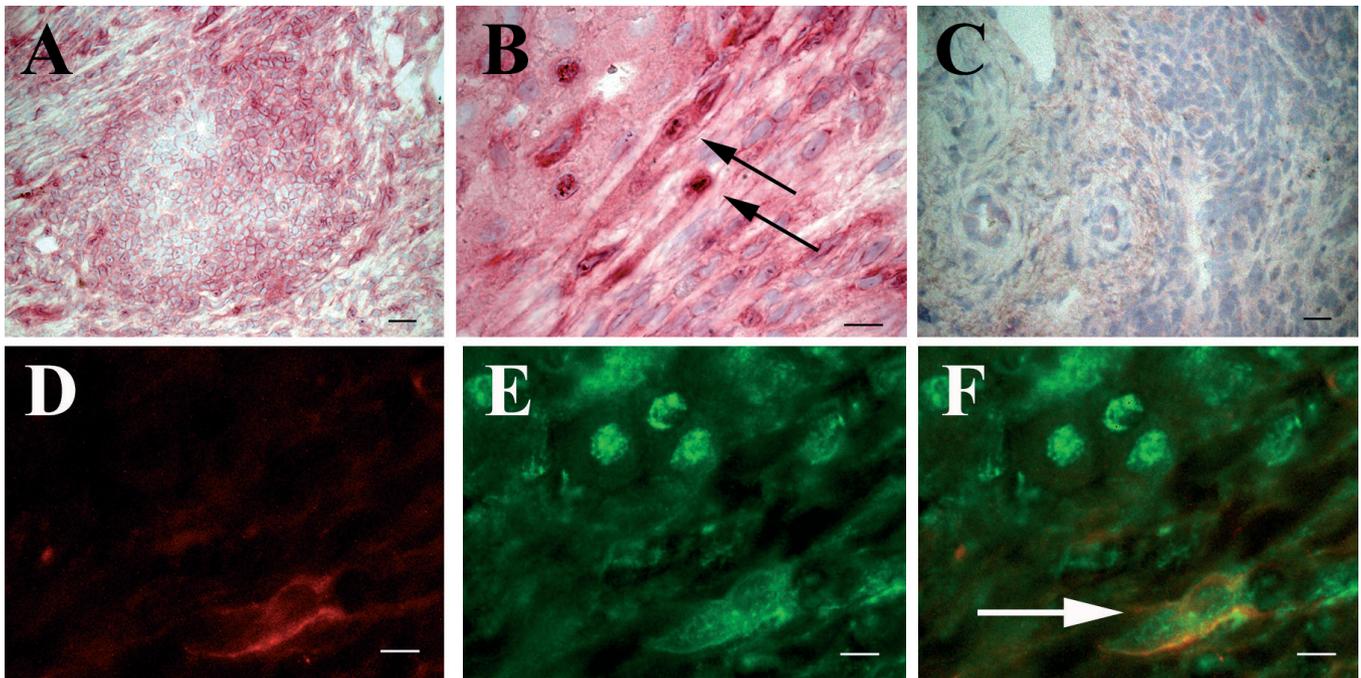
**Fig. 1.** Expression pattern of Trk-A in normal liver tissue. **A, B.** Sections of normal liver tissue were immunostained with antibodies directed against the intracellular domain of Trk-A (Santa-Cruz Biotechnology, see Table 1) and APAAP. Nuclear Trk-A immunostaining was present in some of the hepatocytes (arrowhead) and cells of the hepatic sinusoid (arrow), while other cells were negative at the nuclear level (double arrow). **C.** Negative control for immunohistochemistry. **D, E.** Immunofluorescence with anti-Trk-A antibodies (Santa-Cruz Biotechnology, see Table 1). A portal tract (**D**) and intralobular structures (**E**) are shown. **F.** Negative control for immunofluorescence. Scale bar: 50  $\mu$ m (**A**); 25  $\mu$ m (**B-F**).

liver cells, Trk-A immunostaining is located in the nucleus, and that hepatocytes and activated HSC are among the cell types in which this localization may be observed *in vivo*.

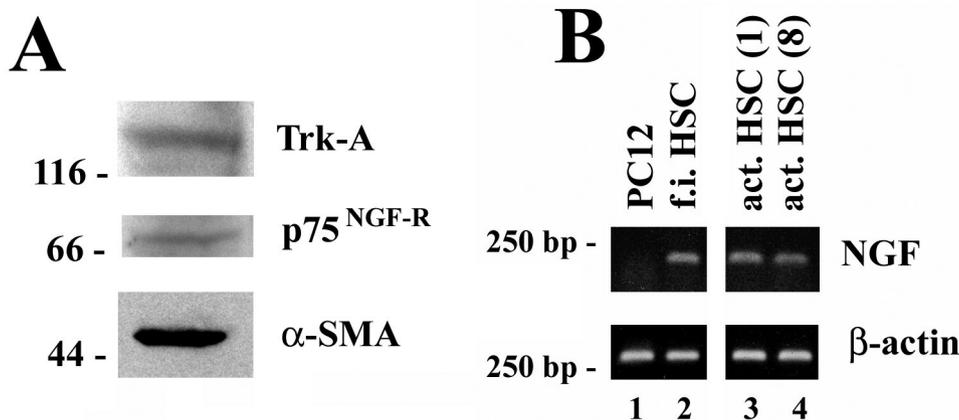
*Cultured HSC express NGF and its receptors*

To obtain further information on the significance of the NGF system in these cell types, we evaluated the expression of the two NGF receptors, namely, the high-

affinity receptor Trk-A and the low-affinity p75, in cultured human HSC. After culture on plastic, a procedure that induces some characteristics of the activated phenotype, expression of Trk-A could be detected by western blot analysis (Fig. 3A). In addition, mRNA transcripts encoding for this receptor were found by RT-PCR (data not shown). Activated HSC also showed a specific signal for the p75 receptor, in agreement with data previously reported by other investigators in both rat and human HSC (Trim et al.



**Fig. 2.** Expression pattern of Trk-A during liver damage and fibrosis **A, B.** Sections of liver tissue with chronic damage and fibrosis due to HCV infection were immunostained with antibodies directed against the intracellular domain of Trk-A and APAAP. An inflammatory lymphoid follicle (**A**) and a septum-nodule interface area (**B**), where nuclear staining of fibrogenic cells is especially present at the interface between septum and nodule (arrows), are shown. **C.** Negative control for immunohistochemistry. **D-E.** Immunofluorescence with anti- $\alpha$ -smooth muscle actin antibodies (**D**) or anti-Trk-A antibodies (**E**) (Santa-Cruz Biotechnology, see Table 1). **F.** Overlay of **D** and **E**, showing an hepatic stellate cell positive for Trk-A (arrow). Scale bar: 100  $\mu$ m (**A**); 25 $\mu$ m (**B, D-F**); 50  $\mu$ m (**C**).



**Fig. 3.** Expression of all components of the NGF axis in cultured human HSC. **A.** Total protein lysates from culture-activated HSC were analyzed by immunoblotting using the indicated antibodies. Migration of molecular weight markers is indicated on the left. **B.** Total RNA isolated from cultured PC12 (lane 1), freshly-isolated human HSC (lane 2) or two lines of activated HSC (lanes 3 and 4) was analyzed for NGF expression by RT-PCR, as described in Materials and Methods.

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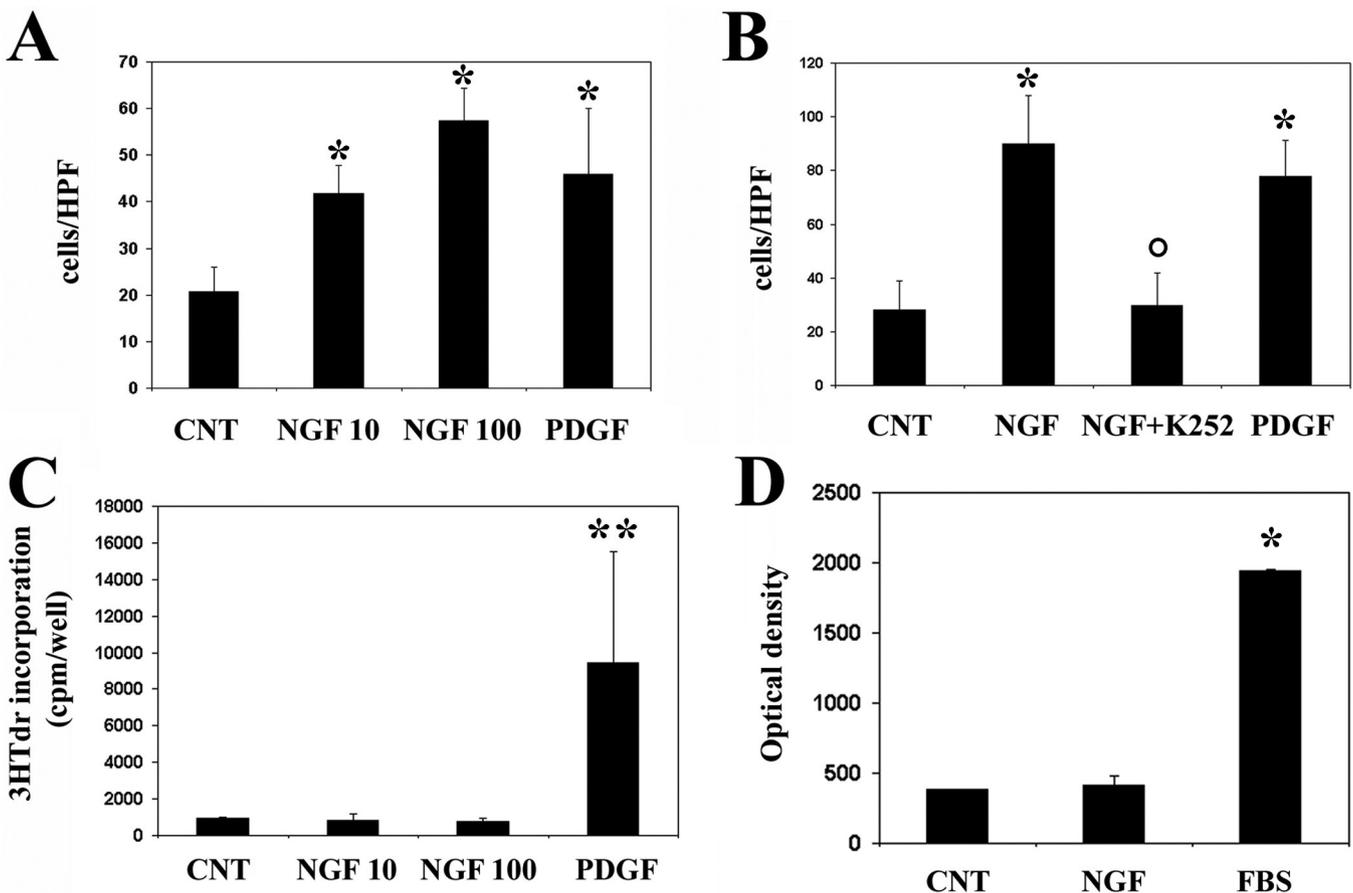
The presence of both receptors was associated with the expression of mRNA encoding for NGF, as shown by RT-PCR in both freshly-isolated (quiescent) and activated HSC (Figure 3B). NGF gene expression was accompanied by protein secretion, as indicated by a specific ELISA assay ( $0.25 \pm 0.009$  ng/ml, mean of 3 separate experiments). Thus, cultured HSC express all components of the NGF axis, indicating the possible occurrence of an autocrine loop.

#### Activation of Trk-A results in biological actions and downstream signaling in HSC

To establish whether expression of Trk-A by HSC is functional, we evaluated the effects of recombinant NGF on biological actions, such as cell proliferation and migration, which in other cell types have been associated with activation of Trk-A (Kraemer et al. 1999).

Exposure of HSC to increasing concentrations of NGF resulted in a 3-fold stimulation of cell migration in modified Boyden chambers (Fig. 4A-B). This effect was comparable to that of PDGF, a well-established chemotactic factor for these cells. To establish the functional role of Trk-A in mediating the chemotactic actions of NGF, HSC were pre-incubated with K-252, an inhibitor of Trk's tyrosine kinase activity (Kafitz et al., 1999; Rosini et al., 2000). At nanomolar concentrations, K-252 completely abolished NGF-induced cell migration (Fig. 4B). In contrast, NGF did not modify the proliferation of HSC, as indicated by analysis of DNA synthesis or evaluation of cell number using crystal violet (Fig. 4C,D).

The intracellular signaling pathways responsible for the transduction of chemotactic signals originated by surface receptors in HSC have been characterized in several studies, and include activation of the PI-3K/Akt and Ras/ERK pathways (Kaplan and Miller, 1997).



**Fig. 4.** Biologic actions of NGF on activated human HSC. **A.** Serum-deprived HSC were analyzed for cell migration in modified Boyden chambers as described in Materials and Methods, using as agonists NGF (10 or 100 ng/ml) or 10 ng/ml PDGF-BB, as indicated. **B.** The experiment was conducted as described for panel A, but cells exposed to 100 ng/ml NGF were pre-incubated with 100 nM K252 or its vehicle before measuring migration. **C.** Serum-deprived HSC were left untreated or incubated with NGF (10 or 100 ng/ml) or 10 ng/ml PDGF-BB, as indicated. After 24 hrs, incorporation of 3H-thymidine was measured. **D.** Serum-deprived HSC were left untreated or incubated with 100 ng/ml NGF or 10% fetal bovine serum for 5 days. At the end of incubation, cell number was evaluated by crystal violet staining. \*,  $P < 0.01$  vs. control; °,  $P < 0.01$  vs. NGF alone; \*\*,  $P < 0.07$  vs. control (Student's t test).

*Trk-A in liver cells*

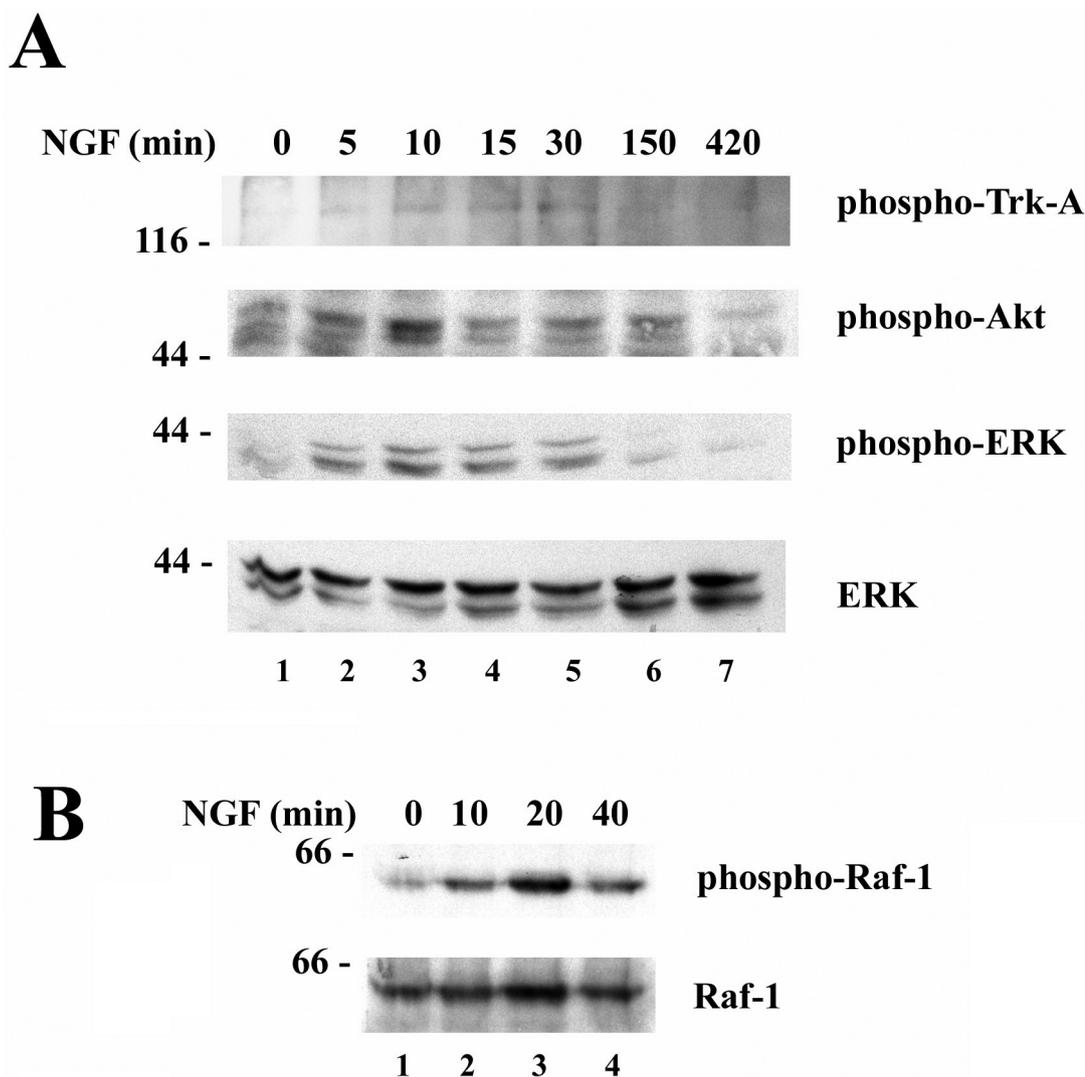
Exposure of HSC to recombinant NGF resulted in an increase in tyrosine phosphorylation of Trk-A, together with increased phosphorylation on activation-specific sites of Akt and ERK (Fig. 5A). Activation of the ERK cascade was confirmed by the ability of NGF to increase phosphorylation levels of Raf-1, a protein that functions as a MAPKKK in the Ras/ERK pathway (Fig. 5B). Of note, ERK and PI-3K/Akt have been shown to be required for NGF-induced migration in other cell types (Kraemer et al., 1999; Ho et al., 2001). Accordingly, inhibition of MEK/ERK or PI-3K using PD98059 or LY294002, respectively, prevented the induction of cell migration by NGF in HSC (data not shown).

*Nuclear localization of Trk-A in cultured human HSC*

The observation that Trk-A is expressed in the nucleus of fibrogenic cells of the liver (Figs. 1, 2) prompted us to investigate this phenomenon in greater

detail using cultured cells. We first focused on cultured HSC, which express a functional Trk-A receptor. Immunocytochemistry using antibodies directed toward TrkA demonstrated the presence of a specific signal localized in the cytoplasm and the nucleus of the great majority of cells in all fields (Fig. 6A,B). This finding was also confirmed by laser confocal microscopy in HSC after short-time culture on plastic, i.e. before complete activation. In this situation, the typical vitamin A droplets present in HSC after isolation are still present and may be visualized by autofluorescence (Fig. 6D,E). Also in this case, the predominant signal detected using anti-Trk-A antibodies was clearly localized at the nuclear level. In contrast, no evidence of nuclear localization was present for the low-affinity receptor, p75 (Fig. 6F), in agreement with the lack of nuclear staining observed by immunohistochemistry in normal and fibrotic liver tissue (6, and our data not shown).

To better define the specificity and significance of

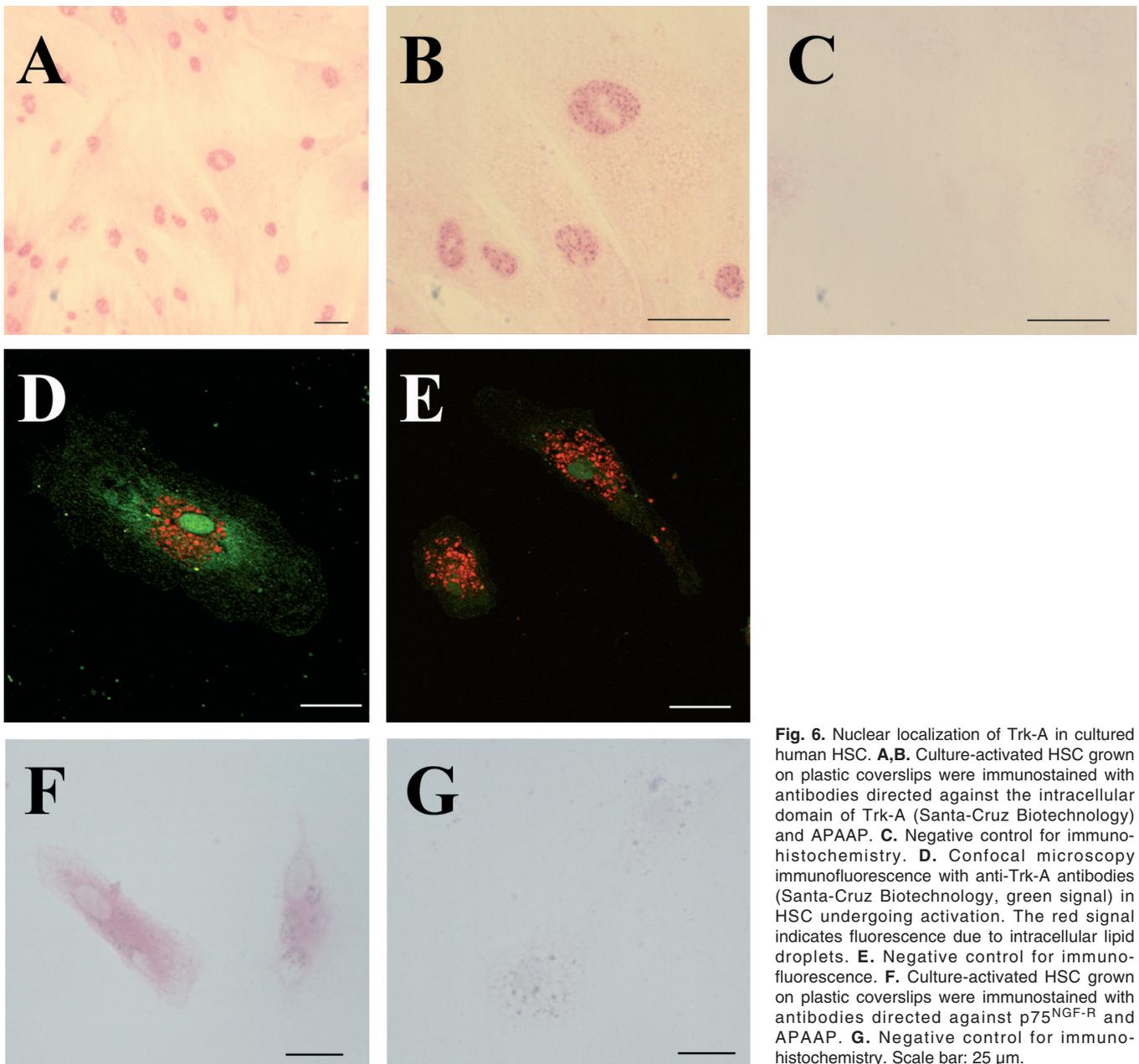


**Fig. 5.** Intracellular signaling elicited by NGF in human HSC. Serum-deprived HSC were incubated with 100 ng/ml NGF for the indicated time points. Total protein lysates were analyzed by immunoblotting using the indicated antibodies.

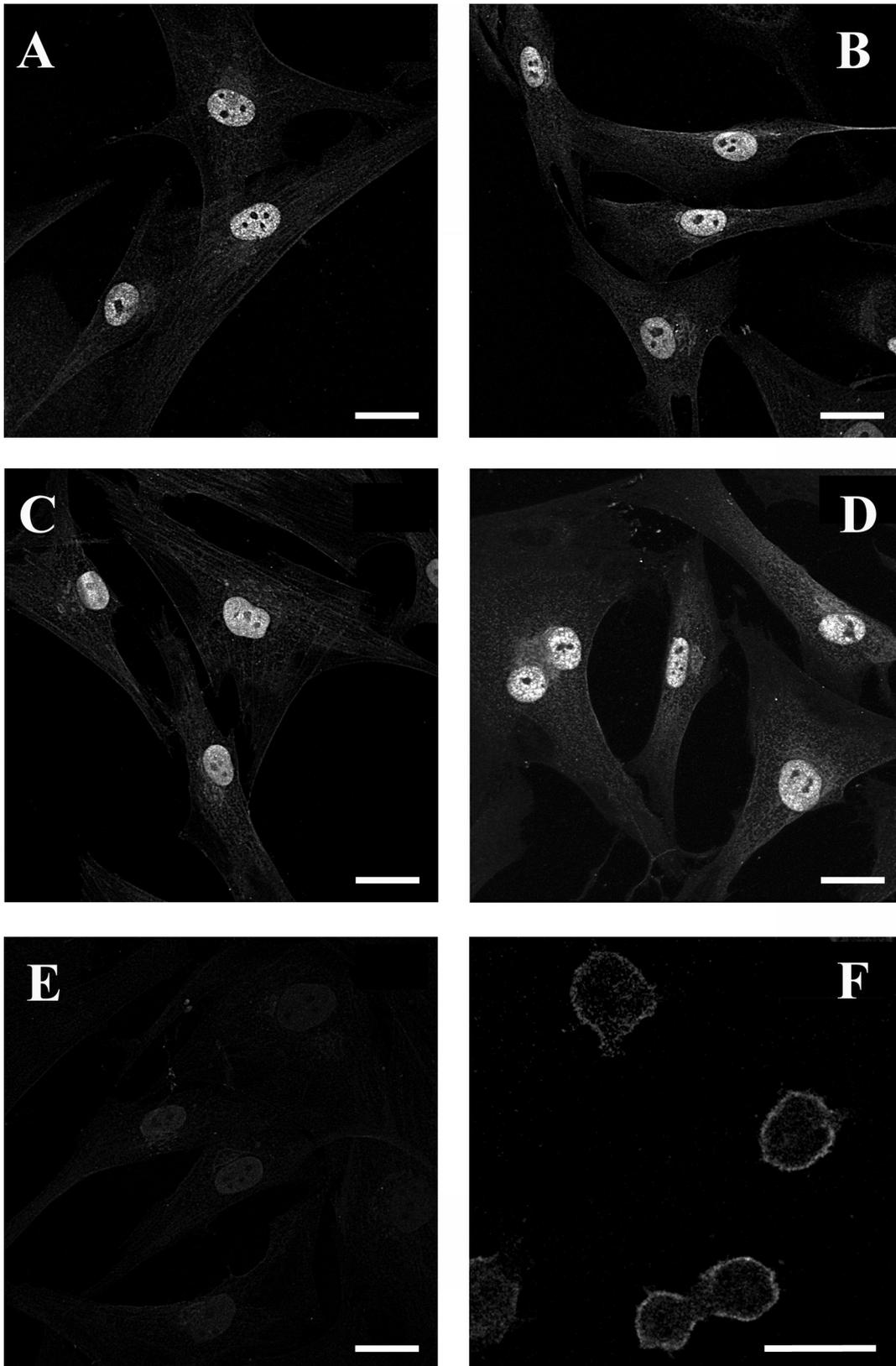
nuclear localization of Trk-A, we utilized laser confocal microscopy using a panel of different antibodies directed against Trk-A (Fig. 7). Two different antibodies confirmed the evident nuclear staining in HSC (Fig. 7A,B). We next employed two antibodies directed against activation-specific tyrosine residues of Trk-A. Both the antibodies recognizing phosphorylated tyrosines 490 or 746 yielded a very similar staining, comparable to that of antibodies directed against the non-phosphorylated form (Fig. 7C,D). These data indicate that at least part of Trk-A present in the nucleus is phosphorylated on activation-specific tyrosine

residues.

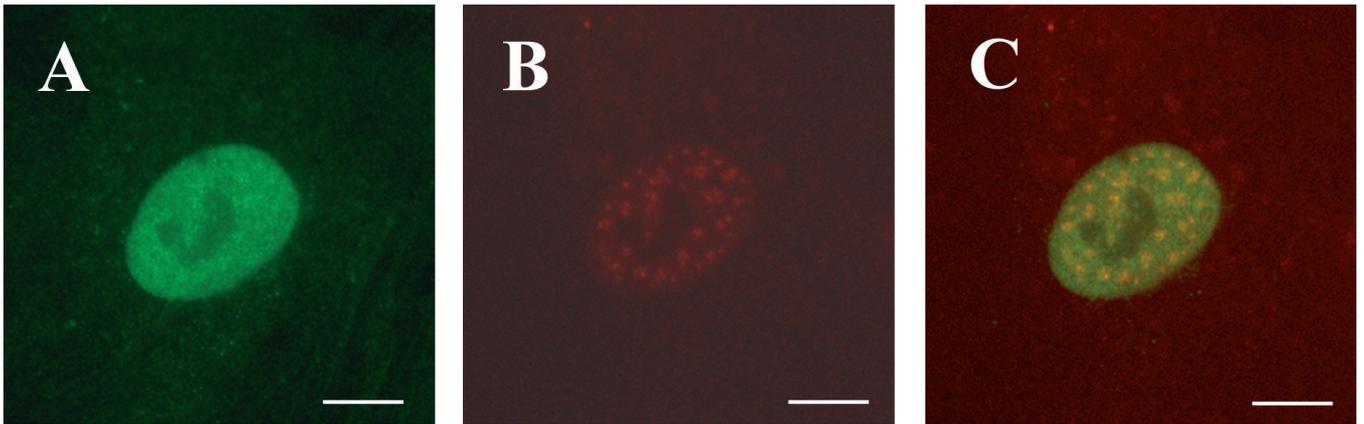
In order to assess whether nuclear localization of Trk-A is a constant finding in cells expressing this receptor, we also analyzed PC12, a pheochromocytoma cell line which expresses Trk-A at high levels, the activation of which mediates differentiation and survival. When these cells were serum-deprived, virtually all signals detected by confocal microscopy were localized at the membrane-cytoplasm level, whereas no evidence of nuclear staining was observed (Fig. 7F). Thus, constitutive nuclear localization of Trk-A is not a general feature of cultured cells.



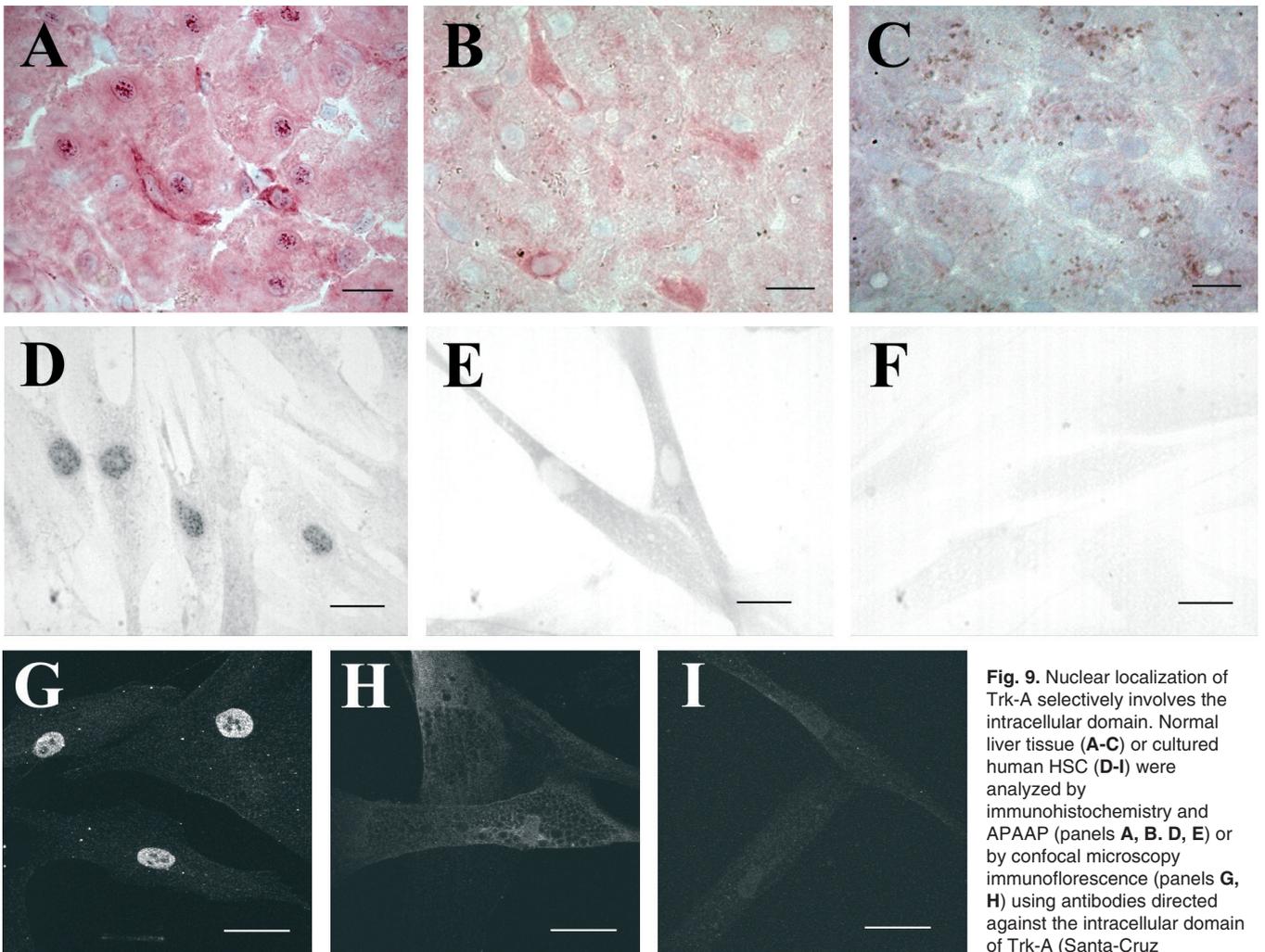
**Fig. 6.** Nuclear localization of Trk-A in cultured human HSC. **A,B.** Culture-activated HSC grown on plastic coverslips were immunostained with antibodies directed against the intracellular domain of Trk-A (Santa-Cruz Biotechnology) and APAAP. **C.** Negative control for immunohistochemistry. **D.** Confocal microscopy immunofluorescence with anti-Trk-A antibodies (Santa-Cruz Biotechnology, green signal) in HSC undergoing activation. The red signal indicates fluorescence due to intracellular lipid droplets. **E.** Negative control for immunofluorescence. **F.** Culture-activated HSC grown on plastic coverslips were immunostained with antibodies directed against p75<sup>NGF-R</sup> and APAAP. **G.** Negative control for immunohistochemistry. Scale bar: 25  $\mu$ m.



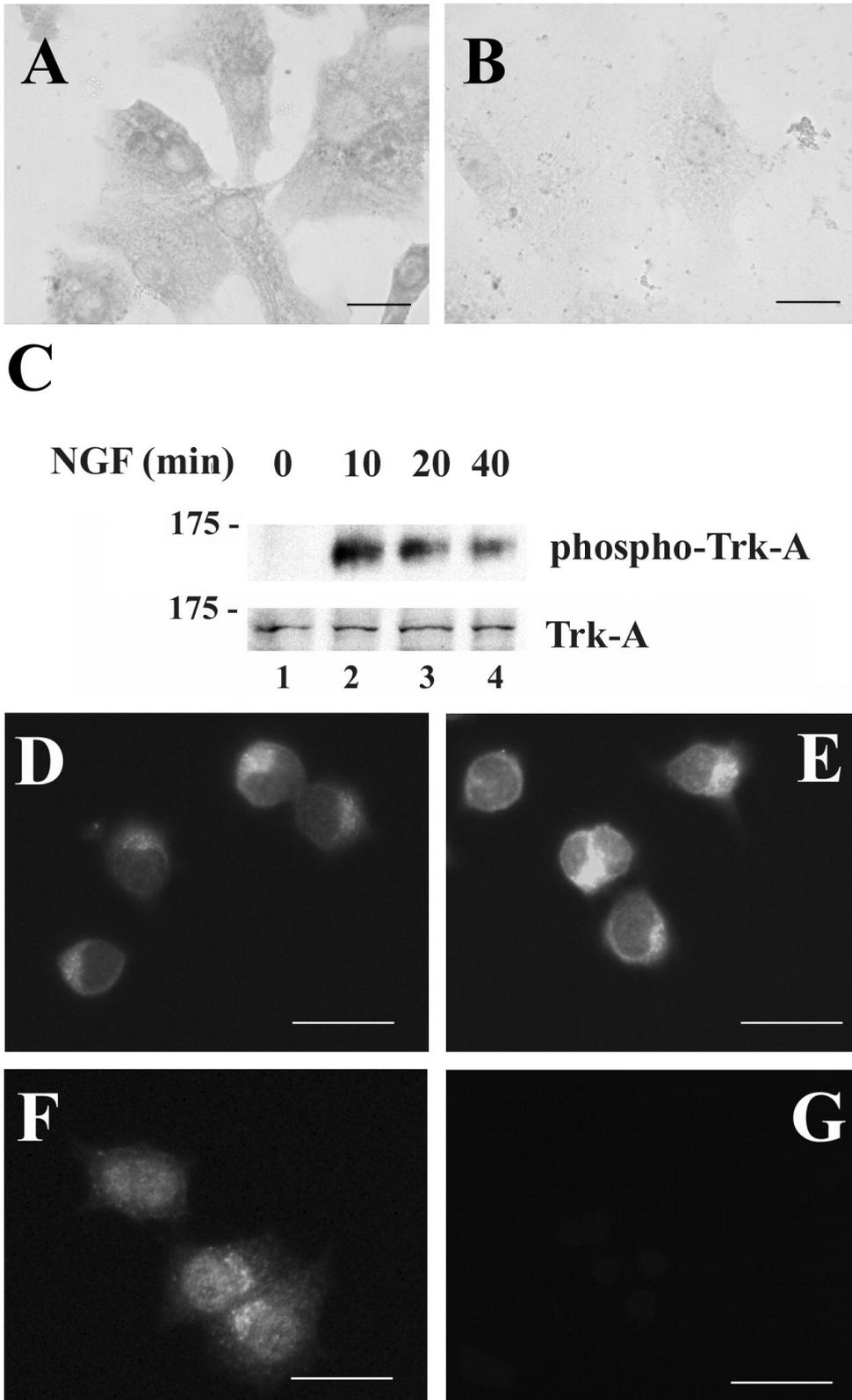
**Fig. 7.** Constitutive nuclear localization of Trk-A in HSC but not in PC12 cells. Confocal microscopy immunofluorescence of cultured human HSC using antibodies directed against the intracellular domain of Trk-A (Santa-Cruz Biotechnology, panel **A**; Cell Signaling Technology, panel **B**, or recognizing the tyrosine phosphorylated forms of the receptor (Y490, panel **C**; Y746, panel **D**). **E.** Negative control for immunofluorescence. **F.** Confocal microscopy immunofluorescence of cultured PC12 cells using antibodies directed against the intracellular domain of Trk-A (Santa-Cruz Biotechnology). Scale bar: 25  $\mu\text{m}$  (A-E); 18  $\mu\text{m}$  (F).



**Fig. 8.** Trk-A colocalizes with the nuclear protein SC-35. Human HSC in complete medium were analyzed by immunofluorescence with antibodies against Trk-A (Santa-Cruz Biotechnology, panel **A**) or with antibodies against the nuclear protein SC-35 (panel **B**). **C.** Overlay of **A** and **B**. Scale bar: 10  $\mu$ m.



**Fig. 9.** Nuclear localization of Trk-A selectively involves the intracellular domain. Normal liver tissue (**A-C**) or cultured human HSC (**D-I**) were analyzed by immunohistochemistry and APAAP (panels **A, B, D, E**) or by confocal microscopy immunofluorescence (panels **G, H**) using antibodies directed against the intracellular domain of Trk-A (Santa-Cruz Biotechnology, panels **A, D, G**) or against the extracellular domain (Upstate Biotechnology, panels **B, E, H**). **C, F.** Negative controls for immunohistochemistry. **I.** Negative control for immunofluorescence. Scale bar: 25  $\mu$ m.



To provide additional evidence for nuclear localization of Trk-A in cultured HSC, we compared immunofluorescence signal for Trk-A with that of the splicing factor SC-35, which is constitutively localized in the nucleus (Spector et al., 1991). Overlay of the two images demonstrated a clear co-localization between the two signals in spotty areas within the nucleus (Fig. 8). These data provide additional, and compelling evidence that in HSC, Trk-A localizes at the nuclear level.

#### *Nuclear localization of Trk-A involves the intracellular domain*

Nuclear translocation of surface receptors has been recently described for members of the Erb family of tyrosine kinases. For some of these receptors, translocation involved only a part of the molecule, and selectively the intracellular domain (Ni et al., 2001). We compared the nuclear staining generated by antibodies directed against the intracellular or extracellular domains of Trk-A in liver tissue and in cultured HSC. While antibodies recognizing the intracellular domain of the receptor clearly decorated the nucleus both *in vivo* and *in vitro* (Fig. 9A,D,G), an antibody directed against the extracellular portion of Trk-A was completely unable to demonstrate a nuclear signal (Fig. 9B,E,H). This observation is further supported by the fact that all 4 antibodies used in confocal microscopy experiments depicted in Figure 7 were directed against epitopes localized in the intracellular domain.

#### *Different modalities of nuclear Trk-A expression in different cell types*

*In vivo* experiments demonstrate that at least a portion of hepatocytes show nuclear localization of Trk-A (see Figs. 1, 2). To evaluate if this aspect was present also in cultured hepatocytes, we immunostained human hepatocytes with anti-Trk-A antibodies directed against the intracellular domain. In this situation, no evidence of nuclear staining was observed (Fig. 10A), indicating that once isolated from the tissue, this cell type no longer maintains nuclear Trk-A localization. To obtain additional information on the intracellular dynamics of Trk-A, we exposed PC-12 to different soluble mediators. As expected, incubation with NGF resulted in a marked increase in tyrosine phosphorylation of Trk-A (Fig. 10B). This effect was associated with a modest increase in nuclear staining using Trk-A antibodies, 1 hour after addition of NGF (Fig. 10D-E). However, when PC12 were exposed to a mixture of soluble mediators, consisting of recombinant NGF, fetal bovine serum, and HSC-conditioned medium, a dramatic increase in nuclear staining of Trk-A was observed (Fig. 10E-F). These data show that nuclear localization of Trk-A may be induced in PC12 by stimulation with soluble factors.

#### **Discussion**

In this study we have characterized the expression of

the neurotrophin receptor, Trk-A in different types of liver cells *in vitro* and *in vivo*, both in normal liver and in conditions of injury. The results of this study demonstrate for the first time the presence of nuclear localization of Trk-A in different types of cells *in vivo*, in both normal liver and during cirrhosis, as a model for chronic damage and repair. This observation suggests a possible physiologic role for nuclear Trk-A localization. Although *in vivo* this phenomenon was evident in both hepatocytes and activated HSC, these cell types showed opposite behaviors once isolated from the tissue. In fact, while nuclear Trk-A localization was no longer evident in isolated and cultured hepatocytes, nuclear staining was consistently present in HSC, even after prolonged maintenance in culture. We have confirmed that human HSC secrete NGF, in agreement with the data of Cassiman et al. (2001), who showed NGF expression by HSC also *in vivo*. In contrast, the role of hepatocytes as a source of NGF remains controversial. While Cassiman et al. showed that hepatocytes are not capable of expressing NGF *in vivo*, and that they did not express NGF mRNA transcripts once isolated from the tissue (Cassiman et al., 2001), Oakley et al. have recently reported that after acute toxic injury in the mouse, hepatocytes represent the major site of NGF expression (Oakley et al., 2003). In PC-12, the appearance of a nuclear staining for Trk-A is evident when the cells are exposed to NGF, as indicated in this study and in the one by Moughal et al. (2004). Based on these data, one may speculate that an autocrine loop involving NGF may be responsible for the constitutive localization of Trk-A in the nucleus of cultured HSC. These data could also possibly explain the fact that hepatocytes, which in intact tissue are located in close proximity to HSC, present nuclear Trk-A localization only *in vivo*. Additional studies are required to validate the hypothesis that an autocrine/paracrine action of soluble mediators expressed by HSC is critical for nuclear localization, and to verify whether soluble mediators are capable to induce nuclear localization of Trk-A in hepatocytes. Expression of Trk-A in the liver was not restricted to hepatocytes and HSC, but was detectable in smooth muscle cells of branches of the hepatic artery, in infiltrating lymphocytes and in cholangiocytes. However, in all these cell types no evidence of nuclear localization was observed, indicating that this phenomenon may not be ascribed to a non-specific binding of the antibodies used for immunodetection. On the other hand, p75, the low-affinity receptor for NGF, was not localized in the nucleus despite its expression in the same types of liver cells in which Trk-A was localized at the nuclear level.

The present study also provides evidence on the functional role of the neurotrophin system in the process of liver fibrosis in humans. Data obtained in animal models of fibrosis and in cultured rodent cells indicate that NGF induces apoptosis of HSC via activation of the p75<sup>NGF-R</sup> (Trim et al., 2000; Oakley et al., 2003). However, NGF was unable to determine apoptosis of human HSC, as demonstrated using concentrations of

NGF as high as 500 ng/ml and four different methods for the detection of programmed cell death (Novo et al., 2006). In that same study, we also demonstrated that human HSC are particularly resistant to apoptotic stimuli, mainly because of up-regulated expression of Bcl-2. Conversely, the present study provides evidence that NGF stimulates chemotaxis of human HSC, a biological effect linked to the development of fibrosis. The specific involvement of Trk-A in mediating this action was confirmed by the ability of K-252, an inhibitor of Trk's tyrosine kinase activity, to block NGF-induced migration. Remarkably, NGF was also capable of inducing activation of intracellular signaling pathways, such as ERK and Akt, which are closely connected to chemotaxis in different cell types, including HSC (Marra et al., 1997, 1999). These data underscore the possibility that the pathophysiology of NGF in liver fibrosis may be different in rodents and in humans, and that a reason for the discrepant findings may be related to the coexisting expression of p75<sup>NGF-R</sup> and of Trk-A in human HSC. This hypothesis is consistent with the data reported by Oakley et al., showing that in normal mouse liver and after induction of acute toxic damage, Trk-A mRNA is not detectable even using highly sensitive techniques (Oakley et al., 2003). Thus, while in rodents NGF appears to be an antifibrogenic mediator via induction of apoptosis (Trim et al., 2000; Oakley et al., 2003), the presence of a NGF/Trk-A axis in human HSC suggests that NGF may even contribute to the recruitment of HSC to areas of damage via chemotaxis. Further studies are needed to establish whether the balance of the two NGF receptors in human HSC may be a factor contributing to individual differences in the progression of fibrosis.

An additional relevant observation of the present study is the fact that while antibodies recognizing the intracellular portion of the Trk-A receptor gave in all cases a clear and consistent nuclear staining, no nuclear signals were detected using antibodies against the extracellular domain. These results suggest that the intracellular portion of the receptor is the major determinant of nuclear Trk-A staining. Nuclear localization of receptors with tyrosine kinase activity has been studied in detail for the Erb family. Studies conducted in the past few years have indicated that Erb receptors may be detected in the nucleus following short-term incubation with the cognate ligands (Wells and Marti, 2002). Remarkably, Erb-1 binds to specific sequences in the nuclear DNA, suggesting that nuclear translocation of growth factor receptors may also directly modulate transcription of target genes (Lin et al. 2001). In parallel, other groups have focused on Erb-4, which is activated by heregulin. While both Erb-1 and Erb-3 are detected in the nucleus as full-length molecules, only a portion of Erb-4, and specifically the intracellular domain, translocates to the nucleus of cells stimulated with heregulin or PMA (Ni et al., 2001). Thus, Trk-A represents the second example of a receptor that is detected in the nucleus only with its intracellular portion, although the exact mechanism underlying the

appearance of this domain in the nucleus remains to be established. It is interesting to note that nuclear staining was evident using antibodies directed against the phosphorylated form of Trk-A, indicating that the receptor present in the nucleus has undergone phosphorylation. This observation suggests the possibility that an enzymatically active portion of the receptor could phosphorylate tyrosine residues on nuclear targets. In addition, Trk-A colocalized with nuclear SC-35 domains (Stein et al., 2000), which contain RNA-splicing factors that generate mature mRNA transcripts. Besides confirming the nuclear localization of Trk-A, this observation could be in keeping with a role of this molecule in the regulation of gene transcription and/or transcript processing.

In conclusion, this study indicates for the first time a constitutive nuclear localization of Trk-A in liver cells such as HSC, while appearance in the nucleus may be induced by soluble factors in other cell types. These findings expand the possible role of this growth factor receptor in the biology of tissue injury and repair in humans.

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