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Neurite Outgrowth Can Be Modulated In Vitro Using a Tetracycline-Repressible Gene Therapy Vector Expressing Human Nerve Growth Factor

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The delivery of neurotrophic factors to the adult nervous system has potential applications for the treatment of neurodegenerative diseases and trauma. In vivo and ex vivo gene therapy offer a means of delivering growth factors and other therapeutic substances to the central nervous system (CNS) in an intraparenchymal, accurately targeted, and regionally restricted manner. Ideally, gene therapy delivery systems should also be regulatable, allowing exogenous control of amount of gene product delivery. In the present experiment, a tetracycline-regulatable gene expression system was generated to determine whether controllable release of nerve growth factor (NGF) and green fluorescent protein (GFP) from primary rat fibroblasts could modulate biological responses (neurite outgrowth) in vitro. Using a tetracycline-repressible construct, it was found that NGF mRNA, NGF protein, and NGF-induced neurite outgrowth could be tightly regulated within a 24 hour period, and in a dose-dependent fashion, by exposure to the tetracycline analog doxycycline. Similarly, levels of green fluorescence could be regulated in GFP-transfected cells. These findings in a neurobiological system lay the framework for future studies using regulated neurotrophin delivery in in vivo models of neurodegenerative diseases and CNS injury. J. Neurosci. Res. 59:402–409, 2000. © 2000 Wiley-Liss, Inc.

Key words: NGF; gene therapy; tetracycline; retrovirus; regulatable expression; spinal cord injury; regeneration

Delivery of trophic molecules in CNS trauma and neurodegenerative diseases is a promising treatment strategy to support neuronal survival and axonal outgrowth in the injured or degenerating CNS (Gage et al., 1987, 1990; Eide et al., 1993; Suhr and Gage, 1993; Fisher and Ray, 1994; Lindsay et al., 1994; Tuszynski and Gage, 1994; Blesch and Tuszynski, 1995; Tuszynski and Gage, 1996; Raymon et al., 1997; Blesch et al., 1998b; Tuszynski et al., 1999). Growth factor delivery, however, must be restricted to specific regions of the brain for bioactive molecules to cross the blood-brain barrier, reach their targets, and prevent undesirable adverse effects that result from non-targeted delivery methods (Winkler et al., 1997). Gene therapy can hypothetically accomplish these goals by delivering molecules intraparenchymally, in a regionally-restricted and well-targeted manner (Gage et al., 1987; Tuszynski et al., 1994b; Blesch and Tuszynski, 1995; Blesch et al., 1998b).

A limitation of conventional gene therapy is the inability to regulate the expression of the transferred gene over time; however, regulating gene expression is of great importance for a number of reasons: 1) with regulatable vectors, expression can be adjusted to obtain maximum biological efficacy while minimizing the risk of adverse effects; 2) sequential activation of gene expression can result in extended temporal and spatial patterns of growth factor delivery, thereby enhancing target sensitivity; and 3) in a clinical setting, regulatable gene expression would allow the discontinuation of treatment if adverse effects occurred. A number of systems have been investigated as potential tools for regulating expression of transferred genes in vitro and in vivo. These include vectors containing steroid- (Delort and Capecchi, 1996; No et al., 1996; Suhr et al., 1998), rapamycin- (Rivera et al., 1996; Magari et al., 1997; Rossi and Blau, 1998; Ye et al., 1999) and tetracycline- (Gossen and Bujard, 1992; Gossen et al., 1993, 1995) regulatable expression cassettes.

We chose to investigate the potential of the tetracycline regulatable system because it has a number of advantages compared to other regulatable expression constructs: 1) it is relatively simple, with only one protein needed for inducing or repressing gene expression; 2) due to the small size of the tet-transactivator and the regulatable promoter, it can be packaged into a single vector; and 3) tetracycline

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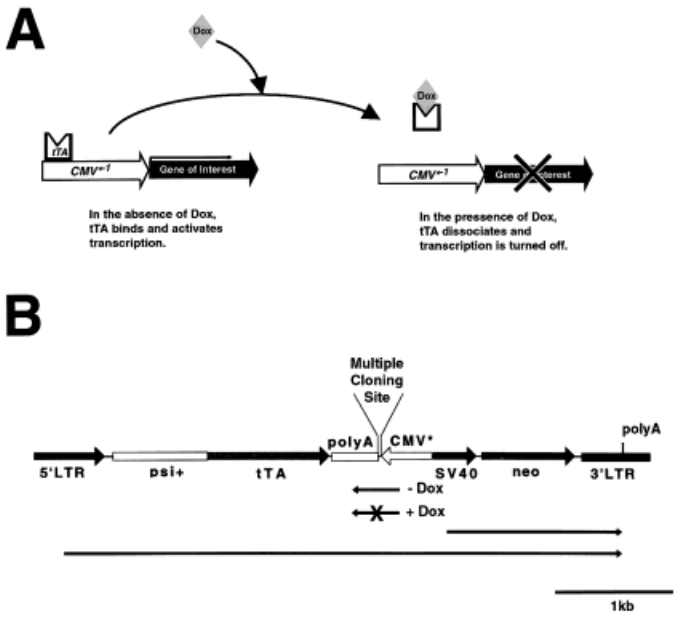

Fig. 1. (**A**) Schematic outline of tetracycline regulatable gene expression (Tet-Off system): The tetracycline transactivator (tTA) binds to the regulatable promoter (CMV$^{\star-1}$) only in the absence of doxycycline and activates transcription. Addition of tetracycline or doxycycline (DOX) results in the dissociation of tTA from the promoter and subsequent loss of transcription. (**B**) Plasmid map of the tetracycline regulatable retroviral expression vector pLN-Tet-Off. Expression of the tetracycline transactivator is driven by the 5′ long terminal repeat (5′LTR), and expression of the neomycin resistance gene (neo) is driven by an internal SV40 promoter. Note that the tet-responsive promoter CMV$^{\star-1}$. is oriented in the opposite orientation to the 5′LTR. A multiple cloning site is added for convenient cloning. Two polyadenylation signals (polyA) are present, one located in the 3′LTR, and the second one behind the multiple cloning site for the regulatable transcript.

and its analogs have very good pharmacokinetics in the CNS, and they can penetrate the blood-brain barrier. Tetracycline has no significant common side effects, and is inexpensive. The originally characterized tetracycline regulatable system uses a tet-repressor from *Escherichia coli* fused to the VP16 transcription activation domain (Fig. 1). This tetracycline transactivator (tTA) stimulates transcription from a minimal CMV promoter fused to the tet operator sequence, but *only* when tetracycline is absent from the system (''tet-off'' system; Fig. 1A) (Gossen and Bujard, 1992). When tetracycline is present, it binds to the tetracycline transactivator, that prevents binding to the promoter. Thus, gene expression is turned off. Expression of a reporter gene can be increased, reaching a maximal 1000-fold activation in some in vitro experiments (Gossen and Bujard, 1992).

In the present study we examined whether gene expression could be regulated in primary rat fibroblasts using a single retrovirus containing all necessary regulatory sequences of the tetracycline transactivator system. We demonstrate that reporter gene expression and nerve growth factor (NGF) expression can be regulated in a dose dependent fashion in primary cells suited for grafting to the CNS. This regulated expression of NGF allows for readily controlled modulation of axonal growth.

MATERIALS AND METHODS

Construction of Retroviral Vectors

The basis for the vector construction was the retroviral plasmid pLXSN (Miller et al., 1986, 1993; Miller, 1990), the plasmid pUHD 10-3 (containing the tet responsive minimal CMV promoter), and the plasmid pUHD15-1 containing the tet-transactivator (tTA) (Gossen and Bujard, 1992; Gossen et al., 1993).

The multiple cloning site (MCS) in plasmid pUHD10-3 containing the tet responsive minimal CMV promoter was replaced with a new polylinker. The plasmid pUHD10-3 was digested with SacII and BamHI, gel purified, and the following linker containing SacI, NotI, ClaI, Eco72I and BglII sites was cloned into these sites resulting in a modified pUHD10-3 (pUHD-10-3mod): 5′ GGCCGC ATCGAT CACGTG AGATCTG 3′; 3′ CGCCGGCG TAGCTA GTGCAC TCTAGACCTAG 5′. The plasmid pUHD10-3mod was then digested with XhoI and NaeI, and the fragment containing the CMV$^{\star-1}$ promoter, MCS and polyadenylation sequence was cloned into the HpaI/XhoI digested retroviral vector pLXSN. The CMV$^{\star-1}$ promoter in the resulting plasmid pLAXCSN was oriented in the direction opposite to the 5′ long terminal repeat (5′ LTR) promoter/enhancer. The tTA coding sequence was amplified by PCR using the following primers resulting in *Eco*RI sites on both ends: 5′ TGTGAATTCATATGTCTAGATTAGAT 3′; 5′ TGTGAATTCCTACCCACCGTACTCGTCAATTTC 3′. The PCR products were then cloned into the *Eco*RI digested pLAXCSN. The resulting plasmid pLtTAXCSN = pLN-tet-off (Fig.1B) was used as a basis for inserting genes coding for nerve growth factor (NGF) and green fluorescent protein (GFP).

For regulatable expression of nerve growth factor (NGF) a HpaI/BamHI fragment containing the cDNA for β-NGF including a Kozak consensus sequence was cloned into the Eco72I/BglII digested pLN-tet-off. For regulatable expression of the control gene green fluorescent protein (GFP), a HindIII/NotI fragment from phGFP-S65-T (Clontech) was blunt ended and cloned into the Eco72I digested pLN-tet-off.

Retroviruses were produced by transient transfection of pLN-tet-off-GFP and pLN-tet-off-NGF plasmids into the Phoenix-ampho retrovirus producer cell lines (Pear et al., 1997). Cultures of primary rat fibroblasts (see below) were infected with supernatants from the Phoenix producer cells. Transfected cells were selected by adding G418 (400 μg/ml) to the cell culture medium.

Cell Culture

Primary cultures of Fischer 344 rat fibroblasts were generated from skin biopsies and cultivated under standard culture conditions as previously described. (Tuszynski et al., 1994a; Grill et al., 1997a).

In Vitro Characterization of NGF and GFP Expressing Fibroblasts

Two genes were separately tested for regulatable expression in the pLN-tet-off vector: green fluorescent protein gene, a non-secreted control gene (pLN-tet-off-GFP vector), and nerve growth factor, a secreted neurotrophic factor (pLN-tet-off-NGF vector). The transcripts from the regulatable CMV$\star^{-1}$ promoter were oriented in the opposite direction to the main reading frame of the 5′LTR to 1) avoid possible activation by the enhancer in the 5′LTR, and 2) to further suppress possible weak expression from the CMV$\star^{-1}$ in the presence of tetracycline or doxycycline (a tetracycline analog), by forming RNA duplexes with the constitutively expressed transcript from the 5′LTR.

Dose Response Curve

Tet-off NGF transfected fibroblasts were seeded into 24 well plates (2.5×10^4/well) in 1 ml cell culture medium and pretreated for 24 hr with doxycycline with different concentrations (0, 0.01, 0.05, 0.1, 1, 5, 10, 100, 500, 1000 ng/ml; 4 wells for each concentration). The medium was removed, fresh medium was added containing the same amount of doxycycline as in the pre-incubation, and 24 hr later medium was harvested and analyzed for NGF by ELISA.

Time Course

Tet-off-NGF transfected fibroblasts were seeded into 6 well plates (1.25×10^5 cells/well), 8 wells received no doxycycline and 4 wells were treated with 1 μg/ml doxycycline. One week later, when cells had reached confluence, medium was replaced 12 hr before the start of the assay with 2 ml cell culture medium with or without doxycycline as in the previous incubation. At Time 0, the first 12 hr supernatant was collected, cells were washed three times, and cells received new medium. One group continued to receive no doxycycline, one group continued to be cultivated with doxycycline (1 μg/ml), and one group was changed from medium without doxycycline to medium with doxycycline. At time points of 12, 24, and 36 hr medium was changed and collected for ELISA analysis of NGF levels. Medium was changed again at 48 hr and the last supernatants were collected at 72 hr.

Northern Blot

NGF gene expression in transduced primary fibroblasts was assessed by Northern blotting. Twenty μg total RNA were separated on a 1% formaldehyde-agarose gel, transferred to a nylon membrane, and hybridized to a ^{32}P-labeled human NGF cDNA fragment. After 2 washes in 1 × SSC (150 mM NaCl; 15 mM sodium citrate) with 0.1% SDS for 1 hr each at 65°C, blots were exposed to X-ray film.

Bioactivity of Secreted NGF

Activity was measured using a PC-12 neurite outgrowth assay, as previously described (Rukenstein and Greene, 1983). Briefly, 24 hr conditioned media (10 ml) were obtained from confluent flasks of doxycycline-treated or untreated tet-off-NGF fibroblasts. Similarly, conditioned medium was collected from doxycycline-treated or untreated GFP-transfected fibroblasts. PC-12 cells primed with NGF were seeded onto collagen coated 6-well plates in 0.5 ml medium, and 2.5 ml volumes of conditioned medium from the above groups was added. After 24 hr, the proportion of cell clumps exhibiting neurite outgrowth (Rukenstein and Greene, 1983) was determined at 100× magnification using an inverted microscope equipped with phase contrast. Measurements were done in triplicates.

NGF ELISA

For determination of NGF protein levels a sensitive NGF ELISA was used, as previously described (Conner and Varon, 1996). Briefly, the capture antibody (rabbit polyclonal anti-NGF; gift of Dr. J. Conner, University of California, San Diego) was diluted 1:500 in carbonate coating buffer (pH9.6) and added to the ELISA plates on day 1 (Costar, 96 well EIA/RIA plates; 50 μl/well). For control wells, purified rabbit IgG (Sigma; 1:2000) was used. After overnight incubation, wells were washed, nonspecific binding sites on the plates were blocked (3% BSA in PBS), and purified NGF standards (1–100 pg/well) or unknown samples were added to the wells (50 μl/well). The plates were incubated overnight at 4°C. On Day 3, samples were removed and wells were washed. Next, anti mouse anti-NGF (monoclonal; Boehringer-Mannheim, Germany) diluted 1:100 in homogenization buffer was added (50 μl/well). The plates were again incubated overnight at 4°C, washed, and the peroxidase conjugated anti-mouse IgG (Dako P-260) diluted 1:1000 in anti-mouse peroxidase buffer was added. On Day 5, plates were washed and the color substrate (OPDA) was added to each well (150 μl/well). This was incubated in a dark box for 5–30 min or until solutions turned bright yellow. Reactions were stopped by adding 50 μl of a 10% H_2SO_4 solution. Plates were read on a microplate reader using a 490 nm filter.

Statistics

Two-group comparisons were made using Student's *t*-test. Multiple group comparisons were made by analysis of variance followed by Fisher's post-hoc analysis, using a 95% significance level in all cases.

RESULTS

To obtain tetracycline-regulatable expression in primary cells, a retroviral vector was constructed that contained all the necessary elements for exogenous control of gene expression, including the tetracycline binding transactivator (tTA) driven by the 5′ long terminal repeat (5′LTR), and the regulatable CMV minimal promoter coupled to the tetracycline operon sequences. Additionally, a neomycin resistance gene driven by an internal SV40 promoter was included to select for transfected fibroblasts (Fig. 1B).

Regulated Expression of GFP

After transfection and selection for neomycin resistance with G418, primary rat fibroblasts infected with pLN-tet-off-GFP showed regulatable fluorescence in vitro: cells cultivated without doxycycline showed strong fluorescence (Fig. 2A), whereas addition of doxycycline at 1 μg/ml to the cell culture medium for 7 days reduced the fluorescence to undetectable levels (Fig. 2B). As GFP has

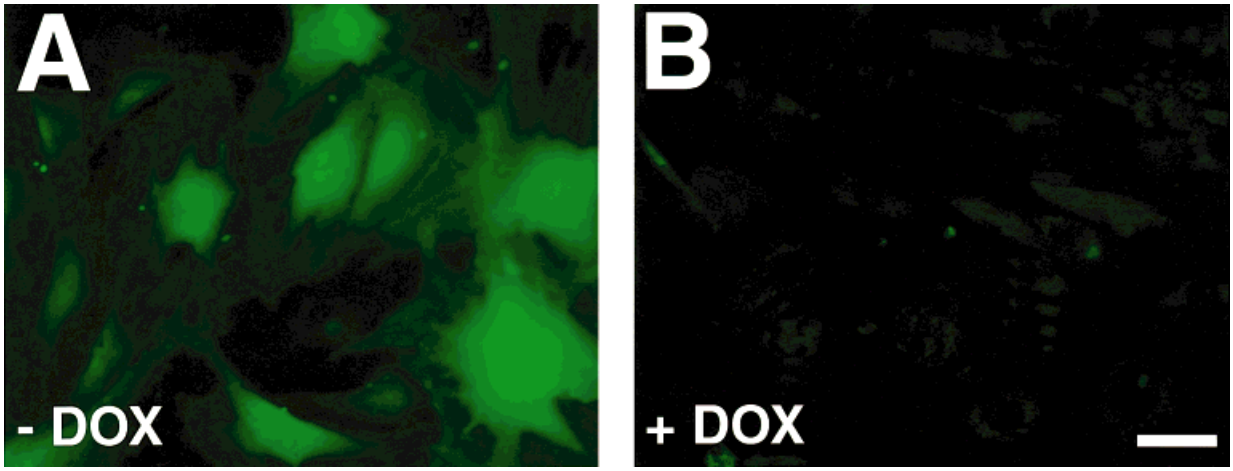

Fig. 2. Expression of GFP is dependent on the dose of doxycycline in the cell culture medium. Fibroblasts transfected with the regulatable GFP expression vector show green fluorescence if cultivated (**A**) without doxycycline in the cell culture medium, whereas (**B**) addition of doxycycline suppresses expression of GFP. Scale bar = 35 μm.

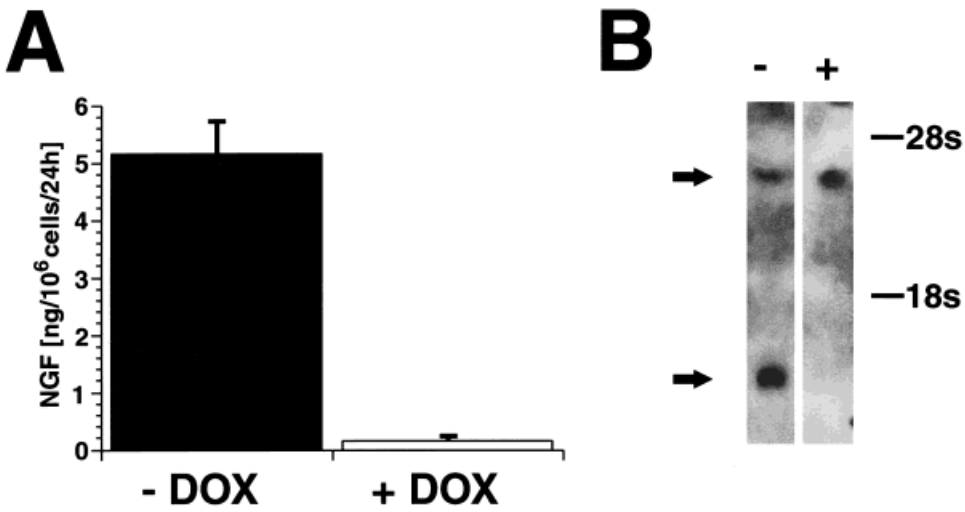

Fig. 3. (**A**) Primary fibroblasts transfected with pLN-tet-off-NGF secrete 5.4 ng NGF/10^6cells/24 hr. NGF production is down-regulated to 0.15 ng/10^6 cells/24 hr after incubation with 1 μg/ml Dox. No NGF is detectable in control GFP transfected fibroblasts (data not shown). (**B**) Northern blot analysis of tet-off-NGF transfected fibroblasts that were either treated with doxycycline (+Dox) or untreated (−Dox), hybridized with a probe for human NGF. The smaller transcript that originates from the regulatable CMV promoter and drives NGF expression is only present in the absence of doxycycline. The large transcript originating from the 5′LTR driving expression of the tetracycline transactivator (tTA) (compare to Fig. 1B) has similar expression levels in untreated and treated cells.

a long intracellular half life and direct measurement of kinetics and dose-dependence of fluorescence are difficult to obtain, more detailed analysis was carried out for regulatable NGF expression.

Regulated Expression of NGF

Primary fibroblasts infected with tet-Off-NGF produced 5.4 ng/10^6 cells/24 hr NGF; however, only 0.15 ng/10^6 cells/24 hr NGF was detected in supernatants from cells pre-treated for 48 hr with 1 μg/ml doxycycline. Thus, NGF expression can be regulated at least 32-fold in primary rat fibroblasts after infection with a single tet-Off retrovirus (Fig. 3A). These results were confirmed by Northern blot analysis (Fig. 3B). The shorter transcript originating from the regulatable CMV promoter was present in cells that were grown in the absence of doxycycline; addition of doxycycline resulted in undetectable levels of gene expression. In contrast, the long transcript originating from the 5′LTR was unchanged by the addition of doxycycline.

The initial characterization of regulatable expression of NGF and GFP was done in the presence or absence of 1 μg/ml doxycycline; however, substantially lower levels of doxycycline could be capable of turning off NGF expression, and ideally various levels of NGF expression should be obtainable depending on the amount of doxycycline present. Analysis of the dose response curve of tet-off-NGF transfected cells exposed to various doses of doxycycline demonstrated that concentrations of doxycycline as low as 1 ng/ml nearly completely shut off the expression and secretion of NGF into the cell culture medium (Fig. 4A). The ID_{50} lies in the range of 20–30 pg/ml doxycycline. Thus, expression of NGF in these cells is dependent on the concentration of doxycycline in the medium and can be accordingly modulated.

The time course of NGF expression was measured in the presence or absence of tetracycline. At Time 0, doxycycline (1 μg/ml) was added to the medium of cells previously grown in antibiotic-free medium. As shown in Figure 4B, NGF secretion was rapidly reduced within 24 hr of exposure to doxycycline, and the medium harvested at the 36 hr time point (containing the protein secreted between 24 and 36 hr) contained no detectable NGF. Parallel cultures that were grown without antibiotic continued to express NGF, whereas cultures grown with constant levels of antibiotic had virtually no detectable NGF expression. The slight increase in NGF expression at the 12 hr time point in cultures grown with doxycycline might be a partial consequence of the washing step at Time 0 causing a brief activation of the regulatable promoter.

The dependence of NGF expression on the presence of the regulator doxycycline was further tested in a neurite outgrowth assay using PC-12 cells. Conditioned media were harvested from primary transfected fibroblast cultures that were cultivated in the presence or absence of doxycycline for 24 hr. PC-12 cells were then exposed to conditioned media from these cells, and showed differential amounts of neurite outgrowth. Neurite growth was significantly higher in PC-12 cell cultures containing conditioned media from fibroblasts cultivated without doxycycline, where NGF secretion was greater. In contrast, PC-12 cells exposed to conditioned media from fibroblasts cultured with doxycycline to suppress NGF gene expression exhibited very little neurite extension (Fig. 5). Similarly, PC-12 cells cultured in supernatants from GFP-expressing fibroblasts treated with or without doxycycline exhibited little neurite extension. Thus, only fibroblasts lacking exposure to doxycycline expressed significant amounts of NGF, and this elicited significant amounts of neurite outgrowth compared to other culture conditions ($P < 0.01$).

DISCUSSION

Regulated expression of therapeutic proteins delivered by gene therapy has numerous potential advantages and applications over constitutive, uncontrolled gene expression. Inserting a molecular "switch" into gene therapy vectors can permit the regulated delivery of therapeutic genes according to the state of a disease, thereby maximizing efficacy while using the smallest amount of bioactive molecule necessary, and providing the possibility of discontinuing treatment by turning off gene expression entirely.

The present results demonstrate that a tetracycline-regulatable system contained in a single retrovirus is functional in primary cells, and is suitable for regulating the expression of therapeutic proteins of the neurotrophin family such as NGF in neurobiological systems. The tetracycline analog doxycycline was used to activate the tetracycline repressor in this experiment, thereby diminishing activity of the minimal CMV promoter ($CMV\star^{-1}$). In cultured fibroblasts containing this regulatable system, NGF expression levels could be rapidly altered within 24 hr. Levels of NGF could be varied over a 2-log range, and concentrations of doxycycline as low as 1 ng/ml were sufficient to achieve a switch in gene expression. This regulation resulted in significant changes in neurite outgrowth from PC-12 cells, indicating that a biological growth response could be altered by regulating NGF release.

The regulatability of NGF gene expression in primary rat fibroblasts in vivo, and duration of gene expression in vivo, remains to be determined. Previous in vivo studies have demonstrated that transcription of reporter

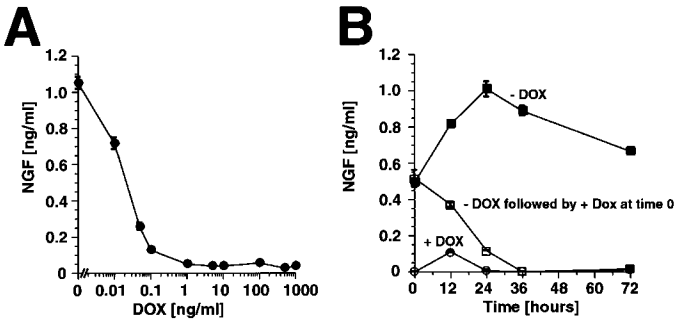

Fig. 4. Dose dependence and kinetics of NGF production. (**A**) With increasing concentrations of doxycycline in the cell culture medium, NGF expression is down-regulated. Concentrations of Dox as low as 1 ng/ml nearly completely shut off gene expression in cultures of primary Fischer 344 rat fibroblasts transfected with Tet-Off-NGF. (**B**) Kinetics of NGF expression. Tet-Off-NGF transfected cells were cultivated for 7 days without doxycycline (**closed** and **open squares**) or with dox (**open circles**). Supernatants were collected beginning at Time 0, and one group (open squares) was changed from doxycycline-free to doxycycline-containing medium. NGF levels were measured by ELISA. Cells that were cultivated in doxycycline-free medium (closed squares) showed continued NGF expression. Cells that were constantly treated with 1 μg/ml doxycycline (open circles) showed very little NGF expression. Cells that were first in doxycycline-free medium and were then changed to doxycycline-containing culture medium at Time 0 (open squares) completely shut down gene expression after 24 hr. This was evidenced by the 36 hr supernatant, that was collected between the 24 hr and 36 hr medium change and contained no detectable NGF. Error bars in (A) and (B) are not always visible due to their small size.

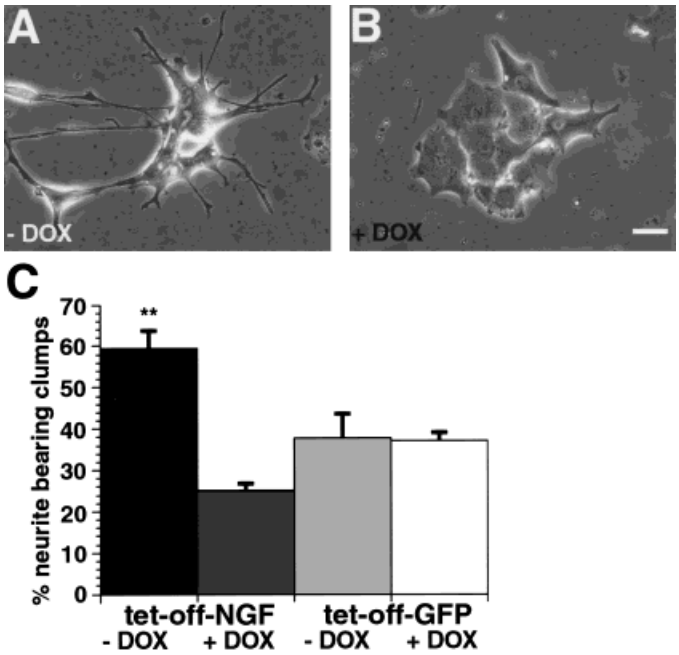

Fig. 5. Biological activity of regulated NGF expression. PC-12 neurite outgrowth is significantly higher in cultures incubated with conditioned medium from (**A**) cultures lacking doxycycline, that therefore produce high amounts of NGF expression, compared to (**B**) cultures treated with doxycycline, that turns off NGF expression in tet-off-NGF-transfected fibroblasts. (**C**) Quantification of the proportion of neurite-bearing PC-12 cell clumps incubated with conditioned medium from untreated- or doxycycline-treated tet-off-NGF and -GFP transfected primary fibroblasts. Supernatants from tet-off-NGF-transfected cells cultured without doxycycline induce significantly more PC-12 neurite outgrowth than supernatants from tet-off-NGF transfected cells cultured in the presence of doxycycline ($P < 0.01$). No significant neurite outgrowth occurs in PC-12 cells grown in the presence of conditioned medium from tet-off-GFP-transfected cells treated with or without doxycycline. Values represent mean ± SEM. Scale bar = 44 μm.

genes in transgenic animals (Furth et al., 1994; Shockett et al., 1995; Kistner et al., 1996) and expression of tyrosine hydroxylase (Corti et al., 1996) or erythropoietin (Bohl et al., 1997) in grafted cells can be regulated by adding tetracycline to drinking water at concentrations that show no evidence of toxicity in animals. One potential concern has been that expression from the minimal CMV promoter used in previous studies might be regulatable for only short time periods. In the present study, expression of the tet-transactivator was driven by the 5′ Long Terminal Repeat (5′LTR) promoter, a promoter that has exhibited potent and long-term activity in fibroblasts after in vivo grafting to the CNS (Tuszynski et al., 1996; Grill et al., 1997a). Thus, gene expression could be regulatable as long as the 5′LTR is active; this possibility is the subject of ongoing studies.

The regulated delivery of neurotrophic factors has several potential applications in CNS trauma and neurodegenerative diseases. For example, neurotrophic factor delivery using genetically modified fibroblasts elicits extensive axonal growth in models of spinal cord injury. Cells producing nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), leukemia inhibitory factor (LIF) and glia cell line-derived neurotrophic factor (GDNF) promote specific regenerative responses after grafting to rats with spinal cord injury (SCI) (Tuszynski et al., 1994a, 1996, 1997; Nakahara et al., 1996; Grill et al., 1997a, 1997b; Blesch et al., 1998a, 1999; Liu et al., 1999; Weidner et al., 1999). Axonal growth in these models, however, is typically restricted to the graft itself or for relatively short distances surrounding the graft. Clearly, achieving longer-distance axonal growth after injury is a highly desirable feature of gene therapy-based regenerative approaches, particularly when considering the greater growth distances that would be required in the injured human spinal cord. Regulatable expression vectors would allow neurotrophin expression to be turned on or off at controllable time periods, thereby potentially providing a means by which regenerating axons could extend *beyond* a lesion site. For example, NGF-secreting grafts placed in a spinal cord lesion site are extensively penetrated by regenerating axons, but the axons rarely exit the graft. By turning *off* NGF expression when axons have penetrated a graft, axons might then leave the graft to reinnervate host structures. Chemotropic attraction of these axons to more distal locations might also be achieved by placing additional grafts in the spinal cord "downstream" from an injury site, particularly if gene expression is turned *on* in a distal graft after expression has been turned *off* in a graft at the lesion site. This approach of regulating gene expression after spinal cord injury could thereby establish expanded chemotropic gradients of neurotrophic factors in the injured spinal cord to elicit longer distance axonal growth.

In neurodegenerative disorders such as Alzheimer's disease, long term delivery of trophic molecules such as NGF is likely to be necessary, rather than the briefer periods of support that could be employed after CNS trauma. In these chronic gene delivery applications, regulatable gene delivery systems are also preferred. Regulatable systems would permit titration of neurotrophic factor "dose," or discontinuation of growth factor delivery in the event that an adverse event attributable to a growth factor occurred. A regulatable gene therapy system in which gene expression is *suppressed* by addition of an orally administered drug would be useful in diseases where expression of a transgene is necessary for extended time periods. Conversely, the optimal design of gene delivery systems for short term or intermittent transgene expression would utilize *inducible* expression systems. A reversed tetracycline-inducible system has been developed to induce gene expression (Gossen et al., 1995), and this system is currently being adapted for ex vivo gene transfer.

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