Tetracycline-regulated gene expression in the NSC-34-tTA cell line for investigation of motor neuron diseases

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Abstract

The motor neuron-like cell line NSC-34 has become a widely used in vitro model for motor neuron biology and pathology. We established a tetracycline-regulated gene expression system in this cell line by stably transfecting pTet-Off, which codifies for the tetracycline transactivator, the regulatory protein tTA. The monoclonal cell lines (NSC-34-tTA) were evaluated for the presence of functional tTA after transient transfection with pBI-EGFP, analyzing the expression of the reporter gene enhanced green fluorescent protein. We evaluated the regulation of tTA function with doxycycline using fluorescence microscopy and quantitative cytofluorimetric analysis on viable transfected cells. The best-regulated cell line (NSC-34-tTA40) had a 66.4-fold induction for the reporter gene fluorescence in comparison to NSC-34. Alpha-tubulin, GAP-43 and phosphorylated medium and heavy neurofilaments, proteins of importance for the motor neuronal phenotype, were evident in NSC-34-tTA40 by Western blot and immunocytochemistry; they were expressed similarly in NSC-34-tTA40 and in NSC-34.

The cDNA of human Cu/Zn superoxide dismutase, a gene of interest for amyotrophic lateral sclerosis, was cloned into pBI-EGFP, downstream of the tetracycline-responsive bidirectional promoter. This plasmid was transiently transfected into NSC-34-tTA40, and the functionality of bidirectional transcription was verified by determining the expression of enhanced green fluorescent protein and of human Cu/Zn superoxide dismutase. Both proteins were regulated by doxycycline.

This novel cell line, NSC-34 tTA40, that permits tetracycline-regulated gene expression may prove useful to unravel the mechanisms of motor neuron degeneration.

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\textit{Theme}: Disorders of the nervous system
\textit{Topic}: Genetic models

\textbf{Keywords}: NSC-34; Tet-Off; Green fluorescent protein; Alpha-tubulin; GAP-43; Neurofilament; Neurodegeneration

1. Introduction

Major motor neuron diseases such as familial spastic paraparesis, amyotrophic lateral sclerosis (ALS), spinal muscular atrophy and spinal-bulbar muscular atrophy are still high-priority disorders because of their relentless progressive course and the invariably fatal prognosis.

Primary cultures of embryonic or newborn tissue or immortalized cell lines of different origin are widely used as cellular models in the study of pathogenic mechanisms.

A few cell lines have been generated that express motor neuron properties [10,26,40]. Among these, the cell line NSC-34 has become the best-characterized and the most frequently used to study motor neuron biology [5,32,45] and pathology [11,16,19,31,35,44]. This line derives from the...
fuson of the murine aminopterin-sensitive neuroblastoma N18TG2 with mouse motor neuron-enriched embryonic day 12–14 spinal cord cells and expresses many motor neuron features without the need for addition of inducing agents to the culture medium [14].

NSC-34 cells are suitable for transfection/infection protocols with human wild type/mutant forms of genes of interest for neurodegenerative disorders [1,2,22,28]. However, in these models, the gene expression level cannot be predicted or influenced. Another limitation is that in stable transfections the toxicity of the corresponding mutant proteins often impairs cell viability during clonal selection, so the surviving clones do not express high levels of transgenic proteins [15].

The major aim of this study was to provide a novel approach to unravel the mechanisms involved in degeneration of motor neurons by applying the tetracycline (Tc)-regulated gene expression system (Tet-Off system) [18] to the NSC-34 cell line in order to modulate the function/toxicity of the genes involved. NSC-34 was stably transfected with pTet-Off that codifies for the Tc-controlled transactivator protein (tTA), a Tc-repressor/virion VP16 fusion protein, with regulatory activity [43]. In the absence of Tc or its analogues, tTA activates transcription of genes placed under the control of a promoter containing Tc-responsive elements. tTA-activated gene transcription is blocked by addition of Tc and its analogues [4].

The monoclonal NSC-34-tTA cell lines were evaluated for the presence of functional tTA after transient transfection with a reporter gene (enhanced green fluorescent protein, EGFP). The line with the best regulation of expression with doxycycline (dox), a Tc analogue, was selected and characterized by Western blot and immunocytochemistry for the presence of alpha-tubulin, GAP-43 and phosphorylated heavy neurofilaments, all proteins of importance for the motor neuron phenotype. In this line (NSC-34-tTA40), we also verified the functionality of bidirectional transcription [3] by determining the expression of EGFP and of human Cu/Zn superoxide dismutase (hSOD1), a gene of interest for the fatal motor neuron disease ALS, cloned into pBI-EGFP downstream of the Tc-responsive bidirectional promoter Pbi-1.

2. Materials

Flasks and plates were obtained from Corning (Corning Incorporated, Corning, NY, USA). Opti-MEM Reduced Serum Medium, LipofectAMINE 2000, genetecin (G418 sulfate) and Alexa Fluor 546 goat anti-rabbit IgG were from Invitrogen (Life Technologies, Paisley, UK). Defined FBS was purchased from Hyclone (Hyclone, Logan, Utah). FluorSave reagent and the sheep polyclonal anti-SOD1 antibody were from Calbiochem (Calbiochem, EMD Biosciences, Inc. La Jolla, CA, USA). The rabbit anti-SOD1 antibody was from Upstate (Upstate, Lake Placid, NY, USA). Dox, donkey anti-sheep peroxidase-conjugated IgG, mouse anti-alpha-tubulin and anti-GAP-43 (clone GAP-7B10) antibodies were purchased from Sigma (Sigma-Aldrich Co., St. Louis, MO, USA). Mouse anti-actin antibody was from Chemicon International (Chemicon International Inc., Temecula, CA, USA). Mouse anti-EGFP was from Zymed Laboratories (Zymed Laboratories Inc., South San Francisco, CA, USA), and goat anti-mouse peroxidase-conjugated IgG SAB-100 was from Stressgen (Stressgen Bioreagents, Victoria, BC, Canada). pTet-Off and pBI-EGFP were purchased from Clontech (Clontech, Palo Alto, CA, USA). ECL detection system was from Amersham (Amersham Biosciences, Buckinghamshire, UK). SMI312 antibody was purchased from Sternberger Monoclonals (Sternberger Monoclonals Inc., Maryland, USA). Mouse AlexaTRITC-labeled secondary antibody was from Molecular Probes (Molecular Probes Inc., Eugene, OR, USA).

3. Methods

3.1. Plasmid construct

The cDNA of hSOD1 was obtained from total RNA of transgenic mice expressing wild type hSOD1 by RT-PCR [42] using the forward primer 5'-CCATGCAACAGCTGTCAGTCTTCGGAACCCAGGA-3' and the reverse primer 5'-AAGGAAAGAAGCTAGCAGGATAACAGAGTGAATAAGGAG-3'. These primers introduce a PvuII and a NheI restriction site (underlined).

Full length cDNA was directionally cloned into the PvuI and NheI sites of the mammalian expression vector pBII-EGFP, and the sequence of the resulting clones was confirmed by DNA sequencing (BMR, CRIBI, University of Padova).

3.2. Tissue culture conditions and transfection protocols

The cell line NSC-34 was routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% heat-inactivated FBS, 1 mM glutamine, 1 mM pyruvate and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin).

Stable transfection: NSC-34 cells were transfected with pTet-Off using LipofectAMINE 2000 reagent. Two days before transfection, 4 × 10⁵ cells/well were seeded in six-well plates and allowed to reach 90% confluence in DMEM, supplemented with 10% heat-inactivated defined FBS, glutamine and pyruvate. Following the manufacturer’s protocol, 6 or 8 μg of plasmid/well was added to a DNA (μg):LipofectAMINE 2000 (μl) ratio of 1:1.6 or 1:1.25 respectively.

To generate stable clones, cells were differentially diluted after 48 h, and G418 sulfate (0.5 mg/ml) was added 24 h later. After 4 weeks, individual G418 sulfate resistant clones were isolated.
Transient transfection: each NSC-34-tTA cell line was seeded at a concentration of 2.5–3.5 × 10^5 cells/well in six-well plates. Cells were transfected with 6 μg of pBI-EGFP following the manufacturer’s protocol and allowed to grow for 48 h then used to evaluate EGFP fluorescence with and without dox.

Transient transfection of NSC-34-tTA40 with pBI-EGFP containing hSOD1 cDNA was done with the same protocol using 6 μg of DNA; hSOD1 and EGFP expression were analyzed after 48 h by Western blotting and confocal microscopy.

3.3. EGFP fluorescence and confocal microscopy analysis

NSC-34-tTA cell lines were transiently transfected with pBI-EGFP in six-well plates (each well containing 24 mm glass cover-slips) as described in the previous paragraph. After 48 h, cells were fixed with 4% paraformaldehyde in 1 × PBS for 1 h at 4 °C. Cover-slips were washed with 1 × PBS then mounted adding FluorSave reagent and observed with a fluorescence microscope (Axioskop-Zeiss, Germany). The excitation and emission wavelengths were respectively 488 and 525 nm. The same wavelengths were also used for confocal microscopy (Zeiss LSM 510 META point-scanning confocal microscope).

3.4. Cytofluorimetric analysis

At 48 h after transient transfection, EGFP fluorescence of selected NSC-34-tTA cell lines was quantified with a two-laser FACSCalibur cytometer (Becton Dickinson) using the same excitation and emission wavelengths as for fluorescence microscopy. Cells were detached from the plates with 1 × PBS containing 1 mM EDTA, pelleted by centrifugation, washed once and resuspended in 1 × PBS.

Cells were stained with propidium iodide (1 μg/ml) 5 min before flow cytometry in order to exclude propidium-iodide-permeable (dead) cells. NSC-34 cells, not transfected with pTet-Off, were transfected with pBI-EGFP and analyzed as negative control.

We considered two arbitrary fluorescence intervals (identified as M1 and M2) for the comparative analysis of NSC-34-tTA lines. M1 was used to compare EGFP fluorescence between the negative control (NSC-34) and NSC-34-tTA lines. M2 was used to compare EGFP fluorescence among the different NSC-34-tTA lines with and without dox. For each cell line, mean EGFP fluorescence intensity and percentage of cells were measured in both fluorescence intervals with and without dox (2 μg/ml).

Induction (I) of EGFP fluorescence in each cell line was calculated applying the following formula to the values obtained for interval M1: 

\[ I = \frac{(F \times N)_{NSC-34-tTA}}{(F \times N)_{NSC-34} \text{without dox}}, \]

or for interval M2: 

\[ I = \frac{(F \times N)_{-DOX}}{(F \times N)_{DOX}}, \]

where: 

\[ F = \text{mean EGFP fluorescence intensity}, \]

\[ N = \text{percentage of cells}. \]

3.5. Immunocytochemistry

NSC-34-tTA40 cells and NSC-34 cells were plated in six-well plates (each well containing 24 mm glass cover-slips) at 70 × 10^3 cells/ml in standard growth medium containing 5% FBS. After 48 h, cells were fixed using 4% paraformaldehyde in 1 × PBS. Immunocytochemistry was done using the following monoclonal antibodies at 1:1000: anti-alpha-tubulin, anti-GAP-43 (clone GAP-7B10) and SMI312. Immunoreactivity was visualized using anti-mouse AlexaTRITC-labeled secondary antibody (1:1000).

SMI312-positive neurites, alpha-tubulin and GAP-43 immunoreactivities were evaluated by fluorescence microscopy, a Zeiss Axiovert microscope, equipped with TRITC filter for fluorescence analysis, was used throughout the study.

For hSOD1 immunocytochemistry, NSC-34-tTA40 cells were transiently transfected with pBI-EGFP containing hSOD1 cDNA, fixed and probed with rabbit anti-SOD1 antibody (1:1000). Immunoreactivity was visualized using Alexa Fluor 546 goat anti-rabbit secondary antibody (1:500) by confocal microscopy (excitation/emission wavelengths: 555/565 nm, Zeiss LSM 510 META point-scanning confocal microscope).

3.6. SDS PAGE and Western blotting of SOD1 protein

Total cellular proteins (10–40 μg) were separated by electrophoresis on a 12% polyacrylamide gel. A sheep anti-SOD1 antibody (1:1000) was used coupled to anti-sheep peroxidase-conjugated IgG (1:1000). Mouse anti-actin (1:2000), mouse anti-EGFP (1:1000), mouse anti-GAP-43 (1:1000), mouse anti-alpha-tubulin (1:1000) and mouse SMI312 (1:1000) were coupled to mouse peroxidase-conjugated IgG (1:1000). Protein bands were detected using the ECL detection system.

4. Results

4.1. Development of NSC-34-tTA cell lines

NSC-34 cells were stably transfected with pTet-Off to obtain clones expressing regulatory protein tTA and G418 sulfate resistance. Out of the 88 monoclonal clones isolated after the 4-week selection with G418 sulfate, 55 became cell lines over a growth period of 4 months. During this time, these lines were evaluated by optical microscopy for their morphology and for their growth rate and ability to adhere to the culture plates; 28 cell lines were selected and used for the subsequent analysis.

4.2. Functional comparison of NSC-34-tTA cell lines

A functional assay was used to assess the presence of the tTA protein in the 28 NSC-34-tTA selected cell lines.
EGFP fluorescence was evaluated with and without dox in the culture medium after transient transfection of the cells with pBI-EGFP. This plasmid contains the Pbi-1 promoter which can potentially co-regulate two genes (the reporter gene EGFP and a cloned gene of interest) (Fig. 1). First, the cell lines were studied by fluorescence microscopy: 17 NSC-34-tTA cell lines showed poor or no fluorescence both in presence and in absence of dox, while 11 were regulated showing good fluorescence without dox and much lower expression with dox. Four of these cell lines (NSC-34-tTA83, NSC-34-tTA53, NSC-34-tTA66, NSC-34-tTA40) showed the best EGFP fluorescence intensity and fluorescence modulation by dox and had a larger number of fluorescent cells (Fig. 2). No fluorescence was observed when the same protocol was applied to NSC-34 cells which were not transfected with pTet-Off (data not shown). NSC-34-tTA83 was not further considered because of its less-than-optimal substrate adhesion and lumpy growth morphology.

For a quantitative evaluation of induction of NSC-34-tTA53, NSC-34-tTA66 and NSC-34-tTA40 cell lines, EGFP fluorescence was analyzed in the viable cell population by flow cytometry, as described in Methods. Fig. 3 shows that the fluorescence profile was heterogeneous. Fluorescence intensity was distributed in more than one cytofluorimetric channel, as expected, because of the inter-cellular variability of transient transfection. Comparing NSC-34 profiles with profiles of NSC-34-tTA lines in intervals M1 and M2, only NSC-34-tTA lines showed EGFP fluorescence expression without dox and EGFP fluorescence regulation with dox. EGFP expression and regulation occurred only in presence of functional tTA protein.

Table 1 shows the cytofluorimetric parameters (obtained from profiles shown in Fig. 3) for each clone with and without dox in the culture medium. Without dox, i.e. in the condition that permits full expression of EGFP fluorescence, NSC-34-tTA40 showed the highest total EGFP fluorescence intensity.

Fig. 1. Doxycycline-dependent gene expression regulation (Tet-Off system) in NSC-34-tTA cells transiently transfected with pBI-EGFP. When doxycycline (dox) is not added to the culture medium of cells that express functional tTA protein (NSC-34-tTA), transcription of genes downstream of the Pbi-1 bidirectional promoter in pBI-EGFP can begin in both directions, owing to the interaction of tTA protein with the tetracycline-responsive elements (seven E. coli operator repeats). When dox is added to the culture medium, it induces a conformational change in the tTA protein structure and reduces the affinity of the interaction, blocking transcription in both directions. TRE: tetracycline-responsive elements; MCS: multiple cloning site; Pcmv: minimal promoter of cytomegalovirus; EGFP: enhanced green fluorescent protein; tetR: E. coli repressor; AD: activator domain.

EGFP fluorescence was evaluated with and without dox in the culture medium after transient transfection of the cells with pBI-EGFP. This plasmid contains the Pbi-1 promoter which can potentially co-regulate two genes (the reporter gene EGFP and a cloned gene of interest) (Fig. 1). First, the cell lines were studied by fluorescence microscopy: 17 NSC-34-tTA cell lines showed poor or no fluorescence both in presence and in absence of dox, while 11 were regulated showing good fluorescence without dox and much lower expression with dox. Four of these cell lines (NSC-34-tTA83, NSC-34-tTA53, NSC-34-tTA66, NSC-34-tTA40) showed the best EGFP fluorescence intensity and fluorescence modulation by dox and had a larger number of fluorescent cells (Fig. 2). No fluorescence was observed when the same protocol was applied to NSC-34 cells which were not transfected with pTet-Off (data not shown). NSC-34-tTA83 was not further considered because of its less-than-optimal substrate adhesion and lumpy growth morphology.

For a quantitative evaluation of induction of NSC-34-tTA53, NSC-34-tTA66 and NSC-34-tTA40 cell lines, EGFP fluorescence was analyzed in the viable cell population by flow cytometry, as described in Methods. Fig. 3 shows that the fluorescence profile was heterogeneous. Fluorescence intensity was distributed in more than one cytofluorimetric channel, as expected, because of the inter-cellular variability of transient transfection. Comparing NSC-34 profiles with profiles of NSC-34-tTA lines in intervals M1 and M2, only NSC-34-tTA lines showed EGFP fluorescence expression without dox and EGFP fluorescence regulation with dox. EGFP expression and regulation occurred only in presence of functional tTA protein.

Table 1 shows the cytofluorimetric parameters (obtained from profiles shown in Fig. 3) for each clone with and without dox in the culture medium. Without dox, i.e. in the condition that permits full expression of EGFP fluorescence, NSC-34-tTA40 showed the highest total EGFP fluorescence intensity.

Fig. 2. EGFP expression in NSC-34-tTA monoclonal cell lines transiently transfected with pBI-EGFP. Each monoclonal cell line was observed by fluorescence microscopy (excitation wavelength 488 nm, emission wavelength 525 nm) 48 h after transient transfection with pBI-EGFP, without (on the left) or with doxycycline (dox) (on the right) in the culture medium. Panel A shows examples of cell lines with low expression of enhanced green fluorescent protein (EGFP) (NSC-34-tTA3 and NSC-34-tTA14) or with lumpy growth morphology (NSC-34-tTA83). Panel B shows the cell lines with the highest EGFP expression and the best regulation (NSC-34-tTA40, NSC-34-tTA53 and NSC-34-tTA66).
Mean EGFP fluorescence intensities and the percentages of cells in intervals M1 and M2 (Table 1) were used to calculate EGFP fluorescence in the respective intervals \((F \times N)\) (Figs. 4A and 4B) and the EGFP fluorescence induction \((I)\) (using formulas described in Methods) in order to compare the cell lines with NSC-34 or among themselves. On the basis of the M1 interval, the NSC-34-tTA40 line was 66.4 times more fluorescent than NSC-34, without dox. The induction values for NSC-34-tTA53 and NSC-34-tTA66 were respectively 35.1 and 43.2. On the basis of the M2 interval, the NSC-34-tTA40 line expressed 31.6 times more EGFP fluorescence than in the presence of dox. NSC-34-tTA53 and NSC-34-tTA66 EGFP fluorescence induction values were respectively 16.5 and 5.5.

Thus, NSC-34-tTA40 also has the best induction value. Only a very small concentration of tTA protein is necessary to obtain the regulatory effect, and, in fact, it was below the detection limit in a Western blot done with a lysate (25–50 µg) of NSC-34-tTA40 cells with a polyclonal rabbit anti-

![Flow cytometric profiles](image)

**Fig. 3.** Flow cytometric profiles of EGFP fluorescence in a control cell line (NSC-34) and in selected NSC-34-tTA lines. Flow cytometry profiles for EGFP fluorescence of NSC-34 and NSC-34-tTA66, NSC-34-tTA53 and NSC-34-tTA40 cell lines after transient transfection with pBI-EGFP. The EGFP fluorescence for each cell line without (black line) or with (gray line) doxycycline (dox) in the culture medium is shown. The arbitrarily selected M1 and M2 fluorescence intervals (see Methods) are indicated.

**Table 1**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Total fluorescence intensity</th>
<th>Percentage of cells in M1 (N)</th>
<th>Percentage of cells in M2 (N)</th>
<th>Mean fluorescence intensity in M1 ((F))</th>
<th>Mean fluorescence intensity in M2 ((F))</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC-34</td>
<td>1</td>
<td>2.03</td>
<td>0.04</td>
<td>126</td>
<td>965</td>
</tr>
<tr>
<td>NSC-34+DOX</td>
<td>1</td>
<td>2.14</td>
<td>0.01</td>
<td>133</td>
<td>1655</td>
</tr>
<tr>
<td>NSC-34Tet-Off-tTA66</td>
<td>4.9</td>
<td>26.2</td>
<td>8.25</td>
<td>422</td>
<td>1540</td>
</tr>
<tr>
<td>NSC-34Tet-Off-tTA66+DOX</td>
<td>1.5</td>
<td>15.4</td>
<td>2.02</td>
<td>255</td>
<td>1155</td>
</tr>
<tr>
<td>NSC-34Tet-Off-tTA53</td>
<td>5.6</td>
<td>25.5</td>
<td>6.9</td>
<td>352</td>
<td>1540</td>
</tr>
<tr>
<td>NSC-34Tet-Off-tTA53+DOX</td>
<td>2</td>
<td>12.9</td>
<td>0.6</td>
<td>184</td>
<td>1075</td>
</tr>
<tr>
<td>NSC-34Tet-Off-tTA40</td>
<td>24</td>
<td>37.5</td>
<td>13</td>
<td>453</td>
<td>1596</td>
</tr>
<tr>
<td>NSC-34Tet-Off-tTA40+DOX</td>
<td>1.8</td>
<td>13.3</td>
<td>0.59</td>
<td>191</td>
<td>1114</td>
</tr>
</tbody>
</table>

The NSC-34 and the NSC-34-tTA66, NSC-34-tTA53, NSC-34-tTA40 cell lines were analyzed for EGFP fluorescence by flow cytometry with and without doxycycline (dox) in the culture medium after transient transfection with pBI-EGFP. The corresponding flow cytometry profiles are shown in Fig. 3. M1 and M2 were arbitrary fluorescence intervals (see Methods). For each culture condition and cell line, total fluorescence intensity in all viable cells, the percentage of cells detected in the M1/M2 interval \((N)\) and the mean EGFP fluorescence intensity in the M1/M2 interval \((F)\) are reported.
4.3. Phenotypic characterization of the NSC-34-tTA40 cell line

The neuronal/motor neuronal phenotype of the NSC-34-tTA40 cell line, in comparison to NSC-34 cell line, was evaluated using cytoskeleton and axonal marker proteins by Western blot and immunocytochemistry. Expression of alpha-tubulin, GAP-43 and phosphorylated medium and heavy neurofilaments (by using SMI312 antibody [30]) (Fig. 5A) and their cellular locations were evident (Fig. 5B) and comparable in both lines. The motor neuron-like morphology of the NSC-34-tTA40, evaluated by phase contrast microscopy, is shown in Fig. 5C and is compared to that of NSC-34.

4.4. Functionality of the bidirectional promoter Pbi-1

To evaluate the functionality of the bidirectional promoter Pbi-1 to drive expression of EGFP and of a non-reporter gene (hSOD1), pBI-EGFP containing hSOD1 cDNA was transiently transfected into the best-regulated cell line (NSC-34-tTA40), and the expression was regulated by the addition of dox (1 μg/ml). Western blot analysis of cells showed the concomitant regulation of EGFP and hSOD1 (Fig. 6A), and immunocytochemical analysis confirmed that both proteins (green and red, respectively) colocalized in the same cell (Fig. 6B). As expected, murine SOD1 expression did not vary in NSC-34 and in NSC-34-tTA40 (Fig. 6A).

5. Discussion

NSC-34 cells were genetically manipulated to obtain a novel clonal cell line, NSC-34-tTA40, expressing a functional tTA protein as demonstrated by induction of EGFP fluorescence and its modulation following addition of dox to the culture medium. This line is the first step in obtaining a Tc-regulated expression system for any gene of interest in a motor neuron-like cell.

This result appears important for the study of motor neuron diseases since, in several of these neurodegenerative conditions, mutations in single genes have been identified. It has been shown in many cases that the mutant protein acquires a toxic function responsible for the selective damage to the motor neurons [9,39]. However, the causal relationships between mutant protein expression and motor neuron toxicity are still unclear, dramatically limiting the possibilities of therapeutic intervention. The genes involved could be cloned under a Tc-responsive promoter and transfected in the NSC-34-tTA40 cell line so that their expression could be controlled by changing the concentration of Tc or of dox. Inducible in vitro systems appear particularly suitable to investigate molecular patterns of neurodegeneration and to relate the toxic phenotype with expression of toxic protein [17]. A further advantage is the possibility to study if suppression of the toxic protein expression, after addition of Tc, can rescue the toxic phenotype(s) [29], as in murine inducible models [48]. This approach could be used to predict potential effects of inactivation of the gene of interest by gene therapy in patients.

We investigated regulation of the expression of the reporter gene EGFP in several NSC-34-tTA cell lines. EGFP protein fluoresces about 35 times more intensely than wt GFP and is optimal for FACS analysis [13]. Therefore, a quantitative cytofluorimetric analysis, which takes into account both fluorescence intensity and the
percentage of fluorescence expressing cells, was optimized to select the NSC-34-tTA cell line with the best induction. This approach permits to overcome the cellular variability in fluorescence due to transient transfection.

Furthermore, we transfected with pBI-EGFP containing Pbi-1, a bidirectional promoter, previously used to obtain co-regulation of two genes [24]. Observing EGFP fluorescence can be a useful way to check the expression also of the protein of interest. We showed the co-expression/co-regulation of EGFP and hSOD1, a house-keeping enzyme that has a role in the pathogenesis of ALS, a motor neuron disease [38]. Thus, pBI-EGFP could be used to establish transient or stable Tet-Off systems for any gene in NSC-34-tTA40.

Both Tet-On and Tet-Off systems are used to develop inducible models [8,20,21,25,46,47]; we chose the Tet-Off system for this in vitro model in light of several considerations. It can mimic more closely the constitutive expression of aberrant proteins seen in patients with familial forms of motor neuron diseases since the system allows continuous expression of the regulated gene without addition of Tc. This system also appears to rapidly repress the expression of the regulated gene of interest upon addition of dox because of the fast cellular absorption of the drug [7]. With the Tet-On system, on the other hand, the time required for induction of gene transcription could be a limiting step because of the slow clearance of dox [36].

Our novel cell line, NSC-34-tTA40, maintained a motor neuron phenotype, as shown by the presence of GAP-43, a neuronal and motoneuronal growth protein [6] and of phosphorylated medium and heavy neurofilaments, expressed at levels comparable to those present in NSC-34. Alpha-tubulin was also expressed well in the cytoplasm.

![Fig. 5. Comparison of neurofilament, alpha-tubulin and GAP-43 expression in NSC-34-tTA40 and NSC-34 cell lines. NSC-34-tTA40 and NSC-34 cells were analyzed by Western blot (40 μg of total proteins). Primary antibodies to phosphorylated heavy and medium neurofilaments (NF-H/NF-M) (by SMI312), alpha-tubulin and GAP-43 were used. Actin level is shown as loading control (A). For the immunocytochemical analysis, the same primary antibodies were used (fluorescence microscopy, 40×) (B). Panel C shows the morphology of NSC-34-tTA40 and NSC-34 cells (phase contrast microscopy, 20×).](image)
and processes of both lines. This protein is essential for the formation of microtubules in every cell type \[23\]; it is particularly important for motoneurons that need to maintain long axonal projections.

The selectivity of damage to motor neurons in some neurodegenerative diseases is still unexplained. This is illustrated for example by the familial forms of ALS involving mutant(s) SOD1: the mutant protein(s) is ubiquitously expressed in the patient's tissues, but the symptoms are certainly due to motor neuron death. Phenotypic peculiarities, which have a largely unknown molecular basis, might explain this selective damage. Motor neurons are large (the somatic diameter is 50–60 \( \mu \)M) non-dividing cells with a long axon and a high degree of differentiation; their membrane phospholipid content is higher than in other cell types, and they also have higher levels of mitochondrial activity and neurofilament proteins and are more vulnerable to damage by oxidative stress and excitotoxicity \[41\]. Primary cells cannot be maintained in culture for long, and this is a great disadvantage for in vitro studies.

As for other types of neural cells, several strategies have been developed to immortalize them, one being the fusion of primary motor neurons with tumor lines, in the attempt to find a compromise between degree of differentiation and capacity for proliferation. The NSC-34 cell line was chosen for this study since it is the best-characterized immortalized motor neuron-like cell culture model so far.

This model is useful to investigate motoneuronal degeneration in the presence of agents that affect voltage-gated ion channels, cytoskeleton organization and axonal delivery, or toxic conditions such as hypoxia or oxidative stimuli \[12,33,37\], or transfected aberrant proteins \[27,34\].

Evidence of the presence of proteins of importance for the motor neuron phenotype coupled to functional tTA in the NSC-34-tTA40 cell line suggests that this line may provide a valuable new tool to investigate the cellular and molecular biology and pathology of motor neurons.

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