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# Variability in RNA interference in neuroendocrine PC12 cell lines stably transfected with an shRNA plasmid

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**Abstract**

RNA interference (RNAi) has quickly become a very powerful technique for specifically suppressing or knocking down the expression of any desired gene. Many fields of research, including neuroscience, have benefitted from RNAi methods. It has been well documented that different small interfering RNAs (siRNAs) and small hairpin RNAs (shRNAs) vary greatly in terms of their effectiveness, and much attention has been focused on guidelines and algorithms for the selection of effective siRNAs. However, it has not been widely appreciated that a single shRNA-expressing plasmid can also produce widely varying levels of knockdown in different stably transfected cell lines derived from the same transfection. Here we report that knockdown of three distinct target proteins varies from minimal to almost complete in independent, stably transfected PC12 cell lines. This variability in knockdown among cell lines emphasizes the importance of characterizing a number of cell lines when attempting to establish stable knockdown cell lines, but also offers the possibility of studying the effects of graded levels of protein expression.
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*Keywords:* RNA interference; shRNA; Gene silencing; Gene knockdown; PC12 cells; Stable transfection

## 1. Introduction

RNA interference (RNAi) has become the method of choice for specifically suppressing the expression of any desired gene. This suppression is commonly called knockdown or silencing. RNAi can be elicited transiently in mammalian cells by transfection with small interfering RNAs (siRNAs) or plasmids expressing small hairpin RNAs (shRNAs) or by infecting cells with viral vectors expressing shRNAs (Dillon et al., 2005; Sandy et al., 2005). Long term RNAi can be achieved by selecting transfected or infected cells for those that have stably incorporated the shRNA plasmid or retrovirus into the genome (Brummelkamp et al., 2002; Cullen, 2006; Paddison et al., 2002). Although there is a substantial investment in time to derive stably transfected cell lines, these lines have several distinct advantages for RNAi experiments: (1) proteins with a long half-life can be knocked down; (2) RNAi experiments can be carried out in cells that are not easily transfected because selection with drugs allows the survival and study only of transfected cells; (3) stably transfected cell lines are homogeneous and maintain their knockdown phenotype for months in culture (Brummelkamp et al., 2002; Choi et al., 2005; Jazag et al., 2005). Stability of the knockdown phenotype means that multiple experiments can be carried out in cells that all have the same reduced level of the targeted protein and that the extent of knockdown does not need to be verified repeatedly for each experiment. Homogeneity of the knockdown cell lines means the extent of knockdown determined by population methods such as immunoblotting can be assumed to represent the knockdown status of each cell in the culture. Thus, studies typically conducted on single cells such as recording ion channel currents by patch clamping or measuring the release of catecholamines by amperometry can be carried out with confidence that each cell has a known extent of knockdown of the targeted protein.

In this study, we used PC12 cells to generate different stable cell lines that continuously express shRNAs. PC12 cells are frequently used to study a range of cellular processes including neurosecretion, neurite outgrowth and oxidative stress, a mechanism of neurodegeneration. These cells are often used in place of neurons because of their flexibility of use with multiple techniques to evaluate changes in single cells and populations of cells. However, the transfection efficiency of PC12 cells is rel-

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atively low. Thus, it can be beneficial to establish stable cell lines when using plasmid-based RNAi in PC12 cells. In the course of deriving stably transfected PC12 cell lines that lack synaptotagmin I (Syt I) or SNAP-25, two proteins involved in neurotransmitter release, we found that knockdown of these proteins varied widely in different cell lines which had all incorporated the same plasmid into their genome. It is well known that different siRNAs and shRNAs designed to target the same mRNA can yield very different levels of knockdown of the target protein (Khvorova et al., 2003; Pei and Tuschl, 2006; Reynolds et al., 2004; Saetrom and Snove, 2004). However, it has not been widely appreciated that a single shRNA can yield very different levels of knockdown of its target in different cell lines stably transfected with the same shRNA-expressing plasmid. We demonstrate that knockdown of three target proteins varies from minimal to almost complete in independent, stably transfected PC12 cell lines, and we test the hypothesis that the level of knockdown depends on the number of copies of the plasmid integrated into the genome.

## 2. Materials and methods

### 2.1. shRNA plasmids

Plasmids designed to express shRNAs targeting SNAP-25 and synaptotagmin I (Syt I) were constructed as described previously (Cahill et al., 2006; Moore et al., 2006). A plasmid targeting EGFP was constructed in a similar manner using nucleotides 804–823 of pEGFP-N1 (GenBank accession no. U55762) as the target region.

### 2.2. Transfection of PC12 cells and selection of stably transfected cell lines

PC12 cells were cultured, transfected and selected with G418 as previously described (Cahill et al., 2006; Moore et al., 2006). G418-resistant cell lines were screened by PCR for presence of the plasmids in genomic DNA (Moore et al., 2006).

### 2.3. Immunoblots

The extent of knockdown was assessed by immunoblotting as previously described (Cahill et al., 2006) or by viewing the fluorescence of EGFP. An antibody against EGFP was obtained from Research Diagnostics (Flanders, NJ). Quantitation of the immunoblots was done using ImageJ (http://rsb.info.nih.gov/ij/index.html).

### 2.4. Quantitative real-time PCR

Quantitative real-time PCR was carried out on an ABI 7900HT Real-Time PCR System using a SYBR Green master mix (AB Gene #AB-1163A) and genomic DNA isolated from each cell line. Plasmid DNA was detected using primers to amplify a 101 bp fragment of the NeoR coding sequence present in all shRNA plasmids. The amount of plasmid DNA in each sample was normalized to the amount of genomic DNA in the sample using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001). The amount of genomic DNA in each sample was determined by amplification of a 113 bp fragment from a single exon of a single copy gene on rat chromosome 3

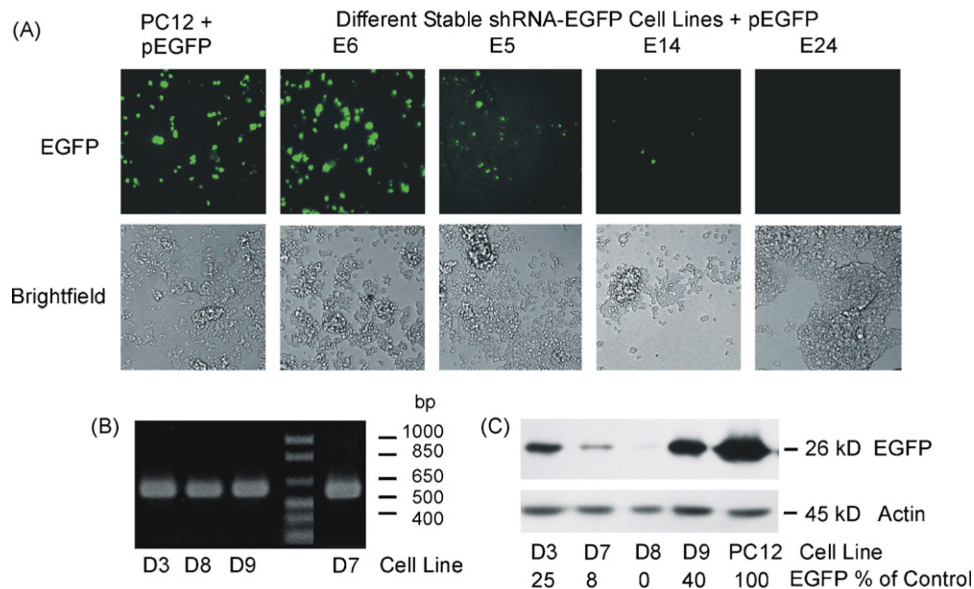


Fig. 1. Knockdown of EGFP protein expression varies in independent PC12 cell lines stably transfected with an shRNA plasmid targeting EGFP. Two independent transfections were performed, and stably transfected cell lines were selected, expanded and tested as described in Section 2. Cell lines derived from the two independent transfections are labelled "D" and "E" lines. (A) Wild-type PC12 cells and each of the stably transfected cell lines were transiently transfected with pEGFP-N1. After 48 h each culture was photographed on a fluorescent microscope and under brightfield illumination. (B) Incorporation of the plasmid into the genome was verified for each cell line by PCR using genomic DNA as the template and PCR primers specific for the shRNA plasmid. (C) An immunoblot confirms varying levels of EGFP expressed 48 h after transient transfection with pEGFP-N1 in the parental PC12 cells and in four cell lines (D3, D7, D8, and D9) stably transfected with an shRNA plasmid targeting EGFP. The amount of EGFP expressed in each cell line was quantified by densitometry and is shown as a percent of EGFP expressed in the parental PC12 cell line (control). Reprobing the immunoblot for actin demonstrates that approximately equal amounts of protein were loaded in each lane.

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