Improved silencing vector co-expressing GFP and small hairpin RNA

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Small interfering RNA (siRNA) is a powerful tool for the specific silencing of gene expression. We developed an improved vector, pG-SUPER, that co-expresses green fluorescent protein (GFP) and small hairpin RNA simultaneously to facilitate analysis of silencing at the level of individual cells. As a test system, we analyzed lamin A/C knockdown in HeLa cells. The GFP signal was a reliable reporter (93%–98%) of strong knockdown (approximately 90%) over a wide range of GFP intensities. The GFP reporter made possible the application of fluorescent-activated cell sorting (FACS) to purify the knockdown cell population. Such populations facilitated Western blotting analysis to determine depletion of the target protein. pG-SUPER was also applied to evaluate gene replacement by exogenous genes rendered refractory to siRNA by introducing silent mutations. Recovery of lamin A was linearly correlated to the expression level of the rescue gene. pG-SUPER will expand plasmid-based siRNA applications through the easy and reliable detection of knockdown and rescued cells.

INTRODUCTION

Small interfering RNA (siRNA) has emerged recently as a powerful method for gene silencing or knockdown of gene expression (1). In this technique, double-stranded RNA induces the degradation of cognate message sequences resulting ultimately in depletion of the encoded protein (2,3).

There are two common ways to introduce siRNA into cells. One is the in vitro chemical synthesis of siRNA followed by transfection (4). The other way is by a DNA vehicle (plasmid or virus) expressing an siRNA precursor (5–18). Expression approaches offer advantages over chemically synthesized siRNA in the greater persistence of the knockdown effect, the molecular biological flexibility, and the potential for genetic rescue. In commonly used expression approaches, a small hairpin RNA (shRNA) fragment is expressed under an RNA polymerase III promoter. Self-complementarity makes the expressed precursor form a hairpin shape, and then DICER removes the loop of the hairpin to generate functional siRNA (9).

One technical issue limiting the utility of expression approaches is that many cell types can be transfected only with low efficiency, resulting unavoidably in a large fraction of nontransfected cells that contaminate the population. For studies of putative knockdown phenotypes in individual cells, transfectants need to be identified easily and unambiguously in mixed populations. This is particularly acute for studies involving transient expression and live cell imaging. For mass analysis, such as Western blot analysis, it is critical to obtain pure transfectant populations since nontransfected cells will otherwise mask the knockdown effect.

Other critical issues involve the design of proper controls and the opportunity to develop a true "cellular genetics" (19). Since RNA interference is very sensitive to base mismatch, an siRNA-refractory gene can be prepared by nucleotide substitution(s) of the target sequence that do not alter the encoded amino acid residues. By introducing an siRNA-refractory gene to cells in which the endogenous gene expression was silenced, several experiments would be possible. One is "rescue," a stringent control for the specificity of targeting by siRNA. Improved cellular genetics may also be possible by analyzing mutations, such as amino acid substitutions or truncations, in the absence of endogenous gene product.

Here we report the construction of an improved vector, pG-SUPER, that co-expresses green fluorescent protein (GFP) and shRNA simultaneously. We tested pG-SUPER by lamin A/C knockdown in HeLa cells and confirmed that the GFP served as a reliable reporter for knockdown. We describe the use of pG-SUPER for single-cell phenotype analysis, fluorescent-activated cell sorting (FACS) purification, and gene replacement.

MATERIALS AND METHODS

Cell Culture and Transfection

HeLa cells were obtained from ATCC (Manassas, VA, USA) and grown as per ATCC's instructions. FuGENE6 (Roche Molecular Biochemicals, Indianapolis, IN, USA) was used for transfection of plasmid DNA. Electroporation was carried out as described previously (20).

Plasmid Construction

DNA encoding enhanced green fluorescent protein (EGFP) was extracted from pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA, USA) with *Eco*RI and *Not*I and then inserted into pME18S-f1 (named pME-EGFP). pSUPER was digested with *Xba*I and *Xho*I and then blunted with Klenow large fragment. The extracted H1 RNA promoter fragment was inserted to pME-EGFP, which was digested with *Ssp*I and *Hin*dIII, and blunted with the Klenow large fragment (named pG-SUPER). pG-SUPER-hLamin A/C and pG-SUPER-mFascin 1 were constructed according to (5). The targeting sequences are nucleotides 820–838 of human lamin A/C (NM_005572) and nucleotides 819–837 of mouse fascin1 (NM_007984).

An expression plasmid of N-terminal myc-tagged human lamin A1 was created by self-ligation of *Nhe*I/*Spe*I-digested pEYFP-lamin A. The resultant plasmid was mutagenized by QuikChange® II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) to substitute the nucleotides C^{828} and G^{831} with T and A, respectively (named pCMV-myc-lamin A*). These point mutations within the target sequence did not change the encoded amino acids. Control empty vector,

pCMV, was prepared by self-ligation of *Age*I/*Bsp*EI-digested pEGFP-C1.

Immunofluorescence

HeLa cells were fixed with 4% formaldehyde for 30 min and permeabilized with 1% Triton®X-100 for 5 min. Mouse monoclonal anti-lamin A/C (clone 636; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-myc antibody (Covance Research Products, Denver, PA, USA) were used as primary antibodies. Tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse immunoglobulin G (IgG) and Cy™5-conjugated anti-rabbit IgG antibodies (both from Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used as secondary antibodies. DNA was stained with 10 µg/mL Hoechst 33258. Cell preparations were imaged with a Diaphot 300 inverted microscope (Nikon, Tokyo, Japan) equipped with a Fluor 20× 0.75 NA dry objective and a slow scan-cooled charge-coupled device (CCD) camera CH350 (PhotoMetrics, Huntington Beach, CA, USA). MetaMorph® Imaging software (Universal Imaging, Downingtown, PA, USA) was used for image acquisition and data analysis. Multispectral fluorescence imaging was performed with a Quad filter set (86000; Chroma Technology, Rockingham, VT, USA). View fields containing GFP-expressing cells were randomly selected, and then DNA, GFP, TRITC, and Cy5 (for the rescue experiment) images were recorded. The excitation and emission filters were changed using a Lambda 10-2 Optical Filter Controller (Sutter Instrument, Novato, CA, USA). Cell nuclei were defined by the DNA staining areas, and then the mean intensity of GFP, TRITC, and Cy5 (if necessary) were measured and corrected by subtraction of the background level (GFP and TRITC) or the mean value of untransfectants (Cy5).

Immunoblotting

Cells were lysed with lysis buffer [31.25 mM Tris-HCl, 2% sodium dodecyl sulfate (SDS), and 10% glycerol, pH6.8]. Protein concentration was determined by the BCA Protein Assay (bicinchoninic acid) procedure (Pierce Biotechnology, Rockford, IL, USA). The protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 4%–20% gradient gels and then transferred to nitrocellulose membranes (Protran®; Schleicher & Schuell, Keene, NH, USA). Mouse anti-lamin A/C (clone 636) was used at a dilution of 1:500. Signal was detected by horseradish peroxidase-conjugated anti-mouse IgG (KPL, Gaithersburg, MD, USA) and the ECL™ Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ, USA). To control for loading on the gels, the same membrane was reassayed by mouse anti-α-tubulin (clone B-5-1-2; Sigma, St. Louis, MO, USA) after treating by Restore Western Blot Stripping Buffer (Pierce Biotechnology).

RESULTS

Construction of pG-SUPER

For easy identification of cells that express siRNA transiently, we constructed a new vector, pG-SUPER (Figure 1A). This vector contains two expression units. One is the expression cassette of shRNA under the human H1 RNA promoter, as originally reported for pSUPER (5), and the other is EGFP under the SRα promoter (21). Therefore, cells that receive pG-SUPER-based silencing constructs can be detected by fluorescence of GFP at the single cell level. A similar strategy was recently reported using viral systems (17,18).

We used lamin A/C silencing in HeLa cells as a test model. We designed and inserted DNA oligoduplex following the criteria of Brummelkamp et al. (5). Six days after transfection to HeLa cells with the resulting targeting construct (pG-SUPER-hLaminA/C), endogenous lamin A/C was assayed by immunofluorescence (Figure 1B). Line scan analysis (Figure 1C) shows that in the GFP-positive cells, the lamin A/C amount was significantly reduced as compared with neighboring GFP-negative cells. The specificity of the knockdown was confirmed by

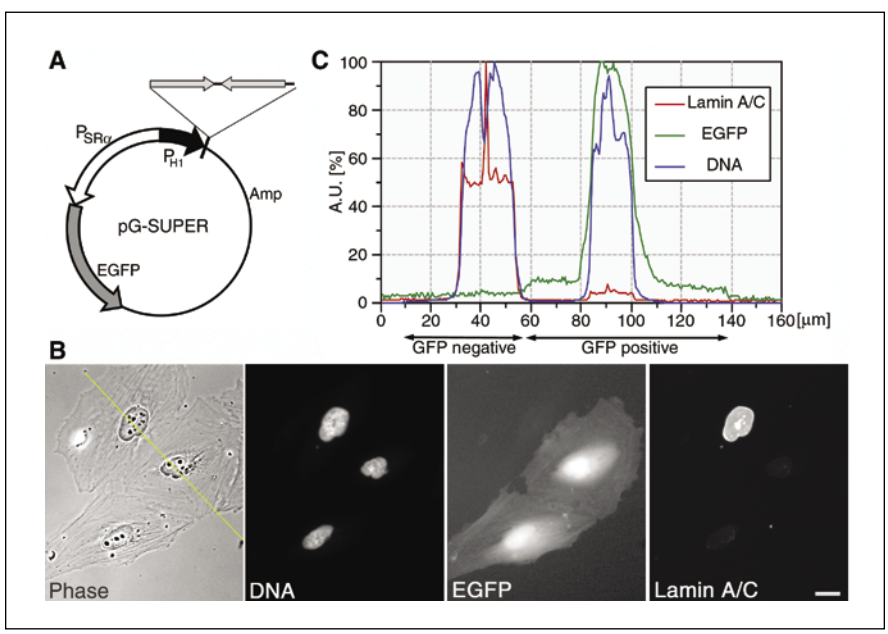

Figure 1. An improved hairpin small interfering RNA (siRNA) vector, pG-SUPER, and its application to silencing of lamin A/C in HeLa cells. (A) pG-SUPER. Total nucleotide length is 3910 bp. Enhanced green fluorescent protein (EGFP) and small hairpin RNA (shRNA) are expressed under SRα promoter and human H1 RNA promoter, respectively. Facing arrows represent oligonucelotide to be inserted after the H1 promoter by *Bgl*II and *Hin*dIII. (B) Immunofluorescence of HeLa cells 6 days after transfection with pG-SUPER-hLamin A/C. A phase contrast and three epifluorescence images [DNA, GFP, and tetramethylrhodamine isothiocyanate (TRITC) channels] are shown. Lamin A/C was stained with mouse monoclonal anti-lamin A/C. Yellow line indicates line scan shown in panel C. Scale bar, 20 µm. (C) Line scan analysis. Blue, green, and red lines represent the intensity profiles of DNA, GFP, and lamin A/C signals, respectively.

using pG-SUPER empty vector and pG-SUPER-mFascin 1 (D. Vignjevic, unpublished data) with a mouse fascin 1-targeting sequence that does not exist in the human genome. In both negative controls, the lamin A/C amounts were indistinguishable between the GFP-positive and GFP-negative populations (data not shown). Thus, on a qualitative basis, knockdown of lamin was readily demonstrable in cells co-expressing EGFP and the silencing construct.

Correlation Between Knockdown and GFP Expression

The relationship between the knockdown effect and GFP expression was analyzed quantitatively. Figure 2A shows a scatterplot of the extent of silencing and the level of GFP expression in individual cells. The amount of lamin A/C remaining is presented as a percentage of the average level expressed in GFP-negative cells [nontransfectants, red dots; <70 arbitrary units (AU)]. GFP-positive cells showed a wide range of fluorescence (70–13,000 AU), indicating that the expression level per cell differed by a factor of 185. However, silencing was virtually equally effective over the entire range of reporter expression, giving a mean level of 12.4% for lamin A/C remaining.

The penetrance of the knockdown effect in the whole population of GFP-positive cells was evaluated statistically. Nontransfected HeLa cells showed heterogeneity in lamin A/C amount with a standard deviation, σ = 22.5% with respect to the mean value, m, defined as 100%. For an objective definition of silencing, we compared the lamin A/C level of each GFP-positive cell to m and σ. Only 2% of GFP-positive cells had a lamin A/C amount within the range (m - σ, m + σ), representing no silencing effect. The rare lack of silencing seemed unrelated to GFP expression level, since it was observed at both low and high GFP intensity values. In contrast, the lamin A/C amounts of 93% of GFP-positive cells were below m-3σ, implying statistically significant reduction of lamin A/C. Therefore the penetrance of pG-SUPER-hLamin A/C was estimated as 93%–98%, indicating strong correlation between knockdown and GFP expression. More importantly, these cells showed fairly uniform silencing over a wide range of GFP intensity. Therefore, the knockdown effect was likely saturated by a rather small amount of shRNA. Correlation between the knockdown effect and the GFP signal was surprisingly persistent, lasting to at least the day 9 after transfection. We conclude that, to a first approximation, GFP-positive cells have homogenous silencing independent of GFP expression level in the pG-SUPER system.

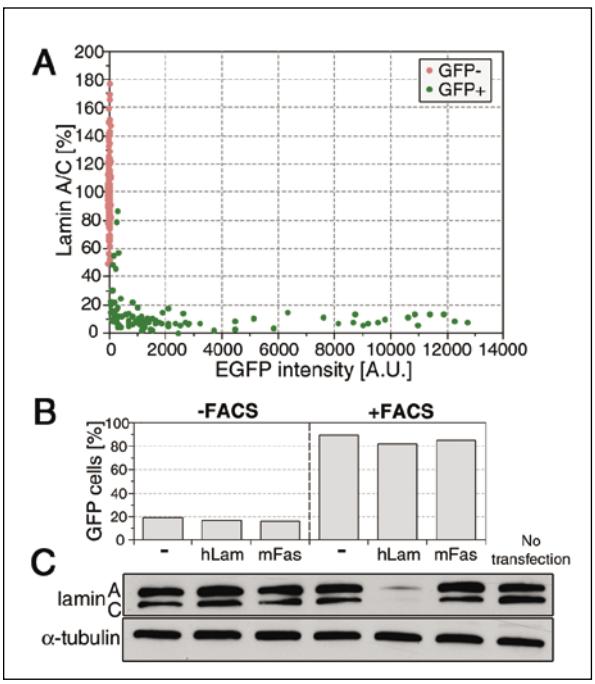

Figure 2. Quantification of lamin A/C silencing by pG-SUPER-hLamin A/C. (A) Scatterplots of lamin A/C amount [%] and green fluorescent protein (GFP) intensity (arbitrary units [AU]). Each dot corresponds to an individual cell. Red and green represent GFP-negative and GFP-positive populations, respectively. Approximately 100 cells were counted for each population 6 days after transfection. (B and C) Fluorescent-activated cell sorting (FACS)-purification of knockdown cells. FACS was carried out 1 day after transfection. (B) Fraction of GFP-positive cells in total population with or without FACS. Eight days after transfection with FuGENE6, 140–300 cells were counted under a fluorescence microscope. –, pG-SUPER empty; hLam, pG-SUPER-hLamin A/C; mFas, pG-SUPER-mFascin 1. (C) Western blot analysis of cell extracts. Four micrograms of total lysate was loaded to each lane. Lamin A/C was detected by mouse monoclonal anti-lamin A/C. The same membrane was analyzed with anti-α-tubulin to confirm that equal amounts of proteins were loaded.

Purification of Knockdown Cells by FACS

FACS was used to purify GFP-positive cells at 24 h posttransfection. Under our conditions, the initially sorted population was >99% pure, and 1 week after FACS, more than 80% of the cells still had GFP signal (Figure 2B). FACS-purification of GFP-positive cells facilitated Western blot analysis to detect substantial depletion of lamin A/C specifically (Figure 2C). In contrast, reduction of lamin A/C was not readily detected in the unsorted population, presumably because nontransfected cells masked the knockdown effect.

Gene Replacement of Lamin A/C

Gene replacement provides an ultimate control for the specificity of silencing as well an approach for substituting mutant protein for endogenous protein. We prepared a myc-tagged lamin A cDNA refractory to siRNA by introducing two silent mutations within the target sequence. Subsequent to knockdown (4 days after electroporation), the myc-lamin A rescue construct was transfected to the cell population and allowed to express for an additional 2 days of culture. Cells were analyzed by immunofluorescence with mouse anti-lamin A/C and rabbit anti-myc antibodies to reveal total lamin A/C and replacement lamin A, respectively (Figure 3A). GFP-expressing cells showed remarkable reduction of lamin A/C as compared with GFP-negative cells, except for rescued cells with positive signal for the myc tag (arrows). As shown in the scatterplot of Figure 3B, these rescued cells expressed myc-tagged lamin A at levels close to the normal range (20%–170% of normal cells; Figure 3B, blue dots in the lower panel) independent of the GFP intensities. Levels of total lamin A/C correlated linearly with levels of the myc-tagged rescue lamin A (Figure 3C). The results

demonstrate that endogenous lamin A/C can be knocked down substantially by pG-SUPER expressing shRNA and replaced to approximately normal levels by a construct refractory to silencing.

DISCUSSION

Properties and Applications of pG-SUPER

In the pG-SUPER system, the correlation between GFP expression and gene silencing was tight (93%–98%), with rare (2%) false positives. Although we have quantified silencing in detail only for HeLa cells, similar properties have been obtained for B16F1 melanoma cells and two other gene products—fascin and capping protein β (D. Vignjevic and M. Mejillano, unpublished results). This suggests that reliable detection of knockdown cells will be generally observed with the pG-SUPER system. The tight correlation of GFP reporter expression and gene silencing permits the use of live cell imaging of knockdown cells to analyze their phenotypes by bright field or fluorescence microscopy. Also pG-SUPER will give a solution when an available antibody against the target protein works only for Western blot analysis. The GFP-positive transfectants can be used for assays of the gene-silenced population after the effectiveness of the silencing construct is checked by Western blot analysis. This method will allow us to expand the target proteins for siRNA experiments. Again relying on the GFP signal as an indicator, FACS becomes a powerful tool to purify the gene-silenced cells for biochemical studies. In our assays, FACS was carried out 24 h after transfection, allowing the collection of transfectants before the silencing effect appears. Thus, FACS-based collection is also applicable to essential genes whose knockdown would not allow continued cell propagation. As a conclusion, since transfection efficiency is not a limiting factor in the pG-SUPER system, gene silencing by transient transfection will be applicable to a wide range of cell types.

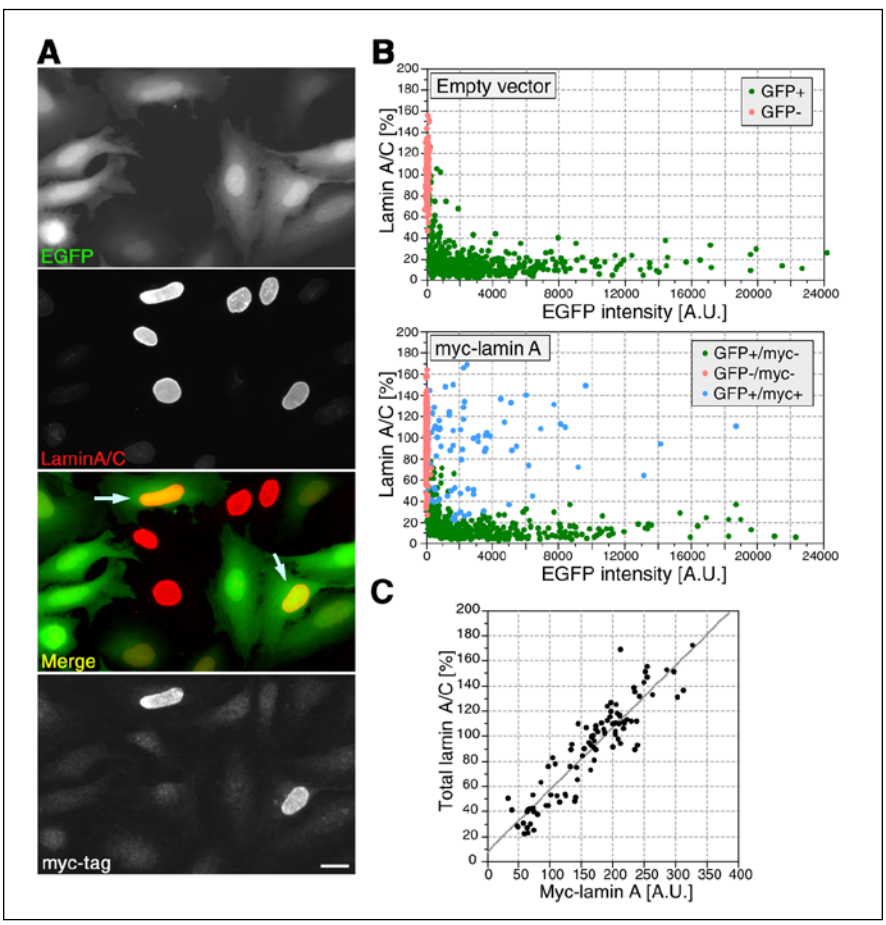

Figure 3. Rescue of lamin A silencing. (A) Immunofluorescence of HeLa cells in the rescue experiment. Four days after electroporation with pG-SUPER-hLamin A/C, the rescue construct (pCMV-myc-laminA*) was transfected by FuGENE6. Transfectants were cultured 2 days more and immunostained with mouse anti-lamin A/C (clone 636) and rabbit anti-myc antibodies. In the merged image, green and red represent enhanced green fluorescent protein (EGFP) and lamin A/C, respectively. Arrows indicate the rescued cells. Scale bar, 20 µm. (B) Scatterplots of lamin A/C amount [%] and EGFP intensity (arbitrary units [AU]). Red and green dots represent GFP-negative and GFP-positive cells not expressing myc-tagged lamin A. Blue dots represent cells that co-expressed GFP and myc-lamin A. (C) Plot of total lamin A/C amounts [%] versus myc-lamin A amount [AU]. Total lamin A/C level [%] was estimated by using mouse anti-lamin A/C antibody that recognizes both endogenous and exogenous lamin A/C. Myc-lamin A level was evaluated from immunostaining with rabbit anti-myc antibody that binds only exogenous lamin A.

Significance of Rescue and Gene Replacement

For a control of siRNA experiments, the use of siRNA mismatched to the target sequence is most desirable. Whereas several groups have shown that a one-base mismatch abrogated siRNA activity (5,22), other reports indicate that in some cases siRNA containing a one-base mismatch is still functional (23,24). Consequently, we employed a two-base mismatch. Recent genome-wide analyses have shown that chemically synthesized siRNA can have unpredicted off-target effects (25–28). Therefore, rescue of the knockdown phenotype by the wild-type offers the ultimate control, as a recent symposium on siRNA has concluded (19). Our results show that a rescue approach using a refractory cDNA is experimentally feasible in a transient expression system.

Expression knockdown and gene replacement with the pG-SUPER system facilitates not only the rescue control, but also provides an equivalent to cellular genetics. By this system, modified gene products will be able to be examined in the absence of endogenous protein. Therefore, gene replacement at the cellular level offers the opportunity to analyze protein function with the specificity previously afforded only by classical genetics.

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NOTE ADDED IN PROOF

We have developed another co-expression vector, pG-SHIN, in which a shorter segment (91 bp) of human H1 promoter is used instead of the one (215 bp) from pSUPER. This vector worked identically to pG-SUPER for silencing of lamin A/C in HeLa cells.

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