Inducible RNA Interference-Mediated Gene Silencing Using Nanostructured Gene Delivery Arrays

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ABSTRACT RNA interference (RNAi) has become a powerful biological tool over the past decade. In this study, a tetracycline-inducible small hairpin RNA (shRNA) vector system was designed for silencing cyan fluorescent protein (CFP) expression and delivered alongside the *yfp* marker gene into Chinese hamster ovary cells using impalefection on spatially indexed vertically aligned carbon nanofiber arrays (VACNFs). The VACNF architecture provided simultaneous delivery of multiple genes, subsequent adherence and proliferation of interfaced cells, and repeated monitoring of single cells over time. Following impalefection and tetracycline induction, 53.1% ± 10.4% of impalefected cells were fully silenced by the inducible CFP-silencing shRNA vector. Additionally, efficient CFP silencing was observed in single cells among a population of cells that remained CFP-expressing. This effective transient expression system enables rapid analysis of gene-silencing effects using RNAi in single cells and cell populations.

KEYWORDS: RNA interference · shRNA · nanostructures · nanofibers · gene silencing · vertically aligned carbon nanofibers · DNA delivery · tetracycline induction · single cell expression · CFP · YFP

Gene delivery to mammalian cells has become a staple of biological studies in the last few decades. A number of nanostructured platforms have been shown to effectively deliver plasmid DNA into mammalian cells, including nanofibers,[1,2] nanotubes,[3,4] nanorods,[5] and nanoparticles.[6,7] Recent demonstrations have shown that vertically aligned carbon nanofiber arrays (VACNFs) grown on a silicon substrate can be used as a parallel gene delivery platform in mammalian cells.[1] In this process, termed impalefection, plasmid DNA is spotted or covalently immobilized on the nanoscale carbon fibers. Subsequently, mammalian cells are impaled onto the VACNFs, recover, and express the introduced genes. During impalefection, single mammalian cells attach and proliferate on the nanostructured platform and can be tracked using spatial indexing of VACNF arrays.[2] Highly parallel introduction of DNA directly into mammalian cells and facile monitoring of the gene expression within individual cells over time are distinct advantages of impalefection when compared with traditional transfection methods. Thus, the VACNF platform also allows for rapid and efficient transient assays of gene expression while tracking individual cells over time. Additionally, DNA immobilized on VACNFs may minimize the potential for incorporation of foreign genes into the chromosomes of manipulated cells.[1,8]

Coupled with nucleotide delivery methods, RNA interference (RNAi) has become a powerful tool for controlling gene expression in many different cell types. First discovered in *Caenorhabditis elegans*, the RNAi pathway is an evolutionarily conserved cellular mechanism that specifically down-regulates gene products post-transcriptionally.[9] The pathway has now been identified in most eukaryotes.[10] The RNAi pathway has been extensively studied and well reviewed in the literature.[11–13] RNAi targets long double-stranded RNAs (dsRNAs), which are cleaved into ~21−24 base pair small-interfering RNAs (siRNAs) by the RNase III enzyme Dicer.[14] The siRNAs are coupled with an enzyme complex referred to as the RNA-induced silencing complex (RISC)[15] and effectively target the RISC complex to complementary mRNA transcripts, which are cleaved and degraded by a process called post-transcriptional gene silencing (PTGS).[16] Although the major function of RNAi seems to be as a viral defense mechanism, siRNAs may also silence transposable elements and repetitive genes to help stabilize the genome.[17] More recently, the RNAi pathway has been exploited for therapeutic approaches in human diseases,[18] including age-related macular degeneration.[19,20]

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Received for review August 28, 2007 and accepted November 29, 2007.

Published online December 27, 2007.
10.1021/nn700198y CCC: $40.75

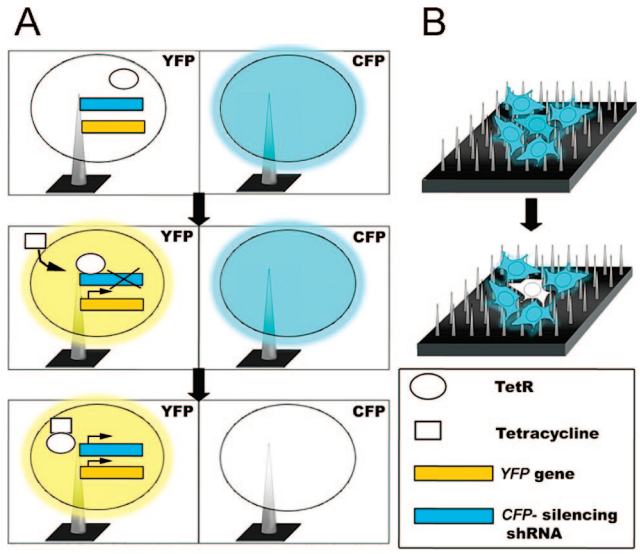

Figure 1. Schematic of the VACNF single-cell gene-silencing platform: (A) Nanofiber-mediated impalefection was used to deliver the *yfp* gene and CFP-silencing shRNA sequence into CHO-K1 cells that constitutively express the *cfp* and *tetR* genes (CHO-K1−CFP-TetR). TetR represses the expression of the CFP-silencing shRNA in the absence of tetracycline. Once tetracycline is added to the system, it binds TetR, allowing expression of the shRNA and induced silencing of CFP expression. Expression of the *yfp* gene allows for direct observation of impalefected cells. (B) This VACNF platform allows for monitored silencing in single cells among a nonsilenced cell population.

Introducing long dsRNAs into mammalian cells can cause a cytotoxic reaction by triggering a type-I interferon response and shutting down genome-wide translation, resulting in cell death.[21,22] The interferon response can be avoided by introducing siRNAs directly to the cells, resulting in efficient silencing of the target genes.[23,24] Additionally, DNA vectors expressing siRNA sequences as short inverted repeats (termed short hairpin RNAs, or shRNAs) have been developed and shown to stably down-regulate gene expression of targeted genes.[25] These shRNA vectors include designs for constitutive and inducible gene silencing in mammalian cells.[26,27] For inducible shRNA expression, a tetracycline induction system is most commonly used[28–31] and is well reviewed in the literature.[32,33]

For tetracycline inducible expression, the RNA polymerase III promoter for the histone 1 (H1) gene can be altered to contain two Tet operator ($tetO_2$) sites and used to express the shRNA sequence. $TetO_2$ sites bind the tetracycline repressor protein (TetR), which represses the transcription of downstream shRNA sequences. However, when tetracycline is administered to the cells, it binds tightly to TetR generating a conformational change that displaces TetR from $tetO_2$ sites. With TetR gone, the H1 promoter is no longer repressed, allowing transcription of the shRNA sequence. This results in generation of "induced" siRNAs that can specifically silence a targeted gene.

Conventional tetracycline-inducible approaches involve generating stable cell lines that express one or more elements of the induction pathway. In addition to a cell line expressing the protein to be silenced, subclones of this line transgenically expressing the TetR protein and the shRNA construct are needed. For negative controls, additional lines expressing nonsense or mismatched shRNA elements are prepared. Positive controls include cells that do not express the repressor protein. The generation and screening of these stable cell lines is not a trivial process, requiring significant time (4+ weeks) and effort prior to evaluating the effects of any inducible RNAi on a cell system.

In this study, we investigated the application of VACNF arrays as a platform for the rapid assay of tetracycline-inducible RNAi-mediated gene silencing using the cyan fluorescent protein (*cfp*) gene expressed in Chinese hamster ovary (CHO-K1) cells. Using the parallel gene-delivery capabilities of VACNFs, the yellow fluorescent protein (*yfp*) gene was simultaneously delivered with an inducible shRNA sequence to allow observation of successfully impalefected cells (Figure 1). Using the VACNF platform for transient assays of gene expression and silencing, we followed both nanofiber-mediated gene delivery and tetracycline induction of gene silencing within individual CHO-K1 cells. Cells were tracked using spatially indexed patterns on VACNF arrays. This is the first demonstration of cotransfection of multiple DNA vectors using carbon nanofibers as a delivery tool, as well as the first manipulation of shRNA-mediated gene silencing on nanostructured architectures.

RESULTS

CFP Temporal Silencing in CHO-K1 Cell Populations by Tetracycline-Inducible shRNAs. Multiple DNA vectors and cell lines were constructed to investigate the tetracycline-inducible gene-silencing capacity of shRNAs against the cyan fluorescent protein (CFP) mRNA in CHO-K1 cells (Table 1). The shRNA sequence had high specificity for CFP and did not silence YFP or GFP. This specificity is due to a two base pair difference within the targeted sequence of these fluorescent variants (data not shown) and is important because the *yfp* gene was used later in this study as a marker alongside the CFP-silencing shRNA vector (pCFPQuiet). The specificity of the pCFPQuiet vector in silencing CFP was also tested by comparing it to pLacZQuiet, a shRNA vector targeting the *lacZ* gene sequence. pCFPQuiet significantly decreased CFP expression levels when transfected into CHO-K1 cells, while pLacZQuiet did not (data not shown).

Next, the inducibility of pCFPQuiet was determined. Figure 2A,B show that pCFPQuiet is induced in CHO-K1 cells (CHO-K1-CFP-shRNA-TetR) using tetracycline (1 μg/mL) and efficiently silences CFP over time. Data were quantified using multilabel counting and inverted fluorescence microscopy. At 24 h, approximately 89.8% of the CFP fluorescence was silenced when compared with the negative controls. When tetracycline was re-

TABLE 1. DNA Vectors and Cell Lines Used in This Study

vector/cell name	characteristics	source
pd2EYFP-N1	mammalian expression vector containing the destabilized yellow variant (d2EYFP) of the enhanced *gfp* gene, constitutive CMV promoter, contains a neomycin G418 resistance gene and pUC origin and Kan resistance for *E. coli*	BD Biosciences (discontinued)
pd2ECFP-N1	mammalian expression vector containing the destabilized cyan variant (d2ECFP) of the enhanced *gfp* gene, constitutive CMV promoter, contains a neomycin G418 resistance gene and pUC origin and Kan resistance for *E. coli*	BD Biosciences (discontinued)
pENTR/H1/TO	mammalian vector for shRNA expression, inducible polymerase III H1/TO promoter, contains the Zeocin resistance gene and pUC origin and Kan resistance for *E. coli*	Invitrogen
pcDNA6/TR	mammalian expression vector containing the *tetR* gene, constitutive CMV promoter, contains the blasticidin resistance gene and pUC origin and Amp resistance for *E. coli*	Invitrogen
pCFPQuiet	pENTR/H1/TO vector harboring a 50 bp sequence from the *cfp* gene downstream of the H1/TO promoter	this study
pLacZQuiet	pENTR/H1/TO vector harboring a 50 bp sequence from the *lacZ* gene downstream of the H1/TO promoter	this study
CHO-K1	derived as a subclone from the parental CHO cell line initiated from a biopsy of an ovary of an adult Chinese hamster ATCC# CCL-61	ATCC
CHO-K1-CFP	CHO-K1 with stable chromosomal insertion of *cfp* gene from pd2ECFP-N1 vector; phenotype is fluorescent cyan color	this study
CHO-K1-CFPshRNA	CHO-K1-CFP with stable chromosomal insertion of CFP-silencing shRNA sequence from pCFPQuiet; phenotype is nonfluorescent	this study
CHO-K1-CFPTetR	CHO-K1-CFP with stable chromosomal insertion of *tetR* gene from pcDNA6/TR vector; phenotype is fluorescent cyan color	this study
CHO-K1-CFPshRNA- TetR	CHO-K1-CFP-shRNA with stable chromosomal insertion of *tetR* gene from pcDNA6/TR vector; phenotype is fluorescent cyan color in the absence of tetracycline	this study
CHO-K1-CFPLacZshRNA	CHO-K1-CFP with stable chromosomal insertion of pLacZQuiet vector; phenotype is fluorescent cyan color	this study

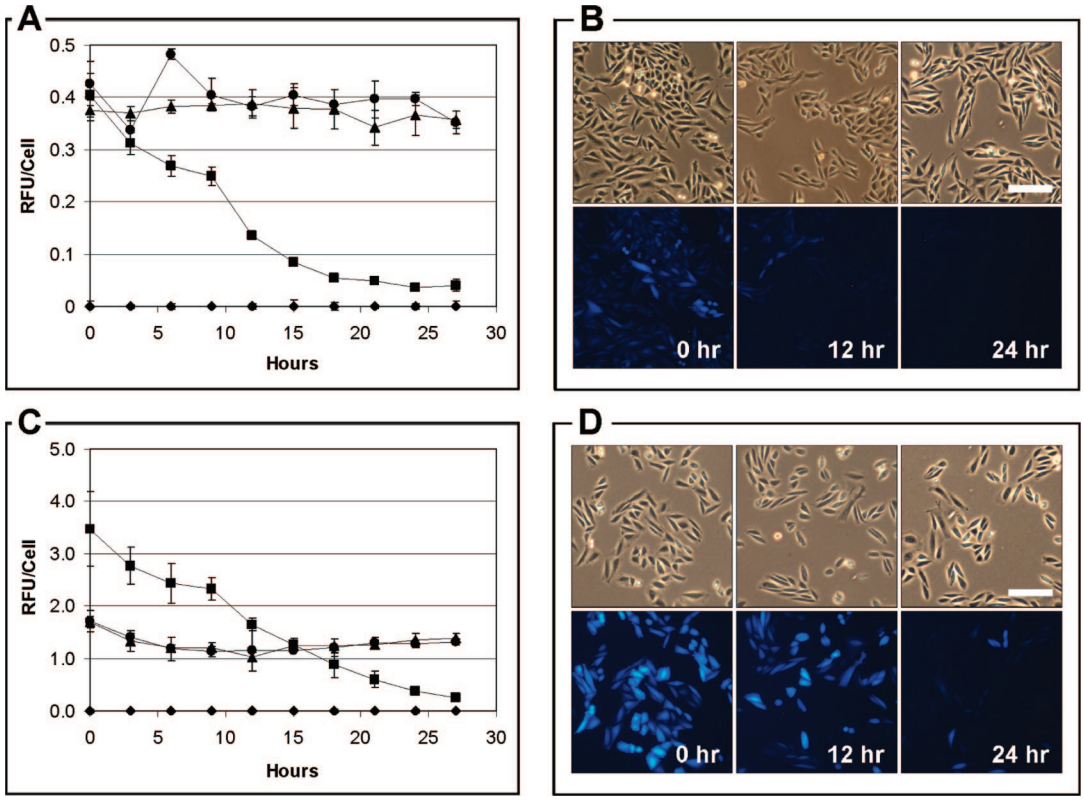

Figure 2. Tetracycline induction of silencing in CHO-K1 cells over time in the absence (A, B) or presence (C, D) of sodium butyrate (NaB). Cell lines used are represented as CHO-K1-CFP-TetR-shRNA (■), CHO-K1-CFP-TetR (▲), CHO-K1-CFP (●), and CHO-K1-CFP-shRNA (◆) in A and C. The average was determined from five individual replicates at each time point. RFU = relative fluorescence units. Standard deviation is shown in bars. Measurements were normalized as described in the text. Fluorescent micrographs of CHO-K1-CFP-TetR-shRNA cells in the absence (B) or presence (D) of NaB are also shown at 0, 12, and 24 h time intervals. Bar represents 100 μm.

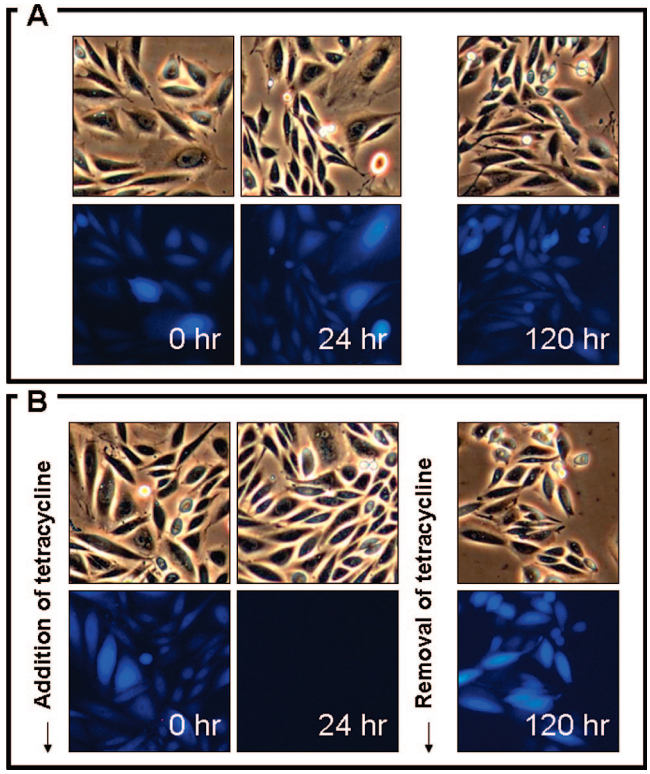

Figure 3. Addition and removal of tetracycline to CHO-K1-CFP-TetR-shRNA cells over time: (A) CHO-K1-CFP-TetR-shRNA cells in the absence of tetracycline over time; (B) CHO-K1-CFP-TetR-shRNA cells in the presence of tetracycline. Tetracycline (1 μg/mL) was added at time 0 h, and cells were monitored for CFP silencing. Once CFP silencing was fully induced at 24 h, tetracycline was removed from the media and cells were allowed to recover CFP expression. Expression was not fully recovered until time 120 h, 96 h after tetracycline had been removed from the media.

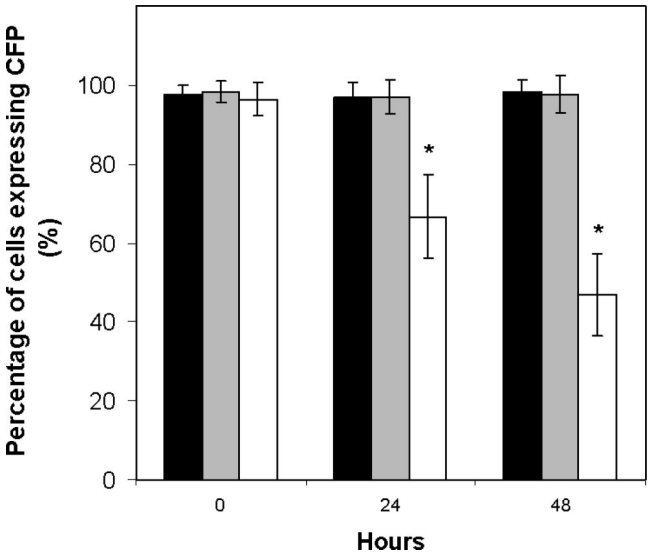

Figure 4. Silencing of VACNF-impalefected cells after tetracycline induction over time. CHO-K1-CFP-TetR cells were impalefected with pd2EYFP-N1 (black), pd2EYFP-N1 and pLacZQuiet (gray), or pd2EYFP-N1 and pCFPQuiet (white). Methods used for calculating cell counts are described in the discussion. The average was determined from ten individual replicates at each time point. Standard deviation is shown in bars. The * denotes statistical significance ($p<0.05$).

moved, CFP expression did not return to wild-type levels again until an additional 120 h after tetracycline removal, which suggests a high level of potency of the siRNAs cleaved from the shRNAs (Figure 3).

In addition, tetracycline induction of shRNA-mediated silencing was carried out in the presence of sodium butyrate (NaB). NaB is a short-chain fatty acid that has been shown to inhibit histone deacetylase activity and can result in enhancement of specific gene expression in CHO-K1 cells,[34] including genes under the control of the cytomegalovirus immediate early (CMV IE) promoter.[35] In this study, NaB was used to enhance the expression of the *cfp* gene on VACNF arrays and to increase the longevity of transient expression after transfection in mammalian cells.[36] NaB temporarily enhanced and prolonged transgene expression resulting in a 4−8-fold increase in CFP fluorescence but did not affect the time constants of CFP silencing (Figure 2C and D). Therefore, NaB was used during all subsequent impalefection experiments.

Silencing of CFP by Tetracycline-Inducible shRNAs in Cell Populations on VACNF Arrays. To examine the use of VACNF arrays as a platform for regulated expression of CFP-silencing shRNAs, pCFPQuiet was impalefected into CHO-K1-CFP-TetR cells, along with pd2EYFP-N1, a vector containing the destabilized enhanced yellow fluorescent protein (*yfp*) gene; *yfp* was used as a marker gene to designate which cells were successfully impaled and received DNA. Codelivery of more than one DNA vector using impalefection had never been addressed prior to this study; thus the optimal DNA concentration of coexpression after impalement was determined. Gene expression was studied by codelivery of either 10 or 100 ng of pd2EYFP-N1 and varying amounts (0.1, 1, 10, and 100 ng) of pd2ECFP-N1. Wild-type CHO-K1 cells were impalefected with both plasmids and counted using inverted fluorescence microscopy. In agreement with previous studies,[1] the impalefected cells successfully recovered within hours and remained viable for multiple days after the initial impalefection experiment. By comparing the number of cells expressing one or both genes to the total number of impalefected cells, we established the optimal concentration as 100 ng of each vector, resulting in 76.7% ± 3.9% codelivery of both genes (*yfp* and *cfp*) (data not shown). This was significantly higher than the cotransfection rate using 10 ng of each DNA plasmid (61.87% ± 10.80%).

While successful impalefections have been seen with DNA concentrations as low as 20 pg and some successful multiplasmid impalefections were seen with DNA concentrations as low as 1 ng (data not shown), our current study shows that higher DNA concentrations will significantly improve the proportion of multiple-vector impalefected cells when using VACNFs. The optimized DNA concentration of 100 ng each was used for codelivery of pCFPQuiet and pd2EYFP-N1 into

CHO-K1-CFP-TetR cells, containing the stably inserted *cfp* and *tetR* genes. Ten replicates of VACNF array chips (3 mm × 3 mm) were used for each variable. pCFPQuiet and pd2EYFP-N1 were impalefected into the cells. pLacZQuiet was used as a negative control. After 24 h of recovery, tetracycline was added, and fluorescent cell counts began (time = 0 h). Significant levels of silencing (53.1% ± 10.4%) were observed 48 h after addition of tetracycline (Figure 4). These data show that VACNF arrays can be used for highly efficient simultaneous delivery of multiple DNA plasmids to mammalian cells. Notably, these levels were not as substantial compared with the results from the multilabel counting method used in Figure 2A,C. This is due to the counting methods used. Using multilabel counting, the estimates of shRNA-mediated silencing were based upon whole population fluorescence measurements. In this experiment, each chip expressing YFP was counted, and then each cell was observed for CFP expression. This method results in a conservative estimate of shRNA-mediated silencing, because a number of the cells exhibiting low-level silencing effects and lower levels of CFP expression may still be counted as CFP positive. In addition, some cells were still expressing DNA up to 7 days after impalefection, which is longer than a typical transient transfection will last, suggesting stable chromosome insertions. Importantly, these data show that inducible shRNA vectors can be incorporated into VACNF-mediated gene delivery systems.

Gene Silencing by Tetracycline-Inducible shRNAs Monitored in a Single Cell on Indexed VACNF Arrays. We additionally investigated whether tetracycline-inducible shRNA regulation of CFP expression could be monitored in single cells over time. Using indexed VACNF arrays, CHO-K1-CFP-TetR cells were impalefected with pCFPQuiet and pd2EYFP-N1 (10 ng of each) using VACNF arrays with nanofibers at a 20 μm pitch and an XY indexing pattern. Controls were performed by impalefecting CHO-K1-CFP-shRNA-TetR with 10 ng each of pCFPQuiet and pd2EYFP-N1 (positive control) and CHO-K1-CFP-TetR with 10 ng of pd2EYFP-N1 (negative control). After 24 h of recovery, tetracycline was added (time = 0 h) and at least 50 individual cell trajectories were photographed and tracked every 24 h. As expected, single CHO-K1-CFP-TetR cells expressing the shRNA and *yfp* marker gene were silenced for CFP expression at 24 and 48 h, while the same cells expressing only the *yfp* marker gene were not (Figure 5). Figure 5 shows representative images where virtually every CHO-K1-CFP-shRNA-TetR cell was silenced at 24 and 48 h. Cells appear at different positions in each image, because CHO-K1 cells adhere to the surface of the substrate but continue to move over time. The relatively long time interval between pictures and the small plane of view (100 μm × 100 μm) give the impression that the cells are moving large distances. Some cells are lost during handling of the VACNF chips between incubators and the microscope. Currently, VACNF array chips are incubated inverted in round-bottom 96-well plates and require transfer to flat-bottom wells for microscopic observation. Due to the high surface area of the carbon nanofibers and poor diffusion of media, the cells cannot survive on VACNF array chips inverted in flat-bottom wells. Efforts are underway to develop better substrates for cell culture and microscopic observations.

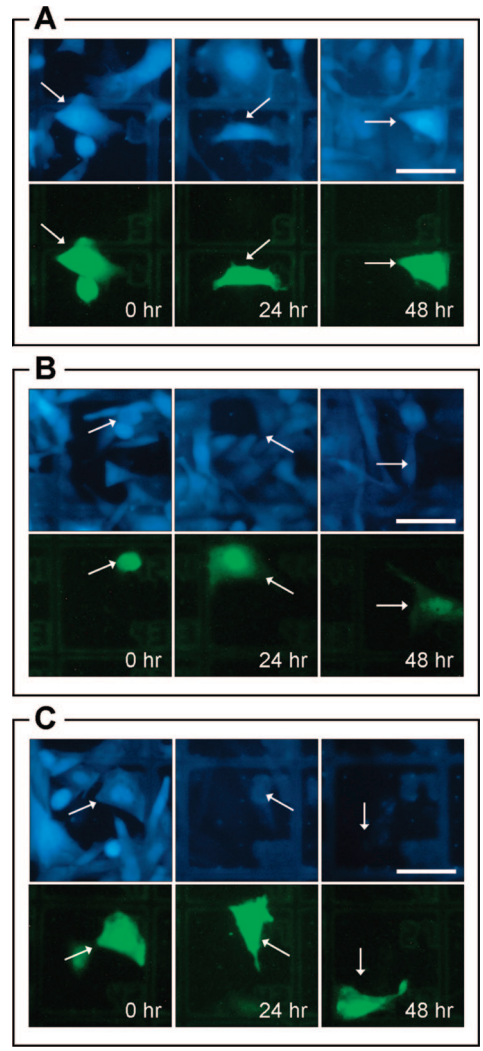

Figure 5. Tetracycline induction and tracking of YFP expression and shRNA-mediated silencing of CFP expression in single impalefected cells on indexed VACNFs over time. CHO-K1-CFP-TetR cells were impalefected with (A) pd2EYFP-N1, (B) pd2EYFP-N1 and pCFPQuiet and (C) CHO-K1-CFP-TetR-shRNA cells were impalefected with pd2EYFP-N1. Tetracycline (1 μg/mL) was added at time 0 h, and cells were monitored for CFP silencing at 0, 24, and 48 h. White arrows denote the original CFP-expressing cell, and subsequent silencing of CFP expression. Bar represents 50 μm.

This study is the first to establish the ability with VACNFs to simultaneously introduce multiple foreign genes into a single cell among a population of cells and track the exogenously controlled expression of genes over time within a 72 h period. This contrasts with other studies that are limited to observing phenotypic changes in a single cell over time or a few studies that

observed phenotypic changes in a single cell among a population of genotypically different cells over time.[37] Additionally, impalefection is a rapid method to generate and track stably transfected cells *en masse*. Using traditional methods, the generation of stable cell lines alone would require a minimum of 10–14 days. While microinjection could be used for this same approach, it would be much more tedious to microinject multiple cells to be tracked over time. In the current impalefection study, approximately 25–30 discrete single cell trajectories were tracked for each VACNF chip (data not shown).

We are currently investigating observations that *yfp* gene expression occurs as early as 75 min after impalefection in CHO-K1 cells. This is remarkable when one considers that the maturation time for wild-type GFP is ~3 h at room temperature.[38] Even when newer fluorescent protein genes with shorter maturation times are considered,[39] our data suggest that the introduction and expression of the *yfp* gene in our system is extremely rapid and efficient. We hypothesize that the impalefection process delivers high gene copy numbers directly to the nucleus, similar to microinjection, allowing for nearly immediate transcription and translation. This may be the fastest method available for assaying gene silencing and could be an important advancement for assays using siRNAs or micro RNAs (miRNA) expressed from shRNA or similar vectors. Other transfection methods have significantly longer periods of time prior to gene expression, due to several factors including the need for internalization of the DNA, localization to the nuclear membrane, decomplexation of the DNA from its carrier, and nuclear import, some or all of which must occur prior to the initiation of transcription. In contrast, we have shown that the fast transcriptional initiation of impalefection, along with the specific tracking of individual cells and longevity of transient expression in the presence of NaB, allows for very rapid and persistent assays of regulated gene expression in mammalian cells without the generation of stable clones. Additionally, preliminary data suggest that impalefection can be applied to a diverse range of cell types with the same degree of efficiency as CHO-K1, including HEK-293, a cell line commonly used for RNAi experimentation (data not shown).

CONCLUSIONS

In this study, the specific target gene suppression of the RNAi pathway was combined with the gene delivery platform of VACNFs to investigate the silencing efficacy in nanostructure-immobilized and spatially indexed cell culture. An inducible shRNA vector (pCFP-Quiet) was designed to efficiently knockdown the expression of a stably expressed reporter gene (*cfp*) in Chinese hamster ovary cells. VACNF arrays provided a mechanism for extremely rapid expression, silencing, and tracking in single cells of interest. The delivery of inducible shRNA vectors to mammalian cells on indexed VACNF arrays provides the ability to not only track specific gene delivery events in discrete single cells over extended periods of time without constant observation under the microscope but also control the expression of the delivered genes by turning them "on or off" using tetracycline induction among a population of cells that remain uninduced. This VACNF-mediated technique can be used for a number of applications as nanoscale probes that provide for gene delivery and modulation of gene expression in a single cell to overcome limitations and help address new types of experimental questions in many fields of study.

METHODS

Plasmid Construction and Maintenance. Strains and plasmids used in this study are listed in Table 1. Plasmids were maintained in *Escherichia coli* strain JM109 and stored at −80 °C. *E. coli* strain JM109 was grown in Luria–Bertani (LB) broth at 37 °C with or without ampicillin (100 μg/mL) or kanamycin (50 μg/mL), depending on the requirements for plasmid maintenance. Plasmids were transformed into chemically competent *E. coli* strain JM109. Briefly, 2–4 μL of plasmid DNA (~100 ng/μL) was added to 100 μL of freshly thawed chemically competent *E. coli* strain JM109 cells on ice. After 30 min, the cells were heat shocked at 42 °C for 45 s and immediately placed on ice for 2 min. Following transformation, 1 mL of 2XYT medium was added, and cells were allowed to recover at 37 °C with shaking at 200 rpm for at least 1 h. Cells were then plated on LB-agar plates with ampicillin or kanamycin, and colonies were selected and screened for the correct insert 18–20 h later.

Plasmid isolation was performed using Wizard mini- or midiprep kits (Promega, Madison, WI). The expression vectors pd2EYFP-N1 containing destabilized enhanced yellow fluorescent protein (*yfp*) and pd2ECFP-N1 containing destabilized enhanced cyan fluorescent protein (*cfp*) were purchased from Clontech (Mountain View, CA). The expression vector for the *tetR* gene (pcDNA6/TR) was purchased from Invitrogen (Carlsbad, CA). pCFPQuiet was constructed using the pENTR/H1/TO vector from Invitrogen. Using the BLOCK-iT Inducible H1 RNAi Entry Vector Kit from Invitrogen, a 50-bp oligonucleotide (5′-CACCGCATCAAGGCCAACTTCAAGACGAATCTTGAAGTTGGCCTTGATGC-3′) containing a complementary sequence to the *cfp* gene from pd2ECFP-N1 was ligated into pENTR/H1/TO and resulted in the pCFPQuiet expression vector. This DNA sequence was selected using the Invitrogen BLOCK-iT RNAi Designer Web site (www.invitrogen.com/rnaidesigner). In addition to the shRNA sequence specific for the *cfp* gene sequence, the pCFP-Quiet vector also contains the Zeocin resistance gene for mammalian selection. A negative control expression vector (pLacZ-Quiet) was constructed from the pENTR/H1/TO vector that contained a 49-bp oligonucleotide (5′-CACCAAATCGCTGATTTGTGTAGTCGGAGACGACTACACAAATCAGCGA-3′) containing a complementary sequence to the *lacZ* gene. This DNA sequence was included in the BLOCK-iT Inducible H1 RNAi Entry Vector Kit. All constructed vectors were confirmed for the correct DNA sequence using M13 forward and reverse primers. DNA sequencing was performed with an ABI Big Dye Terminator cycle sequencing reaction kit on an ABI 3100 DNA sequencer (Perkin-Elmer, Inc., Foster City, CA) at UT-MBRF (Knoxville, TN).

Cell Culture and Reagents. Chinese hamster ovary (CHO-K1BH4) cells were maintained in Ham's F-12 (Gibco-Invitrogen) and Ham's F-12K (ATCC, Manassas, VA) media supplemented with 5% fetal bovine serum (FBS), 100 μg/mL streptomycin, 100 U/mL

penicillin, and 250 ng/mL amphotericin B under an atmosphere of 5% CO_2 at a temperature of 37 °C. Cells were grown to confluence and passed every 4–5 days by trypsinization at a ratio of 1:9. The selection agents blasticidin-s (5 μg/mL) and Zeocin (300 μg/mL) (Invitrogen, Carlsbad, CA) were used on appropriate cell lines. Sodium butyrate was used in the media at a final concentration of 2 mM where mentioned below.

Cell Transfection and Generation of Stable Cell Lines. Cells grown in 35 mm wells were transfected with 1 μg of the pd2ECFP-N1 expression vector. All cell transfections were achieved using the Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) in accordance with the manufacturer's instructions. The cells were then cultured in the presence of G418 sulfate (EMD Biosciences Inc., San Diego, CA), and resistant colonies were passed after 14 days of treatment. Twenty separate single clones from the population were obtained through isolation by limiting dilution in 96 well plates. The cell lines from each clone were examined for CFP expression by fluorescent microscopy. The brightest fluorescent cell line was chosen (termed CHO-K1-CFP), and stocks were made and stored at −80 °C. CHO-K1-CFP was then used to create the cell line CHO-K1-CFP-TetR by transfecting the cells with 1 μg of pcDNA6/TR. The cells were cultured in the presence of 5 μg/mL blasticidin-S, and a single clone was isolated as described above. The cell line (CHO-K1-CFP-TetR) was examined for TetR expression by RT-PCR, and the results were positive (data not shown). CHO-K1-CFP-TetR was then used to create CHO-K1-CFP-TetR-CFPQuiet by transfecting the cells with 1 μg of pCFPQuiet. These cells were cultured in the presence of 5 μg/mL blasticidin-S and 300 μg/mL of Zeocin, and a single clone was isolated as described above. In similar fashion, the CHO-K1-CFP-CFPQuiet cell line was generated from CHO-K1-CFP. An additional cell line (CHO-K1-CFP-LacZQuiet) was generated as a negative control to CHO-K1-CFP-CFPQuiet.

Multilabel Counting Analysis and Tetracycline Induction. Cells were seeded at 1.0×10^5 in 6-well plates and allowed to attach overnight at 37 °C in an incubator with atmosphere of 5% CO_2. After 24 h of growth, tetracycline was added to cells every 3 h for a 27 h time period. At 27 h, all wells were thoroughly washed with PBS three times, and 200 μL of Cell Stripper (Mediatech Inc., Herndon, VA) was added to each well and allowed to incubate for 30–35 min. After dissociation, cells were resuspended and pipetted into each well of a 96-well flat-bottom plate. The plate was covered with a Breathe-Easy sealing membrane (Fisher Scientific Co. L.L.C., Pittsburgh, PA) and centrifuged at 400 × *g* for 15–20 s.

The multiwell plates were assayed as previously described with slight modifications.[40] Briefly, 96-well black clear-bottom plates were read using a Wallac Victor^2 1420 Multilabel Counter (Perkin-Elmer Life Sciences, Waltham, MA). Absorbance was measured at 450 nm for 1.0 s, followed by a fluorescence reading using a CFP filter set with an excitation of 436 nm and emission of 480 nm (Chroma Technology Corp., Rockingham, VT). Readings were taken 8 mm from the bottom of the plates with a 0.5 s measuring time. The lamp control was on a stabilized energy setting, and all reads were obtained at 35 000 V of lamp energy.

The relative fluorescence units described in this study were determined as follows: Fluorescence in each cell line could not be measured on the plate reader from monolayers at optimal levels of confluence (60–80%) due to the low level of CFP fluorescence. Likewise, the cell fluorescence could not accurately be determined at higher levels of confluency (~100%), due to changes in cellular expression that resulted in lower fluorescence of the stably inserted *cfp*. Due to these limitations and variations, cells were maintained at 50–60% confluence in each well for the tetracycline induction experiments and prepared as described above. Standard curves of cell number versus absorbance were generated for each individual cell line between 1×10^4 and 1×10^6 cells, and the slope from each of these standard curves was used to extrapolate the estimated number of cells in each well based on the absorbance measured. Additionally, the fluorescence was measured for each well and normalized using the absorbance to determine the estimated fluorescence per cell, measured in relative fluorescent units (RFU).

Fluorescence Microscopy and Cell Counting. Cells were observed and counted on chips using a Nikon Diaphot 300 or Nikon Eclipse TE300 inverted fluorescent microscope, depending on the experiment. Filter sets on both microscopes for CFP (436 nm excitation, 480 nm emission) and YFP (500 nm excitation, 535 nm emission) were purchased from Chroma Technology Corp. (Rockingham, VT; Cat. No. 31044v2 and 41028). Digital imaging was performed using a MicroPublisher 3.3 CCD camera integrated with QCapture 2.60 imaging software (QImaging Corp., Surrey, BC, Canada), and a Nikon Coolpix 4500 digital camera with a Scopetronix MaxView Plus attachment on the microscope eyepiece lens. For the QImaging system, all images of CFP were obtained using an exposure time of 5 s. Bright field images were obtained using an exposure time of 6.49 ms. For the Nikon Coolpix system, all images of CFP were obtained using an exposure time of 2 s. Images of YFP were obtained using exposure times between 2 and 8 s, due to the varying levels of YFP fluorescence expressed by the cells. This variation did not affect the statistical data of CFP expression, since YFP was only used as a biomarker of gene delivery. For population counting of tetracycline induction of shRNA silencing in CHO-K1-CFP-TetR cells, ten replicate VACNF array chips were used for each variable. For each chip, four images were taken, and the total YFP-expressing cells were counted by hand. Each YFP-expressing cell was then confirmed for the presence or absence of CFP. The presence of CFP was defined as any fluorescence that could be detected in the CFP filter image. At minimum, 250 cells were counted for each variable.

Nanofiber-Mediated Impalefection. Cells were trypsinized and resuspended in 5 mL of Ham's F12K media. Cells were centrifuged at 300 × *g* for 10 min. The media was aspirated, and the cell pellet was resuspended in 0.2–1.0 mL, depending on the size of the pellet and the specific application. For each test sample, 100 μL of the cell suspension was allowed to settle for 5 min on a 5 mm × 5 mm pad of clean room wipes (Durx 770, Berkshire Corp) in multiple wells of a 96-well plate. Once the cells were settled, the nanofiber chip was slowly lowered face down and lightly placed on top of the settled cells. The chip was then sharply tapped with flame-sterilized tweezers and placed face down in 200 μL of Ham's F12K with 2 mM sodium butyrate in a round-bottom well of a 96-well plate. The 96-well plate was placed in an incubator at 37 °C with 5% CO_2 and allowed to recover for 24 h.

Statistical Analysis. All data were analyzed using Microsoft Excel. The student *t* test was used to determine statistical significance.

Nanofiber Array Synthesis. Four inch silicon wafers (100) were photolithographically patterned with 50 nm thick Ni thin films as discrete 500 nm diameter dots at either 5, 10, or 20 μm pitch over the entire surface of the wafer. Nanofibers were synthesized in a custom built DC-PECVD reactor at a temperature of 650 °C, 10 torr, 2 A, using a mixture of a carbonaceous source gas (acetylene) and an etch gas (ammonia). Growth time was selected to provide fibers ranging from approximately 10 to 17 μm tall with tip diameters of approximately 100 nm. Spatially indexed arrays were subsequently spun with a 2 μm thick layer of SU-8 2002 (Microchem Corp., Newton, MA) and patterned with a grid and numerical indexing using contact photolithography and development (SU-8 Developer, Microchem). Following growth and index patterning, wafers were spun in a protective layer of photoresist (Microposit SPR220 CM 7.0, Shipley Corp., Marlborough, MA) and diced into 2.2 mm square pieces. Following dicing, the protective photoresist was removed by soaking in Microposit Remover 1165 (Shipley), followed by copious rinsing in water.

Acknowledgment. The authors are grateful to T. Subich, D. Hensley, D. Thomas, S. Randolph, and P. Fleming for nanofiber fabrication. We thank S. Ripp and J. Fleming for valuable input in editing the manuscript. This study was supported in part by Grant R01EB006316 (NIBIB) and by the Material Sciences and Engineering Division Program of the DOE Office of Science (Grant DE-AC05-00OR22725) with UT-Battelle, LLC, and through the Laboratory Directed Research and Development funding program of the Oak Ridge National Laboratory, which is managed for the U.S. Department of Energy by UT-Battelle, LLC. M.L.S. acknowledges support from the Material Sciences and Engineering Division Program of the DOE Office of Science. A portion of this research was conducted at the Center for Nanophase Materials Sciences, which is sponsored at Oak Ridge National Laboratory by the Division of Scientific User Facilities (DOE).

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