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The construction of plasmids for expressing an siRNA resistant human cofilin

By Imarhia Enogieru, Alisa E. Shaw, O'Neil Wiggan and James R. Bamburg
Harriet L. Wilkes Honors College of Florida Atlantic University and Colorado State University

Abstract

Human cofilin is an essential 19 kDa actin assembly regulatory protein that enhances actin dynamics through depolymerization and severing of filamentous actin.[1] In the cell, it plays a major role in processes such as cytokinesis and cell migration.[2] When cofilin expression is silenced in HeLa cells using siRNA, cellular defects occur. Rescuing normal cell function by expressing cofilin from a mRNA that is resistant to the silencing RNA provides supporting evidence that the cellular defects are the result of cofilin silencing and not off-target effects of the siRNA. To this end we constructed a plasmid for expressing siRNA resistant human cofilin mRNA. To construct the plasmid we inserted four mis-matched nucleotides into the part of the cofilin cDNA sequence targeted by siRNA. The base substitutions selected were silent mutations, meaning that the cofilin sequence became siRNA resistant without changing the amino acid sequence of the encoded cofilin. Oligonucleotides containing the modified cDNA sequence were synthesized and used as primers in a polymerase chain reaction (PCR)-based site-directed mutagenesis. After sequencing confirmed the desired silent mutations were inserted into the human cofilin cDNA, the cDNA was inserted into a mammalian expression plasmid and tested for expression. After expression of the mutant cofilin was verified, the siRNA resistance of the expressed cofilin mRNA was also confirmed, making it a useful reagent for cofilin silence and rescue experiments.

Introduction

Human cofilin is an essential actin binding protein that is responsible for directly severing actin filaments and enhancing turnover of actin.[1] Cofilin is required for many of the normal cellular functions which require dynamic actin assembly/disassembly such as maintaining cell shape and driving cytokinesis and cell migration.[2] Cofilin is activated by Serine 3 dephosphorylation.[3] Endogenous cofilin expression in HeLa cells can be silenced by small interfering RNA (siRNA). siRNAs are 21-23 nucleotide long double stranded RNA sequences that act as mediators in the process known as RNA interference.[4] siRNAs bind to target mRNA through complimentary sequence alignment and signal the formation of the RISC complex, which leads to the degradation of the mRNA and silencing of the gene.[5] siRNAs can be delivered directly through transfection or expressed as hairpin RNAs from an expression vector.[5] In order to demonstrate that the cellular defects arising from cofilin mRNA silencing were caused by the absence of cofilin protein and not off-target effects of the siRNA, it is necessary to rescue the defects by re-expressing cofilin. Previous studies have shown rescuing of a cofilin silencing phenotype in rat cells by expression of a cofilin isoform from a different species (Xenopus) but the ideal silence and rescue experiment would involve re-expressing the same isoform and species of cofilin that was silenced.[6] This requires having an siRNA resistant human cofilin expression plasmid available. To construct this plasmid, a human cofilin cDNA was mutated in the siRNA targeted region by inserting base mutations in the nucleotide sequence while maintaining the amino acid sequence (silent mutations). This mutant was tested for expression and siRNA resistance of its transcribed mRNA.

Methods and Results

Construction of Plasmid: Two versions of human cofilin cDNA were used. One version, called "tagged" had its translation stop codon mutated to encode a translatable linker region for later addition of red fluorescent protein (RFP). The other version called "untagged" has the normal stop codon. An siRNA resistant human cofilin oligonucleotide was designed to contain four silent mutations in which the bases were changed but the amino acids that they encode remained the same (Figure 1).

The oligonucleotides were obtained from Integrated DNA Technologies (IDT) and used as primers in a PCR-based site-directed mutagenesis using methods of the Stratagene QuikChange site-directed mutagenesis kit (catalog #200518). A portion of the PCR reaction was electrophoresed on a 1% agarose gel, stained with ethidium bromide, and the PCR reaction products with the brightest band from both the tagged and untagged vectors were selected for subsequent steps (Figure 2).

Another portion of the selected PCR reactions was transformed into DH5α competent E. coli that had been made competent for heat shock-induced transformation by

The siRNA target sequence of human cofilin:

A T F V K M L
CC ACC TTT GTC AAG ATG CT

The siRNA resistant sequence in human cofilin:

A T F V K M L
CG ACG TTT GTG AAA ATG CT

Figure 1. Wild type human cofilin cDNA and the altered bases creating an siRNA resistant cofilin cDNA. This diagram shows the four silent mutations introduced into the human cofilin cDNA sequence that is targeted by siRNA. The underlined nucleotides replaced the corresponding nucleotides in the siRNA target sequence of human cofilin. The amino acid sequence remained the same.

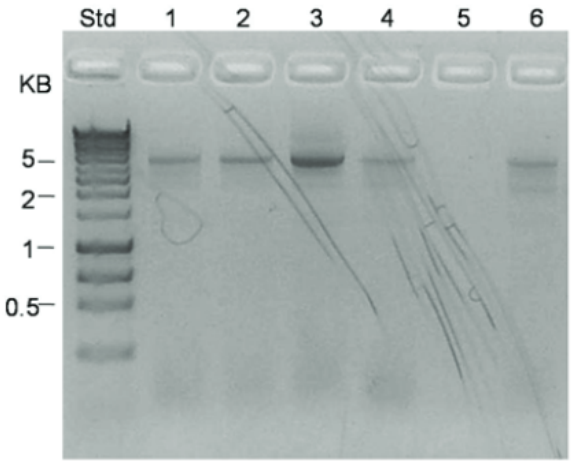

Figure 2. The PCR product from the site directed mutagenesis electrophoretically separated. Ethidium bromide stained agarose gel (1%) electrophoresis of the site directed mutagenesis PCR product. Lanes are as follows: 1) 40 ng untagged template, 2) 16 ng untagged template, 3) 40 ng untagged template with 1 mM $MgSO_4$, 4) 40 ng tagged template, 5) 16 ng tagged template, 6) 40 ng tagged template with 1 mM $MgSO_4$. Untagged cofilin cDNA in lane 3 and the tagged cofilin cDNA in lane 6 were used for the rest of the construction process.

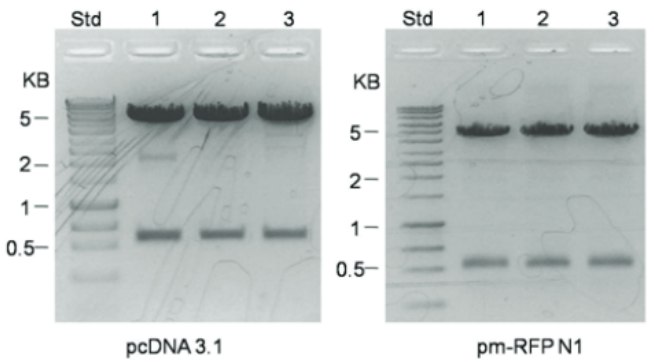

Figure 3. Confirmation of cofilin siRNA resistant mammalian plasmids by restriction enzyme digest. To determine if the ligation worked, a test digest using restriction enzymes that release the siRNA resistant human cofilin cDNAs from their respective mammalian expression plasmids was performed. The pcDNA3.1 test digest shows the release of untagged siRNA resistant human cofilin cDNA from clones 1-3. Human cofilin runs around 0.5 kb on the gel while the plasmid runs ~6 kb. The pmRFP-N1 test digest shows the released of the tagged siRNA resistant human cofilin cDNA from clones 1-3. The pmRFP-N1 plasmid runs at ~5 kb.

treatment with 0.1 M $CaCl_2$.[7] Four colonies from the plates of bacteria transformed with each type of cofilin were individually inoculated into solutions of Luria broth containing 0.05 µg/µL ampicillin, grown up overnight and the DNA plasmids isolated using GenElute® Mini Prep Kit (Sigma-Aldrich). Sequencing of each plasmid confirmed the siRNA resistant mutations had been inserted into the human cofilin cDNAs and that no other mutations occurred in the cofilin open reading frame. A single clone for each (tagged and untagged) was selected for use in subsequent steps.

Subcloning: The siRNA resistant human cofilin cDNAs were subcloned into mammalian expression plasmids with CMV promoters, which will drive strong, constitutive expression in HeLa cells. The tagged siRNA resistant human cofilin cDNA and pmRFP-N1 plasmid8 were digested with *Bam*HI and *Eco*RI whereas the untagged siRNA resistant human cofilin cDNA and pcDNA 3.1 plasmid (Invitrogen) were digested with *Hind*III and *Xba*I. The DNA was electrophoretically separated and purified from the agarose gel using GeneCleanIII® kit (MP Biomedical). The concentration of each sample of DNA was estimated from the ethidium bromide staining intensity.

The cofilin cDNAs were ligated into their respective mammalian expression plasmids with T4 ligase (New England Biolabs). Plasmids were transformed into DH5α bacteria, colonies were selected and plasmid preparations were performed as previously described. Test digests were performed on each of the plasmids using the restriction endonucleases described above for their subcloning. The test digest confirmed the siRNA resistant human cofilin cDNA were inserted into the mammalian expression plasmids (Figure 3).

Test for Expression: HeLa cells were grown to 70% confluence in high glucose Dulbecco's Minimal Eagle's Medium (HG-DMEM) containing 10% fetal bovine serum (FBS) and transfected with Lipofectamine (Invitrogen) in Opti-MEM (Invitrogen) medium for 4 hours at 37°C according to the manufacturer's directions. One culture was transfected without DNA (control), and two others were transfected with plasmids for expressing siRNA resistant cofilin either RFP-tagged or untagged. After the incubation, the medium was changed to HG-DMEM-10% FBS. Expression was checked 48 h after transfection using microscopy (for RFP expression) and western blots (for increased cofilin expression or RFP-cofilin expression). Fluorescence microscopy showed red fluorescence of cells transfected with RFP-tagged cofilin and no fluorescence of cells transfected with no DNA or cells transfected with untagged cofilin (Figure 4).

Cultures of the mock transfected and transfected HeLa cells were washed free of medium and lysed using SDS-lysis buffer made of 2% SDS, 10mM Tris pH 7.5, 10 mM NaF, 5 mM DTT, 2 mM EGTA. Cell extracts were heated in a boiling water bath for 3 minutes and the concentration of total protein in each sample was determined using a filter paper protein assay with ovalbumin as a standard.[9] Once concentrations were determined, volume of each sample that contained 6 µg of protein was loaded onto a 12.5% polyacrylamide gel for SDS-PAGE. The proteins were transferred to nitrocellulose and the western blot was probed by immunostaining for total cofilin (rabbit antibody 1439)[10] and GAPDH as a loading control (mouse anti-GAPDH antibody (Millipore)). Secondary antibodies were conjugated to 700 and 800 nm fluorescent dyes (Dylight, Thermo Scientific). The Odyssey Li-COR scanner was used to visualize cofilin and GAPDH, respectively (Figure 5).

The GAPDH blot showed that there was an equal amount of protein loaded on each well. Both the mock transfected and the untagged cofilin transfected cells contain cofilin immunoreactivity that appeared as a band slightly

below the 20 kDa marker. Quantification of the band intensity by densitometry of the digital scans normalized to the GAPDH loading control showed about a 25% increase in expression of cofilin in cells transfected with untagged cofilin over the mock transfected cells. It is expected to have an increase in cofilin expression in these cells because they are expressing both endogenous cofilin and the siRNA resistant human cofilin. The moderate 25% overexpression is most likely due to the low transfection efficiency. HeLa cells transfected with the RFP-tagged cofilin showed a cofilin immunoreactive band at about 48 kDa.

Test for siRNA-resistance: HeLa cells were transfected at the time of plating with double-stranded human cofilin siRNA oligonucleotides (target sequence CCACCTTTGTCAAGATGCT, Qiagen) using RNAiMAX (Invitrogen) following the manufacturer's directions. The day after this transfection, the cells were re-transfected again, this time using Lipofectamine 2000 (Invitrogen), with the human cofilin siRNA as well as the plasmid expressing siRNA resistant human cofilin mRNA. Two days after the second transfection, the cells were lysed and extracts were used for SDS-PAGE and western blotting as described in the previous section (Figure 6).

Mock transfected cells contained endogenous cofilin (band at ~19 kDa), which disappears when the cells are transfected with only cofilin siRNA. In cells treated with the cofilin siRNA and the plasmid for expressing untagged siRNA-resistant human cofilin, there is a ~19 kDa band present, indicating the siRNA resistant plasmid was expressing in the presence of the cofilin siRNA. In cells transfected with the cofilin siRNA and the RFP-tagged siRNA resistant human cofilin, there is a ~ 48 kDa band present that represents the chimeric cofilin-RFP protein. These results indicate that the four silent mutations in the siRNA targeted region of the human cofilin cDNAs are sufficient to allow expression of cofilin in the presence of cofilin siRNA.

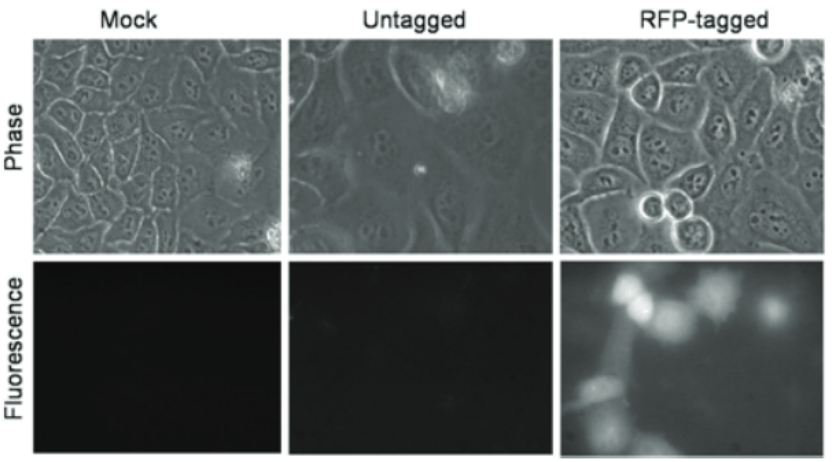

Figure 4. Fluorescent microscope images confirming RFP-tagged siRNA resistant human cofilin cDNA expression. HeLa cells transfected with different cofilin cDNA plasmids. Mock transfected cells were treated with reagents but no plasmid DNA. Untagged cells were transfected with siRNA resistant human cofilin in pcDNA3.1. RFP-tagged cells were transfected with siRNA resistant RFP-tagged human cofilin in pmRFP-N1. Both phase and fluorescent images were taken with a Nikon Diaphot microscope. As expected, fluorescence was only present in cells transfected with the RFP-tagged plasmid. Based upon the percentage of RFP positive cells, transfection efficiency was about 30%.

Discussion

The ability to silence specific genes using siRNA has led to major advances in understanding the specific roles of proteins in cell function. Cofilin, the major actin dynamizing protein in cells, has been implicated in many essential aspects of cell function, including cytokinesis and polarized cell migration.[2] However, cofilin has also been implicated in various neurodegenerative diseases. In Alzheimer's disease, over-activation of cofilin in cells leads to formation of cofilin-saturated actin filaments that bundle into rod shaped structures that can form in axons and dendrites to block transport.[11] In Huntington's disease, stress-induced nuclear rods of cofilin-actin are not cleared rapidly and may have an impact on gene expression or other events requiring proper nuclear-cytoplasmic transport.[12] To study the role of cofilin in cellular models of these diseases, it would be advantageous to be able to silence endogenous cofilin expression and then rescue with either wild type cofilin or mutant forms of cofilin that will not bundle actin filaments into rods or will not enter the nucleus.

Previously, cofilin silencing in rodent cells has been achieved using either siRNAs introduced by transfection or by using a plasmid or adenovirus from which is obtained expression of a hairpin RNA capable of forming a double stranded siRNA when processed in the cell. To demonstrate the specificity of the cofilin silencing to the phenotype obtained, rescue experiments have utilized plasmids encoding cofilin from a different species[6], which are resistant to the siRNAs because of their altered nucleotide sequence. However, often minor changes in the amino acid sequence of protein are sufficient to alter its affinity for other binding partners. Thus, to achieve the maximum rescue it would be beneficial to have a reagent that will allow us to express the same cofilin amino acid sequence from an mRNA sequence that uses alternative codons.

Two types of siRNA resistant human cofilin cDNA plasmids were constructed, one type expressed only cofilin while the other expressed RFP-tagged cofilin. It was important to construct both types in case the presence of RFP interferes with the activity of tagged cofilin. Untagged cofilin could then be used instead, but the RFP tagged cofilin has the advantage of being able to directly visualize cofilin expression so analysis of rescue is confined to only RFP positive cells. Both plasmids expressed cofilin, which was confirmed by using microscopy and western blots. Both plasmids were also able to express cofilin in cofilin siRNA-treated cells that were transfected with these siRNA resistant plasmids.

Using the same methodology described here, we also made siRNA-resistant expression plasmids encoding two RFP-tagged forms of mutated cofilins. The mutations were made to encode either alanine (S3A) or glutamate (S3E) in place of serine at position 3. The S3A mutant behaves as a constitutively active form of cofilin because it is not subjected to phosphoregulation. The S3E version behaves as an inactive phosphomimetic form. Both forms have been useful in past studies of cofilin behavior and function through their overexpression[13,14] but now, coupled with our ability to silence endogenous cofilin, these plasmids will be important tools to further investigate the functional roles of cofilin in the many cellular activities in which it is involved.

Acknowledgments

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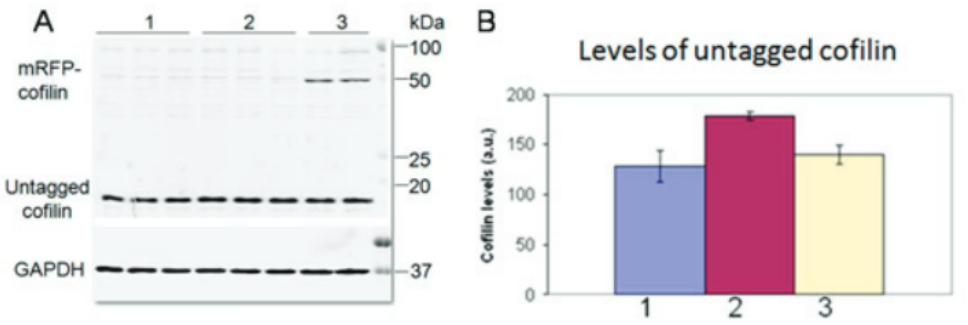

Figure 5. Western blot results confirm expression of both RFP-tagged and untagged siRNA resistant human cofilin. Protein was extracted from HeLa cells with SDS for electrophoresis onto 12.5% polyacrylamide gels and immunoblotting with antibodies to cofilin or GAPDH. The HeLa mock treated cells (1) express endogenous human cofilin. The cells transfected with siRNA resistant untagged cofilin (2) express both endogenous and siRNA resistant human cofilin and thus are expected to have brighter bands. It is easy to see the expressed RFP-cofilin protein (48 kDa band) in the cells transfected with RFP-tagged cofilin (3). The bands were quantified using TotalLab (Non-Linear Dynamics, Newcastle, UK). The bar graph shows that there is more cofilin expressed in cells transfected with the siRNA resistant plasmid relative to mock transfected HeLa cells.

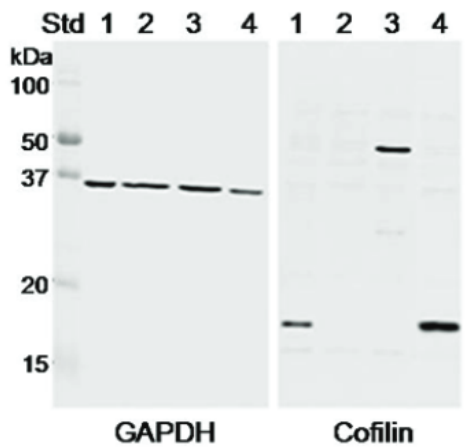

Figure 6. Expression of human cofilin siRNA resistant plasmids in the presence of cofilin siRNA. Protein was extracted from transfected HeLa cells with SDS for SDS-PAGE electrophoresis and immunoblotting with antibodies to cofilin or GAPDH. GAPDH was used as a loading control to demonstrate that approximately equivalent amount of cellular protein was loaded in each lane. HeLa mock cells (1) express endogenous cofilin. Cofilin siRNA transfection (2) results in a loss of cofilin expression. HeLa cells transfected with both the cofilin siRNA and either RFP-tagged (3) or untagged (4) siRNA resistant human cofilin show cofilin expression in the presence of the cofilin siRNA. Cofilin expressed at the expected molecular weight for RFP-tagged and for untagged cofilin.

sible but also enjoyable to finish. Thank you to Aaron Sholders, Yvonne Bridgeman, Marti Stokes and Paul Laybourn for organizing the summer REU program. Also for funding from the National Science Foundation REU Grant DBI-0852017and NIH grants K01NS064217 (OW) and R01NS40371 (JRB).

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