Confirmation of radiation-induced RET/PTC chromosome rearrangements in human thyroid cells

BY HILLARY W. BEDELL AND CHRISTINE L. R. BATTAGLIA, F. ANDREW RAY, AND SUSAN M. BAILEY
ALLEGHENY COLLEGE AND COLORADO STATE UNIVERSITY

Absract

Environmental exposure to ionizing radiation has been linked to incidence of thyroid cancer. Genetic modifications have been shown to play a role in thyroid cancer occurrence, specifically chromosome arrangements involving the *RET* (Rearranged During Transfection) /*PTC* (papillary thyroid carcinoma) gene. The most common *RET/PTC* variants are *RET/PTC1* and *RET/PTC3*—both which are thought to involve intrachromosomal inversions. The goal of this study was to determine if ionizing radiation-induced *RET/PTC* rearrangements in human thyroid cells, which have been associated with thyroid cancer, could be identified molecularly by RT-PCR and confirmed cytogenetically with chromatid painting. After confirming that HTori-3 cells possessed specialized functions of human thyroid cells (*NIS* and *Tg* expression), cells were irradiated with 0, 1.25, 2.5, and 5 Gy gamma rays, and RT-PCR was run to detect *RET/PTC1* and *RET/PTC3*. *NIS* was used as a control for PCR with HTori-3 cells irradiated at 5 Gy and a strong band seen repeatedly around 200 bp, confirming that the cells retained the specialized functions after irradiation. Faint potential *RET/PTC1* products were noted after gel electrophoresis; however, these products were not confirmed and additional products were unaccounted for. Further studies will include a cell-by-cell cytogenetic analysis, by single-stranded chromatid painting.

Introduction

Numerous reports document an increased risk of thyroid cancer linked to environmental radiation exposure. The survivors of the Chernobyl nuclear accident exemplify this association between irradiation and thyroid cancer.[1] In 1986, the Chernobyl nuclear power plant accident in the Ukraine released a great amount of iodine isotope which resulted in extensive radiation exposure - including exposure to the human thyroid.[2] Incidence of thyroid gland tumor development increased following the accident.[3-5]

One of the genetic alterations confirmed to be involved in these radiation-induced thyroid cancers involves chromosomal arrangements of the RET gene.[1] RET is a proto-oncogene located on chromosome 10q11.2 that spans 21 exons and encodes a single pass transmembrane tyrosine kinase that functions as a receptor.[2,6] When the RET gene recombines with one of its 11 fusion partners, it becomes known as *RET/PTC*.[7] *In vitro* studies have indicated that this chromosomal rearrangement is frequently associated with exposure to ionizing radiation.[3] Various mechanisms including unscheduled expression of *RET*, ligand-independent kinase activation, subcellular relocalization and functional alteration of *RET*-fused genes, contribute to *RET/PTC*'s oncogene characterization.[2] The *RET/PTC* rearrangement has been found in both sporadic and radiation-induced tumors and has been detected in post-Chernobyl tumors. The prevalence of *RET/PTC* rearrangement is approximately 20-30% in sporadic thyroid papillary carcinomas and significantly higher in tumors from patients exposed to radiation.[1] The most common *RET* variants are *RET/PTC1* and *RET/PTC3*—both of which are suggested to be intrachromosomal inversions.[1,8] These variants of *RET/PTC* account for more than 90% of all rearrangements in sporadic and radiation-induced thyroid tumors. *RET/PTC1* results from recombination between *RET* and *CCDC6*, and *RET/PTC3* results from recombination between *RET* and *NcoA41*.[9-12] Because these are known rearrangements induced by ionizing radiation and are suggested to be inversions, I aimed to create these rearrangements to use for tests for chromatid painting - a new method of detecting tumor specific chromosomal inversions.[13,14] In this study, I sought to discover if ionizing radiation-induced *RET/PTC* rearrangements in human thyroid cells, which have been associated with thyroid cancer, can be identified molecularly by PCR and confirmed cytogenetically with chromatid painting.

Materials and Methods

Cell Line

HTori-3 cells, human thyroid epithelial cells which have been transfected with an origin-defective SV40 genome, were used in this experiment. These cells are immortalized and retain specialized functions of differentiated cells.[1] The cells were a kind gift from the Nikiforov lab, University of Cincinnati. Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin streptomycin.

Detection of thyroglobulin (Tg) and sodium-iodide symporter (NIS)

Total RNA was isolated from HTori-3 cells using a RNeasy kit (QIAGEN, Valencia, CA). Then, mRNA was extracted using the Oligotex mRNA mini-kit (QIAGEN). RT-PCR was performed using the Superscript II Reverse Transcriptase kit and random hexamer priming (Invitrogen, Carlsbad, CA). PCR was performed to detect *NIS* and *Tg* in separate tubes using the following primers: 5'-CCTCGCAGTTCAATCAGTCA-3'(*Tg* forward), 5'-TGGCTGAAGTAGCCTGAGGT-3' (Tg reverse), 5'-CTCCCTGCTAACGACTCCAG-3' (*NIS* forward), and 5'-GAGGTCCCACCACAACAATC-3' (*NIS* reverse). For each PCR, 2 uL RT mixture and 2 mM $MgCl_2$ was amplified in a final volume of 50 uL using 40 cycles of denaturation (94C for 40 sec), annealing (59 C, 1 min for *NIS*; 57 C, 1 min for *Tg*), and extension (72 C for 1 min). PCR products were electrophoresed in a 1.5% agarose gel and visualized by ethidium bromide staining. The expected size of *NIS* was 207 bp and 303 bp for *Tg*.

Cell Irradiation

Sixteen hours after passage into 75-cm^2 flasks, actively growing cells were exposed to γ-irradiation from a ^{137}Cs Sealed Source, with the turntable in, at a dose rate of 3.9 Gy/min. The 75-cm^2 flasks that were exposed to higher doses of irradiation were more confluent to account for more cell killing due to irradiation. Cells were exposed to 0, 1.25, 2.5, and 5 Gy γ-irradiation. Cells were passaged once in the 9 days after irradiation. Cells were cultured in an incubator at 37° in 95% air/ 5% CO_2.

Detection of RET/PTC rearrangements

Total RNA was then extracted 10 days after irradiation from each flask using a RNeasy kit (QIAGEN). Then, mRNA was isolated using the Oligotex mRNA mini-kit (QIAGEN). RT-PCR was performed using the Superscript II Reverse Transcriptase kit and random hexamer priming (Invitrogen). PCR was performed to detect *RET/PTC1* and *RET/PTC3* concurrently in one tube using the following primers:

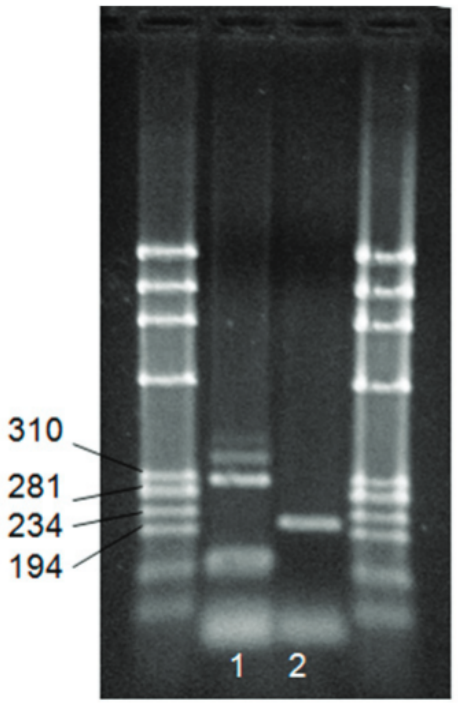

Figure 1. Characterization of HTori-3 cells. RT-PCR analysis demonstrating expression of *NIS* and *Tg*—differentiated thyroid genes. Expected size of *NIS*: 207 bp; *Tg*: 303 bp (*). Lane 1: *Tg*; Lane 2: NIS. L, 147-bp Ladder.

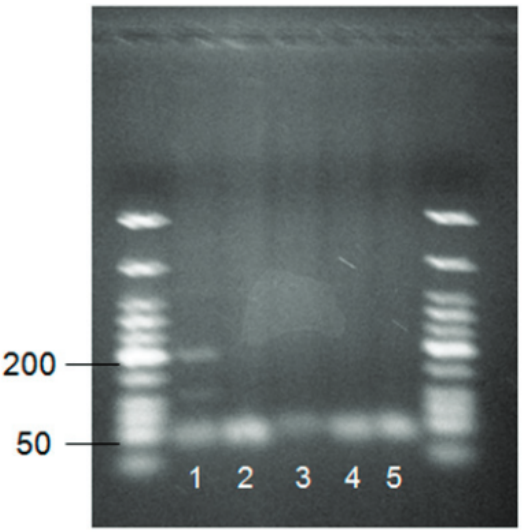

Figure 2. Detection of *RET/PTC1* and *RET/PTC3* rearrangements in HTori-3 cells; 5 Gy. RT-PCR analysis with temperature gradient using specific primers to detect specific *RET/PTC* products. Lane 1: control *NIS* (202 bp) at 59°; Lane 2: 60°; Lane 3: 59°; Lane 4: 58°; Lane 5: 57°. L, Low MW Ladder.

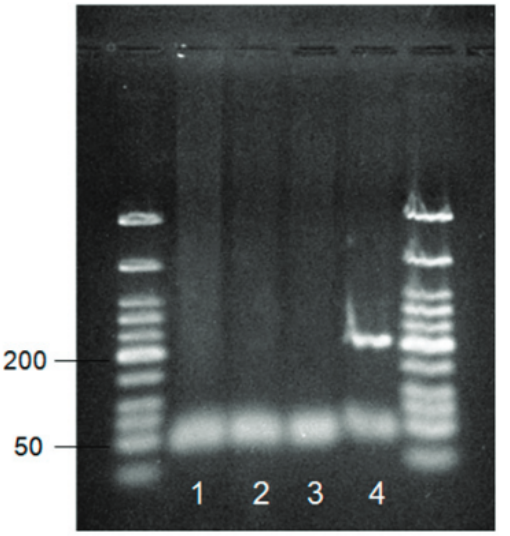

Figure 3. Confirmation of primer dimers around 60 bp. RT-PCR analysis comparing cells irradiated at 5 Gy with cells irradiated at 0 Gy using specific primers to detect *RET/PTC* products. Lane 1: 5 Gy; Lane 2: 5 Gy; Lane 3: 0 Gy; Lane 4: 5 Gy with control, *NIS* (202 bp). L, Low MW Ladder.

5'CAAGAGAACAA-GGTGCTGAAG-3' (RET/PTC1 forward), 5'-CGGTATTG-TAGCTGTCCCTTTC-3' (*RET/PTC3* forward), and 5'-GCAGGTCTCGAAGCT-CACTC-3' (common reverse). For each PCR, 2 uL RT mixture and 2 mM $MgCl_2$ was amplified in a final volume of 50 uL using 40 cycles of denaturation (94C for 40 sec), annealing (58 C for 1 min), and extension (72 C for 1 min). PCR products were electrophoresed in a 1.8% agarose gel and visualized by ethidium bromide staining. A BLAST search confirmed that the expected size of *RET/PTC1* was 137 bp and RET/PTC3 was 66 bp.

Results

Detection of thyroglobulin (Tg) and sodium-iodide symporter (NIS)

Bright bands around 202 bp and 303 bp confirmed that the HTori-3 cells retained expression of *NIS* and *Tg*, respectively (Fig. 1).

RT-PCR with Temperature Gradient to Detect RET/PTC Rearrangements

Figure 2 demonstrates the products of RT-PCR performed on HTori-3 cells irradiated at 5 Gy at different temperatures. A band in lane 1 at about 200 bp confirms *NIS* expression as a control. Products are also evident at about 60 bp in all five lanes (Fig. 2). In Figure 2, a temperature gradient for PCR was run because the annealing temperature for the primers was not known; however, it was estimated from the melting temperature to be 58-59°. The bands at about 60 bp in lanes 2-5 have the possibility to be the *RET/PTC3* product because *RET/PTC3* has an estimated size of 66 bp; however, it is believed that they are primer dimmers for two reasons: the band at about 60 bp is also detected in lane 1 using the specific primers to detect *NIS*; also, *RET/PTC1* is more common than *RET/PTC3* so one would expect to visualize the *RET/PTC1* band (137 bp) before visualizing the *RET/PTC3* product (Fig. 2).

RT/PCR to Confirm Primer Dimers

The products of HTori-3 cells irradiated at 5 Gy and 0 Gy are depicted in Figure 3. There is a bright band at about 60 bp in all 4 lanes including lane 4, which *NIS* was run as a control. A band at about 200 bp in lane 4 confirms *NIS* expression (Fig. 3).

RT/PCR Detection of RET/PTC at 5 Gy

A faint band is present in Figure 4 between the 118 bp and 194 bp ladder bands as well as the regular bright band at about 60 bp. Figure 5 depicts the results of RT-PCR using Isis DNA Polymerase (MP Biomedicals, Santa Ana, CA). Lane 1 demonstrates a couple bands from ~270-140 bp and the primer dimer around 60 bp. The NIS run in lane 2 reveals several bands at ~600, 320, 230, 180, 120, 60 (primer dimer) bp (Fig. 5).

RT/PCR Detection of RET/PTC at 0, 1.25, 2.5, and 5 Gy

HTori-3 cells irradiated at 0, 1.25, 2.5, and 5 Gy produced T-PCR products at about 50-60 bp. Bands around the expected sizes of RET/PTC1 (137 bp) are absent (Fig. 6).

Discussion

The HTori-3 cell line has preserved the most specialized functions of differentiated human thyroid cells, even after irradiation (Fig. 1; Fig. 2). To confirm it is product dimers, RT-PCR was performed on cells irradiated at 5 Gy and 0 Gy using specific primers to yield *RET/PTC* rearrangement products. Because the bands at about 60 bp are present in all the lanes, these bands are confirmed to be primer dimers (Fig. 3). If the band at about 60 bp was the *RET/PTC3* rearrangement, then the band would

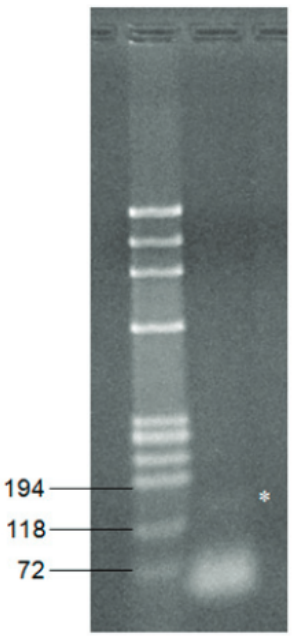

Figure 4. Detection of *RET/PTC1* rearrangements in HTori-3 cells; 5 Gy. RT-PCR analysis using specific primers to detect specific *RET/PTC* products. Expected size of *RET/PTC1*: 137 bp (*). Lane 1: *RET/PTC*. L, 147-bp Ladder.

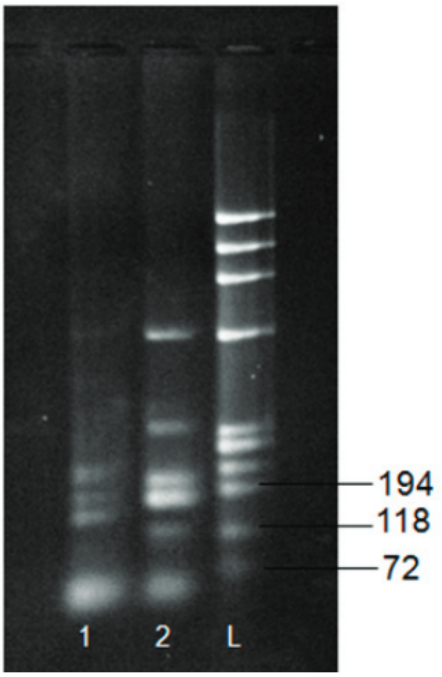

Figure 5. Detection of *RET/PTC1* rearrangements in HTori-3 cells with Isis DNA Polymerase; 5 Gy. RT-PCR analysis using specific primers to detect *RET/PTC* products. Expected size of *RET/PTC1*: 137 bp (*). Lane 1: *RET/PTC*; Lane 2: NIS (202 bp). L, 147-bp Ladder.

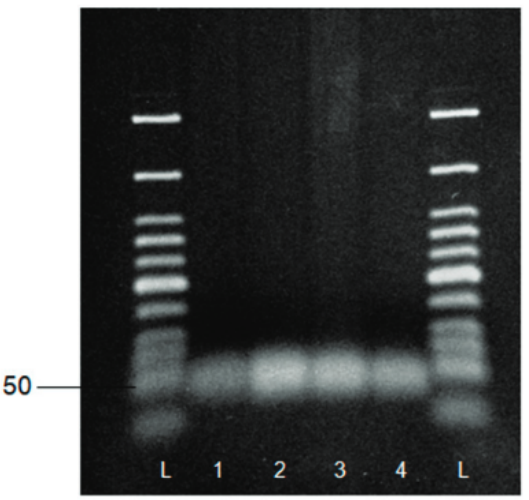

Figure 6. Detection of *RET/PTC1* rearrangements in HTori-3 cells . RT-PCR analysis using specific primers to detect *RET/PTC* products. Lane 1: 5 Gy; Lane 2: 2.5 Gy; Lane 3: 1.25 Gy; Lane 4: 0 Gy; L, 147-bp Ladder.

not be in lane 3 because the rearrangement would not be present in cells that have not been irradiated.

RT-PCR was performed again with HTori-3 cells irradiated at 5 Gy at 58° and a dim band between 119 bp and 194 bp could suggest a *RET/PTC1* product (137 bp) (Fig. 4). Assuming the band is the desired product, it was decided that greater amplification was needed. Thus, Isis DNA Polymerase, a highly thermostable DNA polymerase, was incorporated into the next PCR mixture and the number of cycles was increased to 60 cycles. The bottom band of lane 1 lies between 119 bp and 194 bp, thus it has the potential to be a *RET/PTC1* product (137 bp). However, there are other bands present which are not accounted for. This PCR also resulted in *NIS* to not yield the correct product. Instead of *NIS* resulting in a single band at 207 bp, it resulted in many additional products, most likely because of the increased number of cycles. Also, *NIS* did not depict a band right at 200 bp which could hinder the validity of the potential *RET/PTC1* product (Fig. 5). Even though faint bands around 137 bp were not confirmed to be *RET/PTC1* products for HTori-3 cells irradiated at 5 Gy, RT-PCR was run on cells irradiated at 0, 1.25, 2.5, and 5 Gy; bands at 137 bp were absent. Thus, the *RET/PTC* rearrangements were not identified with RT-PCR at this time.

One reason for limited or absent *RET/PTC* products detected by PCR is that they are reported to be low in frequency (3.0 per 106 irradiated cells).[1] Thus, future studies will include a cytogenetic cell by cell analysis for RET/PTC detection. A developing technology for high resolution inversion detection involves strand-specific chromatid painting. Strand-specific chromatid paints hybridize to one chromatid of a metaphase chromosome, so if an inversion is present, a switch in labeling from one side to the other occurs within the inverted region.[13,14] Thus, if a *RET/PTC* rearrangement is present, a switch in labeling will confirm the inversion.

Acknowledgements

Colorado State University, especially the Department of Environmental and Radiological Health Sciences, for their guidance and support.

Everyone involved with the 2010 Molecular Biology REU at CSU--both the program coordinators, mentors, and participants for providing this educational opportunity and many unforgettable experiences.

Christine L. R. Battaglia, F. Andrew Ray, and Susan M. Bailey for advising me throughout the summer and always being there when I needed assistance

The NSF and the NIAID for funding my research pursuits.

Allegheny College, especially the Biology Department, for demanding academic success and providing a strong framework in the sciences which has enabled me to have research experiences such as the CSU REU.

To my parents for their endless love and support in all I do.

References

[1]Caudill, C. M., Zhu, Z., Ciampi, R., Stringer, J. R., Nikiforov, Y. E., (2005) *J. Clin. Endocrinol. Metab.*, 90(4) 2364 - 2369.

[2]SantoRo, M., Melillo, R. M., Fusco, A., (2006) *European Journal of Endocrinology*, 155 645-653.

[3]Pignalosa, D., Ritter, S., Durante, M., (2010) *Radiation Research*, 174 14-19.

[4]WILLIAMS, D., (2002) *Nat Rev Cancer*, 2:543–549.

[5]Tuttle, R. M., Becker, D.V., (2000) *Semin Nucl Med.*, 30:133–140.

[6]Grieco, M.., Santoro, M., Berlingieri, M. T. , Melillo, R. M.,

Donghi, R., Bongarzone. I., Pierotti, M. A., Della Porta, G., Fusco, A., Vecchio, G. (1990) *Cell*, 60:557–563.

[7]Nikiforov, Y. E., (2002) *Endocr Pathol*, 13 3–16.

[8]Tallini, G., Asa, S. L., (2001) *Adv Anat Pathol*, 8:345-354.

[9]Santoro, M., Dathan, N. A., Berlingieri, M. T., Bongarzone, I., Paulin, C., Grieco, M., Pierotti, M. A., Vecchio, G., Fusco, A ., (1994), Oncogene 9:509–516.

[10]Bongarzone, I,. Butti, M. G., Coronelli, S., Borrello, M. G., Santoro, M., Mondellini, P., Pilotti, S., Fusco ,A., Della Porta, G., Pierotti ,M. A., (1994) Cancer Res 54:2979–2985.

[11]Pierotti, M. A., Santoro, M., Jenkins, R. B., Sozzi, G., Bongarzone, I., Grieco, M., Monzini, N., Miozzo, M., Herrmann, M. A., Fusco, A., Hay, I. D., Della Porta, G., Vecchio, G. (1992), Proc Natl Acad Sci USA 89:1616–1620.

[12]Minoletti, F., Butti, M. G., Coronelli, S., Miozzo, M., Sozzi, G., Pilotti, S., Tunnacliffe, A., Pierotti, M. A., Bongarzone, I. (1994), Genes Chromosomes Cancer 11:51–57

[13]Bailey S. et. al., unpublished

[14]Goodwin, E. H. (1999) DTIC Online Information for the Defense Community. Annual rept. 30. http://oai.dtic.mil/oai/oai?verb=getRecord&metadataPrefix=html&identifier=ADB249668. (Accessed 03/04/2011).

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.