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DNA damage repair Neil3 gene Knockout in MOLT-4

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Abstract: *RNAi is superannuated cellular mechanism that protect organism against viruses that replicate through double- stranded RNA. RNAi can be used to diminish gene expression from plasmid expressing and inserted sequence repeat. A stable harpin would be expressed after the vector was integrated into the genome. In this paper a shiRNA expressing vector for Neil3 was designed and developed which is capable of replication in MOLT-4. This shiRNA vector had the ability to arose the RNAi pathway, and reduce the gene expression of Neil3. This was assessed by using pSilence 4.1CMV as a vector, and Gapdh as positive control.*
Key words: *Neil3, Gapdh, shiRNA, MOLT-4, gene knockout*

I. Introduction

An evolutionary preserved pathway, the base excision repair (BER) , a reformatory repair pathway to prevent mutagenesis and cytotoxicity1 . Five key enzymatic steps are involved in the repair response which aim to abstract the initial DNA damages and restore the genetic material back to its original state1,2 (1) excision of an inadequate base , (2) incision of phosphate backbone at a resulting abasic site, (3) authorization unabated repair synthesis and , or nick ligation by termini clean-up , (4) gap-filling to substitute the excised nucleotide, and finally (5) sealind of the final DNA nick1,2.

First enzymes in BER are DNA glycosylases, which distinguish the wrong bases, excise the damage and stock substrate for the later enzymes in the pathway 3. Five DNA glycosylase that are specific for oxidative DNA base damage have been assigned in Human cells, OGG1, NTH1, Neil1, Neil2, Neil33,4. OGG1 , is responsible for excision a mutagenic base byproduct which is the result of DNA exposure to ROS (hydroxyl radical OH.), the 8-oxoguanin.5 NHT1 removes oxidized pyrimidine from dd DNA.4 NEIL1 removes oxidized pyrimidines and some purines from ddDNA , Neil 2, Neil3 preferred ssDNA4.

The glycosylase activity of human NEIL3 is on both single-stranded and duplex DNA containing Tg and Sp [59]. NEIL3 a proteins exhibit very weak lyase activity which occurs primarily via β-elimination, leaving an α, β-unsaturated aldehyde at the 3'terminus . In contrast, most other Fpg/Nei family members exhibit robust lyase activity acting primarily via β,δ-elimination leaving cleavage products with 3′ phosphate termini 6.

Few literatures worked on NEIL3 knockout in cell line, especially MOLT-4. In this paper I focused on Neil3 genotyping in MOLT-4 cells, and transfection these cell with pNeil3 vector by using pSilencer 4.1 CMV (gene silencing technique), pGapdh was used as a positive control.

II. Material and Methods

Reagents and there composition are listed in table (1)(All reagents all analytical grade from Sigma)
Table (1) reagent composition

Composition	Reagents
0.89M Tris. , 0.89M boric acid, 20M EDTA (pH 8.3)	10x TBE buffer
(w/v) bromophenol blue, 025%(w/v)xylene cyanol, 30%(v/v)glycerol, 10M EDTA	Agarose gel DNA loading buffer
50mMNaCl, 10mM Tris HCl, 0.1mM EDTA (pH8.3)	Annealing buffer
10x stock solution (Invitrogen) , diluted with dd H_2O to 1x (pH7.4).	PBS

Restriction Enzyme

Restriction enzymes were accommodated from New England Biolabs (NEB), stored at -20. All restriction endonuclease used for diagnostic restriction digestion, and cloning the siRNA vector are listed in table (2)

Table (2) Restriction Enzymes: The annotation in the restriction site sequence is the position the restriction endonuclease cuts.

Restriction site	Restriction Endonucleases
5′...A▼AGCTT...3′ 3′...TTCGA▲A...5′	HindIII-HF
5′...G▼GATCC...3′ 3′...CCTAG▲G...5′	BamHI-HF

Oligonucleotides

An inverted complementary DNA sequence must be expressed within the cell for stable reduction of gene expression . A hairpin loop should be performed , once this inverted sequence is expressed, double –strand RNA imitated, know trigger for RNAi. Only target sites specific for gene insert were chosen, these target sites appeared at least two out of the four computer programs which were checked for homology to other sequence in the human genome(7-11). The target sequence for Gapdh was the sequence used by Ambion as the positive control in the pSilencerTM 4.1-CMV puro siRNA kit. The oligonucleotide inserts for pshRNA were designed to have the sense target (18-20 nucleotides) and the corresponding inverted antisense sequence separated by a 9 nucleotide loop sequence (5′-TTCAAGAGA-3′)7-12 .The inverted repeats were flanked at the 5′ end by an BamH I site, and at the 3′ end with a RNA polymerase termination signal of 6 thymine residues and a Hind III site for ease of cloning into pshRNA , fig (1). PCR oligonucleotide that used for genotyping MOL-4 cell line and PCR nucleotide are showed in table 3.

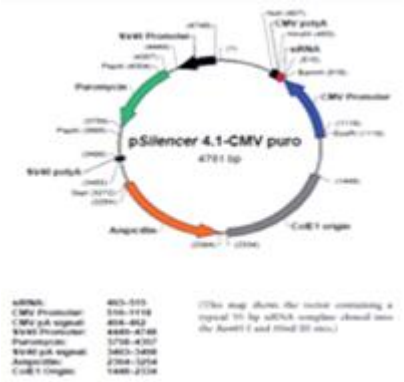
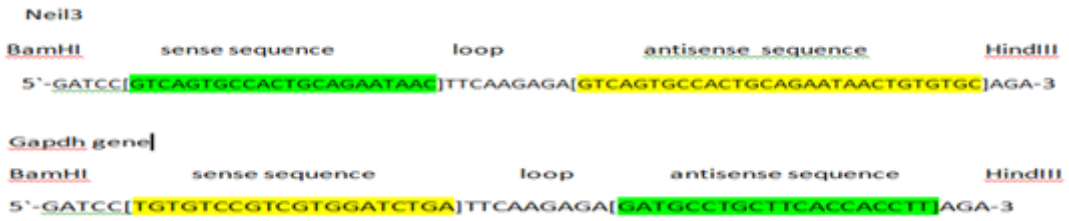


Fig (1) A: pSilencer 4.1 CMV puro



Fig(1) B: Sequence of (˜ 55bp) , the sense and antisense sequence for neil3 and Gapdh, loop, cloning site BamHI & HindIII.

Table (3) DNA oligonucleotides used in genotyping and PCR

Sequence 5′ to 3′		**Genotype**
ACTGAAT GGAGAGAAGATCCGGG	**Forward**	**Neil3**
CAGCTCCTTCCCTAA GGTTTCCA	**Reverse**	
AACTTTGGCATTGTGGAAGG	**Forward**	**Gapdh**
ACACATTGGGGGTAGGAACA	**Reverse**	
Sequence 5′ to 3′		**PCR**
GGGCAACATCATCAAAAATGAA	**Forward**	**Neil3**
CTGTTTGTCTGATAGTTGACACACCTT	**Reverse**	
TCGTCCCGTAGACAAAATGGT	**Forward**	**Gapdh**
CGCCCAATACGGCCAAA	**Reverse**	

Cell culture

Molt-4 cell lines were examined in this study for gene silencing. MOLT-4 cells are T-4 cell lines which were initially derived in 1971 from T-cell acute lymphoblastic leukemia patients12. Memorialization was done by continuous growth at 20% O2. MOLT-4 cell line were preserved in RPMI 1640 medium (Invitrogen), supplemented with 10% FBS (Invitrogen), and 2 mM L-Glutamine (Invitrogen) at 37C0, 5% CO2, 3% O2. Cells were subcultured every 2-3 days, by pipetting the cell solution into universal flask, centrifuged for 5minutes at 125g. Supernatant were removed, and the cells were re-suspended in fresh medium. Cells were counted by a haematocytometer .

Optimizing Antibiotic Selection Conditions

Concentration of the antibiotic (puromycin) that killed non–transfected cells were determined. MOLT-4 cells were platted in 2x104 , and 4x104 cell/well of 12 well plate and incubated for 24 hours. Puromycin was added to the wells at the concentration range from 0 – 4 µg/ml .

Cell were cultured for 14 days, replacing the puromycin–containing medium every 3 days.Wells were examined for viable cells at 100x magnification every 2 days to identify the lowest puromycin concentration that began to give massive cell death in approximately 5-7 days ,and kills all cells within 2 weeks. This concentration was used to select cell containing the pSilencer 4.1CMV puromycin (DNA plasmid) after transfection.

Annealing the hairpin siRNA template oligonucleotides

Both sense and antisense siRNA for (NEIL3 and GAPDH)(1µg/µl) were annealed through mixing with 1xDNA annealing solution , reaction mixtures were completed to 50µl, heated 90 to C0 for 3min, then placed in 16 C0 overnight incubation. The annealed hairpin siRNA template insert can either be ligated into a pSilencer 4.1-CMV vector immediately or stored at –20°C for future ligation.

Ligation of annealed siRNA template insert (NEIL3 and GAPDH) into the pSilencer 4.1-CMV vector

An 8ng/µl of the diluted annealed hairpin siRNA insert (NEIL3 , GAPDH) were use in a two set reaction. The annealed were added to a 10x T4DNA ligase buffer, pSilencer CMV vector (Ambion , Cambridge shire, UK) ,and (5U/µl) T4DNA ligase. The reactions were incubated overnight at 16C0 for high ligation efficiency. Plasmid DNA was transformed in E.coli strains.

Transforming E. coli with the ligation products (pNeil3 and pGapdh) vectors

NovaBlue (Novagen) competent E.coli strains were used for DNA plasmid transformation . The medium composition for growing E coli strains is LB agar (10 g/L tryptone, 5g/L yeast extract, 17 mM NaCl , 20 g/L bacto –agar).

For transformation of NovaBlue competent strains, 20µl of aliquots of cells were pipetted in pre-cooled tube in ice for 5min to thaw. Plasmid DNA solution (1 µl) was added directly to the cells and mixed, incubated in ice for approximately 5 min. Tubes were then heated for exactly 30sec in 42 C0 water bath without shaking, and replaced in ice for 2 min to entitle the uptake of DNA plasmid into the bacterial cells. At room temperature, (80 µl) of SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, and 20 mM glucose) were added to the miture. Transformation were accomplished by platting the cells on LB agar medium containing ampiciline (50µg/ml) and incubated overnight at

Inoculating a Liquid Bacterial Culture:

To isolate the transformed E.coli cells with plasmid DNA which is resistance to antibiotic, five colonies from LB agar plate were picked, mixed with LB broth (5ml) containing ampiciline (50µg/ml) and incubated 24 hours at with shaking (250rpm). After incubation growth was checked, which was characterized by a cloudy haze in the medium

Plasmid Purification

To purify the plasmid DNA from the transformed E.coli cells, mini-probe (Promega) plasmid DNA purification protocol was used . The bacterial cell were lysed using a modified alkaline lysis solution, which will lysis the cell and denature the plasmid DNA. DNA solution then neutralized to snap back the plasmid DNA into double –stranded. The precipitant were removed by centrifugation. The supernatant was then applied to the silica –gel membrane for further purification of the plasmid DNA. dsDNA will stick to the membrane, and eluted from the membrane by using a low – ionic strength buffer or nuclease free water. The plasmid DNA concentration was determined by absorption at 260nm using ND-2000 spectrophotometer (Thermo scientific).

Restriction Enzyme Digests.

Routine diagnostic endonuclease enzyme digest were performed for both plasmid GAPDH, and plasmid NEIL3 . plasmid DNA (a 300 µg/ µl), 2 µl of enzyme buffer, 0.5U Hind III, 0.5 BamH I, the reaction volume was completed 20 µl by free nuclease water, incubation for 1.30hrs at 37 C0. Inactivation of the reaction was accomplished by heating at 60 C0 for 20 min.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to isolate plasmid DNA (GAPDH , NIEL3). Agarose gel was dissolved 0.5gm of the gel (for DNA fragments less than 1kd)in 1x TBE, procceded by adding 4 µg/ml ethidium bromide. Each sample loaded per gel well consists of plasmid DNA, loading buffer, and diluted with free nuclease water. DNA (hyperladder) (promega) was used to identify the size of the DNA fragments. Electrophoresis(Labnet) was performed in 1x TBE at 90 volt for 60min. DNA bands were visualized under UV trasilluminater.

Generation of stable knockdown mammalian cells (MOLT-4) (Plasmid transfection for both gene GAPDH and Neil3)

Before stable knockdown for both genes (Neil3 and GAPDH), the reaction conditions for MOLT-4 cell line were optimized to ensure best transfection results. The recommended starting conditions were: **cell density,** MOLT-4 cell density was ≥ 80 %, DNA samples were highly purified with A260/ A280 absorbance ratio of 1.8-2.0 and using appropriated cell culture medium. Other conditions like **ratio of TransIT-LT1 reagent to DNA complex formation condition**, and post- **transfection incubation time were** sated in transfection protocol as mentioned later.

Immortalized MOLT-4 were plated at a cell density of 2x105 cell/well in a 24 well plate and incubated at 37C0 , 5%CO2, 3%O2 until 90% cell confluency . For transfection, 1µg of plasmid vector DNA was diluted with 500µl of complete growth medium, (opti-MEM reduced medium) (with 5 min incubation) a 1.5µl TransIT-LT1 reagent, 50 µl of serum free medium was added respectively to the mixture, mixed and incubated for 30min in RT. Mixture was added to the MOLT-4 cells drop-wise to different area of the well within 2 min and incubated for 48 hours. Cells were harvested and re-plated in 24 well /plate and 4µg/ml of puromycin were added to the medium to select for resistant cells. Fresh pyromycin containing medium was added to the cells every 3-4 days until wells containing individual colonies could be identified. Individual clones were expanded for ~ 5 passages in pyromycin containing medium to ensure the clones were antibiotic resistant.(a negative control with no plasmid were needed to check that all the cells have been killed by the puromycin).

Extraction of total RNA

Extraction of total RNA were performed for both non- transfected and transfected MOLT-4 cell line, first one was to identify the existence of Neil3 gene and the second was to test the efficiency of gene silencing achieved by the target vector, the level of target gene expression in the stable knockdown cell lines was assayed by PCR. RNA extraction was performed by using PureLink RNA Mini Kit (Ambion)

Molt-4 cell (1x10 6 cell) were translated to an RNAase – free tube and centrifuged for 2000xg for 5 min at 5 c0 , the supernatant was discarded. A fixed volume of lysis buffer was added to the cells, mixed, until the cells appeared to sample was lysed. Ethanol 70 % were added to homogenate and mixed. Sample was added to a silica column centrifuged at 12000xg for 15

sec. at rt., this will insure that all RNA molecules would bind to the silica. , the silica mini columns were washed thoroughly with washing buffer I and washing buffer II respectively. RNA samples were collected by washing the silica mini tubes with RNAase free water and the concentration of the RNA samples were determined by absorption at 260nm using ND-2000 spectrophotometer (Thermo scientific).

First strand cDNA synthesis:

Reversal transcription reactions was performed to form c DNA from m RNA that consisted in the cellular total RNA samples for both non –transfected and transfected MOLT-4 cell line. A (500 ng/ml) of oligo (dT)12-18 were added to specifically (19.5 ng/µl) of total RNA samples, followed by (10 mM) of dNTP(mix) , the reaction mixture were completed to 12µl with sdH2O. After heating the mixture at 65 C0 for 5 min and quiche chilled on ice later. A 5 X first – strand buffer, 0.1 M DTT, 40U/µl of RNase OWT were added to the mixture, and mixed gently, incubated at 42 C0 for 2 min. SuperScript II RT (200 Units) were added to the mixture , incubated at 42 C0 for 52 min. Finally the reactions were deactivated by heating at 70 C0 for 15 min.

PCR:

PCR was first performed to the non- transfected MOLT-4 to examine the expression of Neil3 gene, in the association with housekeeping gene (GAPDH)

PCR for non – transfected MOLT-4 cell line (genotyping for Neil3, and Gapdh):

PCR reactions were performed in 50 ul using 200ng of cDNA , 20uM of each primers(table 3), in DNase free PCR tubes, 2 X of MY TaqMix (BioLine. UK)13 was added to the mixture, and reactions were completed to the exact volume with DNase free water. PCR protocol was 1 cycle at 95C0 for 1 min, 30 cycle of at 95C0 for 15sec, 57C0 for 15 sec, and 72 C0 for 30sec, and 1 cylce at 72 C0 for 1 min. the PCR products were assessed by agarose gel electrophoresis(2.5% agarose gel in 0.5 x TBE) and performed at 80 volts for 20 min . DNA bands were visualized under UV trasilluminater.

PCR for transfected MOLT-4 cell line by (pNeil3, pGapdh, psilencer 4.1 CMV):
PCR reaction were performend as with the non – transfected MOLT-4 . Primers are mentioned in table(3)

III. Result and discussion

Genotyping the Neil3 and housekeeping gene Gapdh in MOLT-4

To study the inhibitory effect of hsiRNA, MOLT-4 cell line was used for this study. To confirm the existence of Neil3 and Gapdh, total RNA was extracted from Molt-4 and cDNA were formed for genotyping, finally PCR reaction was performed by using designed primers to amplify the genes. Fig ( ) revealed the amplified fragment of DNA from Neil3 and Gapdh, thus confirming the expected genotype.

Neil3 has been shown to be highly expressed in various tumor types and to synchronic with organogenesis. Taking in regards to these observations , and the truth that no durable DNA glycosylase activity has been detected, elucidate that Neil3 is a protein involve in the process but not a typical DNA glycosylase. Neil3 take place in extensive proliferative process, it is a hallmark substance as tumor developing, progressing and brain development (14, 15.16).

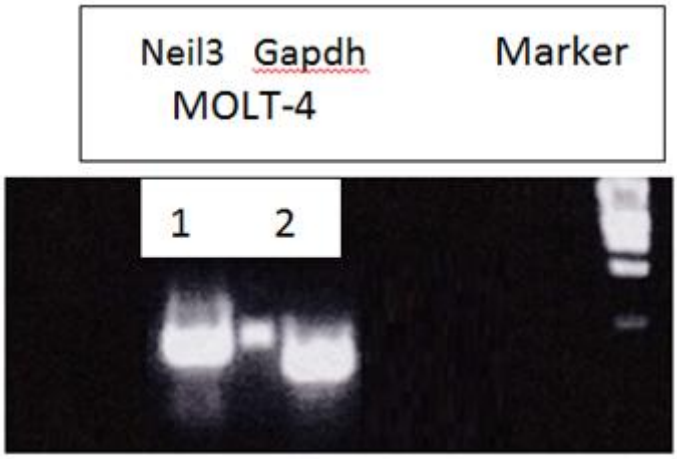


Figure 2: PCR to confirm the genotype Neil3, Gapdh

Lane 1: Neil3 gene in MOLT-4 cell, Lane 2 : Gapdh in MOLT-4 cell, Lane 3: Marker

Establishment the optimum condition for MOLT-4 transfection .

MOLT-4 cell line would be transfected with shiRNA expression vector that modulate the expression of both Neil3 and Gapdh genes. This vector contains puromycin resistance gene, therefore cells containing shiRNA vector can sustain in the presence of puromycin. The preserver concentration of puromycin was determined by adding increasing concentration of puromycin to the cell culture medium and utilized to MOLT-4. 0.5 µg/ml was the concentration of puromycin that result in approximately 70% of MOLT-4 death (eye examination) after 3 days, and full cell death after two weeks. This concentration was chosen for the isolation of resistant cell colonies after transfection.

A 0.75 µg/ml of puromycin was used as an ultimate concentration to initiate the generation of stable gal3 knockout cell line (17).

Generation of plasmid DNA : pNeil3 and pGapdh

For long Neil4 and Gapdh knockdown, siRNA were designed for these two genes, cloned into pSilencer 4.1 CMV puro (Ambion), as approximately 55bp hairpin loop between restricted site HindIII and BamH1. E. Coli. Cells were transformed with plasmid DNA for gene Niel3, and housekeeping gene GAPDH (plasmid DNA : pSilencer cmv4.1 + insert(Neil3, and Gapdh) respectively , by using heat-shock method. The transformation was approved by further growth of selected transformed Ecoli in liquid LB broth medium containing 50ug/ml of carbancilin. Plasmid DNA samples were isolated from confirmed transformed Ecoli , a

restriction digest was performed with HindIII HF, and BamHI HF , two bands were distinguished in the gel , first with higher nucleotides 4535nt , and the second 22nt, Fig (3).

Nox4 was knokdown successfully by using pSilencer 4.1 CMV and resulted in lowering vascular endothelial growth factor transcription, and decreasing the transforming growth (18). MRAP expression in cells was knoked down by the iRNA using siRNA duplexes cloned into BamH1 and HindIII digest pSilencer 4.1 CMV neo expression vector (19) , pSilencer was further used to create plasmid expression miR-17-5P (20).

Stable Transfection of MOLT-4 with pNeil3 and pGapdh

MOLT-4 cell line was stably transfected with pNeil3 and pGapdh to generate MOLT-4 with pshRNA vector. The transfection on MOLT_4 was performed with pshRNA of Neil3, Gapdh, or pSilencer 4.1 CMV without insert. Cell culture medium containing 0.5 ug/ml puromycin was used to select positively transfected MOLT-4. Individual colonies were isolated to assure homogenous population, and developed for a number of passages in puromycin medium. As the MOLT-4 + psh Neil3 and MOLT-4 + psh Gapdh colonies was confirmed. To prevent unusual gene expression resulting from the existence of puromycin , the antibiotic was removed from the culturing medium.

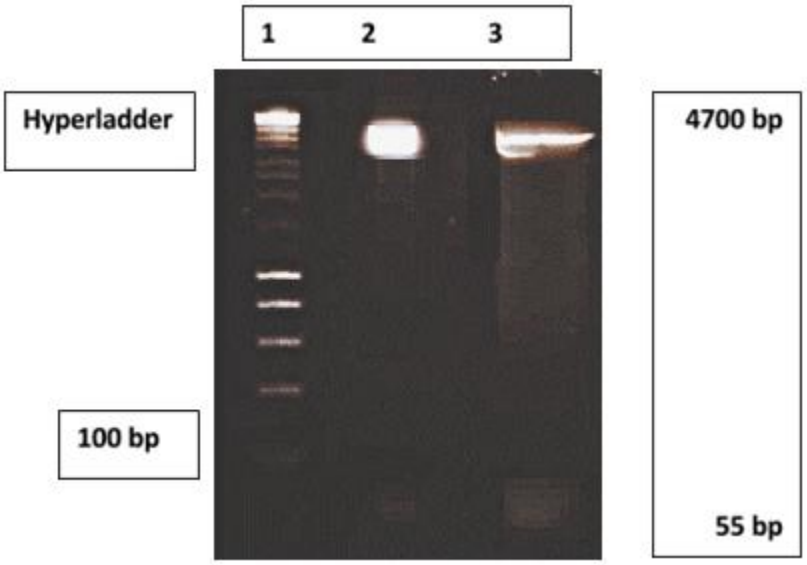


Fig (3): Purified plasmid DNA for Neil3, Gapdh from transformed E.coli
Lane 1: Hyperladder, Lane2 : pNeil3, Lane 3: pGapdh

Potential knockdown of Neil3 and Gapdh genes .

RNA was extracted from transfected MOLT-4 +pNeil3, and MOLT-4 +pGapdh clones, and cDNA were generated by reverse transcription PCR to project for potential reduction in Neil3 and Gapdh gene expression. The oligonucleotide primers characteristic for Neil3 and Gapdh genes were mixed with cDNA samples and subjected to PCR to decide any alteration in the levels of mRNA for both neil3 and Gapdh genes. Fig(4) , PCR screen showed reduced intensity for both Neil3 and Gapdh genes as compared to MOLT-4 with empty vector cDNA. This represents a potential Neil3 and Gapdh knockdown target. This result revealed that Neil3 knockdown underscore the usefulness of this knockdown on human tumor prolifrative and developing, (no references were found that support this result) Further studies is needed on MOLT-4 phenotyping to show the effect on Neil3 transfection the cancer proliferation and prognosis.

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