February 2016

Ph.D. Thesis

miR-145 regulates non-homologous end joining by targeting DNA-PKcs

Graduate School of Chosun University

Department of Bio-Materials

Muddenahalli Srinivasa Sudhanva

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miR-145에 의한 비상동말단 결합 조절 기전 연구

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Advisor: Prof. Ho-Jin You

A dissertation submitted to the Graduate School of Chosun University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Science

October 2015

Graduate School of Chosun University

Department of Bio-Materials

Muddenahalli Srinivasa Sudhanva

The Ph.D. dissertation of

Muddenahalli Srinivasa Sudhanva is

certified by

Chairman :

Chosun Univ. **Prof. In-Youb Chang** _____

Committee Members:

Chosun Univ. **Prof. Young-Jin Jeon** _____

Jeju Univ. **Prof. Yoon Sang Pil** _____

Chosun Univ. **Prof. Jung-Hee Lee** _____

Chosun Univ. **Prof. Ho Jin You** _____

December 2015

Graduate School of Chosun University

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LIST OF ABBREVATIONS

ASO	Anti sense oligonucleotides
ATCC	American type collection center
cDNA	Complementary DNA
DAPI	4, 6-diamidino-2-phenylindole
DMEM	Dulbeco modified eagle's medium
DNA	Deoxy ribo nucleic acid
DNAPKC'S	DNA dependent protein kinase catalytic subunit
DSB	Double strand break
EDTA	Ethylene diamine tetra acetic acid
EMEM	Eagle's minimum essential medium
FBS	Fetal bovine serum
GFP	Green fluorescent protein
GY	Gray
HR	Homologous recombination
IR	Irradiation
mRNA	Messenger RNA
miRNA	Micro RNA
NAOH	Sodium hydroxide
NACL	Sodium chloride
NHEJ	Non homologous end joining

NT	Nucleotide
NC RNA	Non coding RNA
PBS	Phosphate buffer saline
Pri miRNA	Primary miRNA
Pre miRNA	Precursor miRNA
PVDF	Polyvinylidene fluoride
RFP	Red fluorescent protein
RIPA	Radio immuno precipitation assay
RISC	RNA induced silencing complex
RNA	Ribo nucleic acid
RNA	RNA interference
RT PCR	Reverse transcription polymerase chain reaction
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
TBS-T	Tris buffer saline-tween
UTR	Untranslated region
γ-ray	Gamma ray
%	Percentage
°C	Degree centigrade
Co2	carbon dioxide

국문초록

miR-145에 의한 비상동말단결합 조절기전 연구

무데나하일 스리니와사 수단와

지도교수: 유호진

조선대학교 일반대학원

생물신소재학과

DNA-PKcs 단백질은 이중나선절단에 의한 DNA손상 자극시 비상동말단결합 활성에 중요한 역할을 하는 단백질중의 하나이다. DNA-PKcs 단백질의 기능 소실은 DNA손상복구 결함을 야기함으로써 유전체 불안정성을 유도한다. 그러나 이런 DNA-PKcs단백질 자체의 발현 조절기전 연구는 미흡한 실정이다. 본 연구를 통해 DNA-PKcs 발현을 조절하는 microRNA로 miR-145를 동정하였다. miR-145는 DNA-PKcs의 3`UTR에 결합하여 DNA-PKcs발현을 억제하고 이중나선절단에

따른 DNA-PKcs 활성을 저해함을 comet assay와 γ-H2AX foci 형성을 통해 확인하였다. 또한 miR-145 저해제와 DNA-PKcs cDNA 과발현을 통해 miR-145가 DNA-PKcs의 발현과 활성을 조절함을 재확인하였다. 이상의 결과를 토대로 miR-145가 DNA-PKcs 단백질 발현을 조절함으로써 비상동말단 결합에 관여함을 증명하였다. 또한 본 연구는 DNA 손상복구를 조절하는 새로운 분자적 기전을 제시한다.

I INTRODUCTION

1. Importance of NHEJ in DSB repair mechanism

The human genome is constantly under immense stress from agents generated from both inside and outside of the cell. These damages on the genome results in the generation of thousands of DNA lesions per day in the cells like base insertion, base deletion, pyrimidine dimmers, single strand break and double strand break, with the most deleterious of these lesions being the DNA double strand break (DSB). Introduction of a DSB can be mediated by a variety of means including those by endogenous sources like reactive oxygen species generated by cellular metabolism and replication associated errors and exogenous sources including ionizing radiation and chemotherapeutic agents. Unrepaired or misrepaired DSBs can result in senescence, induced apoptosis, or chromosomal aberrations including translocations and deletions which can result in a loss of heterozygosity. These chromosomal aberrations are associated with genomic instability and can ultimately result in carcinogenesis; therefore, it is of the utmost importance that cells have a mechanism(s) to quickly repair DSBs. A number of highly efficient DSB repair pathways have evolved in eukaryotic cells with non-homologous end-joining (NHEJ) and Homologous recombination (HR), NHEJ likely playing the largest role in DSB repair in humans. The NHEJ pathway is

also essential for V (D) J recombination during T- and B-cell lymphocyte development.

NHEJ mediates the direct religation of the broken DNA molecule. It has the potential to religate any type of DNA ends and unlike the other classically studied DSB repair mechanism, homologous recombination (HR), NHEJ does not require a homologous template for repair of the DNA lesion. Since NHEJ does not require a homologous template, theoretically it is not restricted to a certain phase of the cell cycle, whereas HR is believed to be only active during S and G2 phases of the cell cycle when a homologous template via the sister chromatid is available. The general mechanism of NHEJ can be broken down into individual and sequential steps which are: (I) DNA end recognition and assembly and stabilization of the NHEJ complex at the DNA double strand break; (II) Bridging of the DNA ends and promotion of end stability; (III) DNA end processing; and (IV) Ligation of the broken ends and dissolution of the NHEJ complex. DNAPKC's known to be an important candidate in NHEJ repair which plays a major role in repair of double strand DNA breaks. DNAPKC's involved in many cellular processes, DNA damage response and in DNA repair. DNAPKC's can interacts with lot of proteins other than DNA repair proteins which involves regulation of apoptotic process, cellular senescence, cellular protein modification T-cell differentiation, telomere maintenance, fibroblast and smooth cell proliferation, innate immune response and VDJ recombination, spleen and thymus development and others…

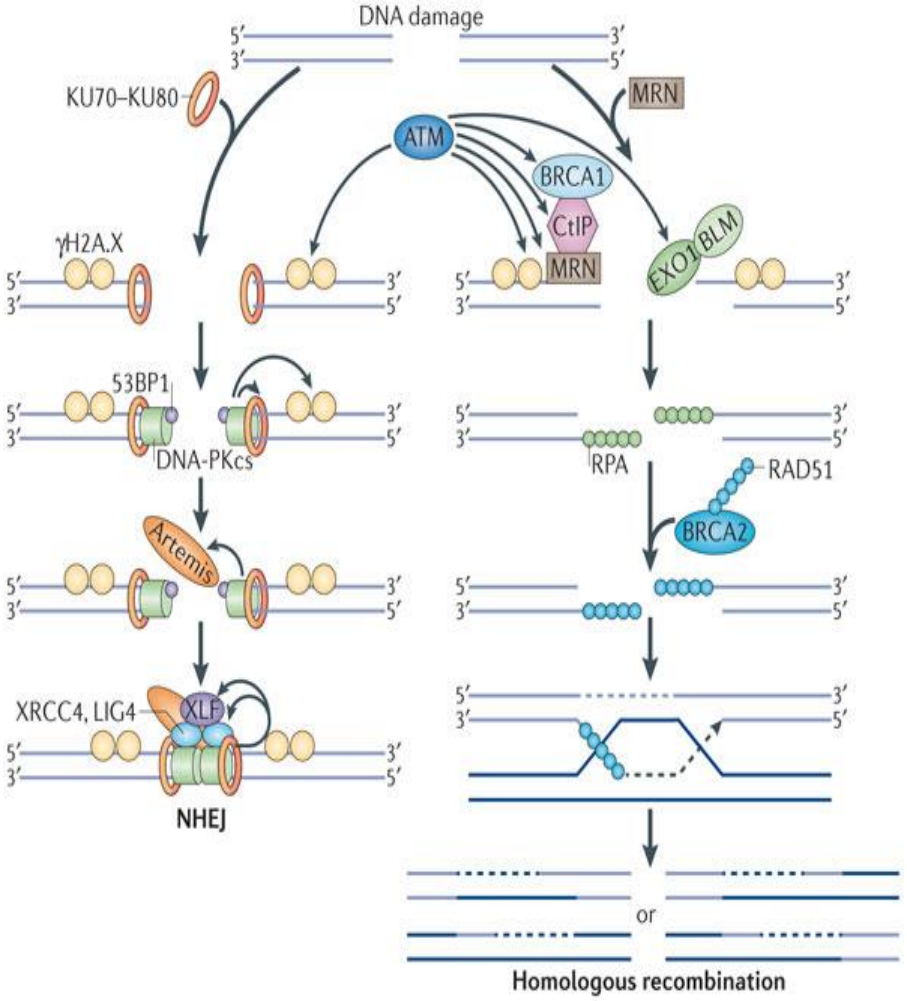


Figure 1. The two major mammalian pathways for double-strand break repair.

2. Micro RNA biogenesis and its role in regulation of DNA repair proteins and cancer metabolism.

MicroRNAs (miRNAs) are short non-coding RNAs, which are viewed as fundamental regulators of cell function. MiRNAs are approximately 20–25 nucleotides (nt) long and are generated from double stranded RNA precursors. One strand is selected and interacts with a member of the Ago protein family to form a miRNA induced silencing complex (miRISC). The miRNA guides such complexes to partially complementary target RNAs. Most of the target sites are located within the 3' untranslated region (UTR) of mRNAs. However, functional miRNA binding sites in the 5' UTR have also been reported. Nucleotides 2–8 of the miRNA are particularly important for pairing with the target mRNA. This sequence motif is referred to as the miRNA seed sequence. Depending on the recognition site, binding of miRISC to the cognate target can have different outcomes. In case the target site is perfectly complementary to the miRNA, the miRNA functions like short interfering RNAs (siRNAs) and the target is sequence-specifically cleaved by miRISC. This is very rare in mammals but more frequent in plants. Binding to partially complementary target sites is the rule in mammals and results in translational repression or degradation of the target transcript (mRNA decay). This degradation process, however, is different to RNAi (RNA interference) like mechanisms and involves the recruitment of various complexes to the mRNA to remove or shorten the poly (A) tail. Poly (A) tail shortening induces the removal of the 5' cap of the mRNA, a process referred to as de-capping. Uncapped mRNAs are

rapidly removed from the cell by 5' to 3' exoribonucleases. Ribosome profiling experiments in conjunction with mRNA level measurements revealed that mRNA decay accounts for approximately 85%, while translational repression contributes only about 15% to miRNA-guided gene silencing effects in mammals.

MiRNAs are also involved in regulating various proteins which are involved in DNA repair during the cell stress condition, cells expresses various miRNA to regulate protein expression and varies the repair mechanism. MiR-145 is a tumor suppressor micro RNA and its expression will be suppressed in many cancer cell types. MiR-145 plays major role in NHEJ by targeting most fundamental protein in NHEJ (Non Homologous End joining mechanism) DNAPKC's. Overexpression of miR-145 results in suppression of NHEJ activity in cells.

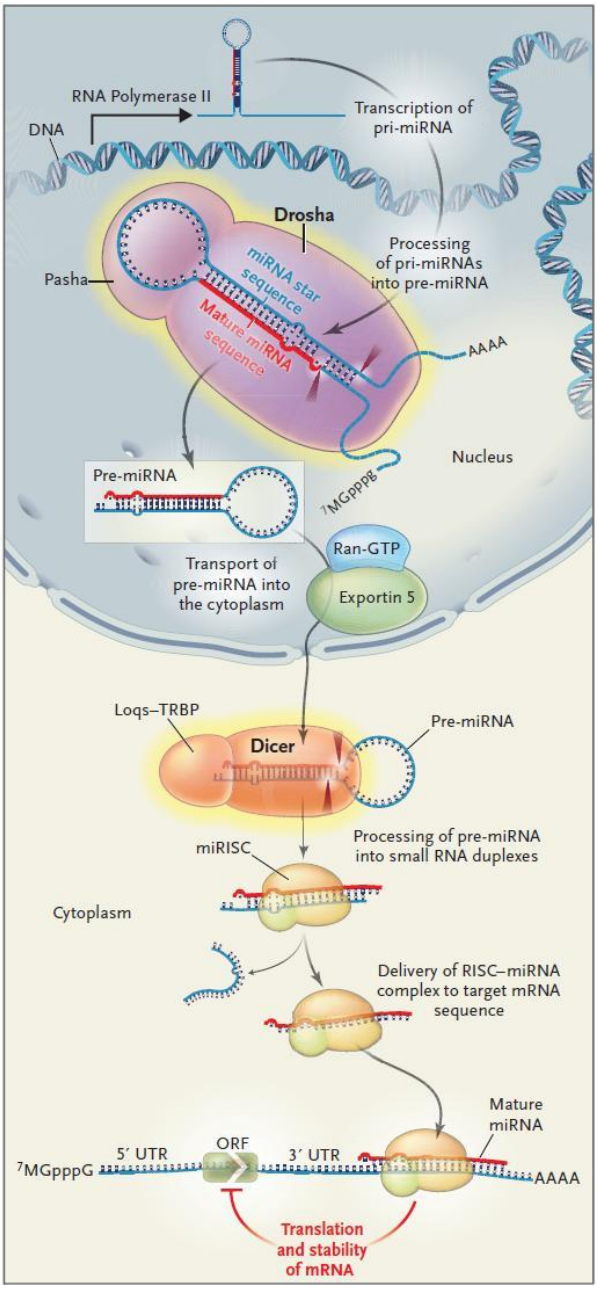


Figure 2: MiRNA biogenesis and its regulation: miRNAs will be processed by many ways the schematic diagram representing its biogenesis through RNA

polymerase and its processing by drosha inside the nucleus and by dicer in the cytosol and combines with the ago to form a complex called RISC (RNA induced silencing complex). The matured miRNA along with RISC complex finally leads to translation repression sometimes mRNA degradation by decapping and deadenylation.

II MATERIALS AND METHODS

Cell culture and IR treatment

The human cervix adenocarcinoma cell line HeLa, the human embryonic kidney cell line HEK293T and U2OS the human bone osteocarcinoma cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Grand Island, NY, USA). The MCF-7 human mammary gland adenocarcinoma cell line was cultured in Eagles minimum essential medium (EMEM) (Gibco-BRL). In both cases, the media was supplemented with 10% heat-inactivated fetal bovine serum (Cambrex Corp., East Rutherford, NJ, USA), 100 units/ml penicillin, and 100mg/ml streptomycin sulfate (Invitrogen, Carlsbad, CA, USA). All cells were maintained in a humidified incubator containing 5% CO2 at 37°C. HeLa and U2OS and MCF-7 cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), and the HEK293T cell line was obtained from the Cornell Institute for Medical Research (New York, NY, USA). To induce DNA breaks, exponentially growing cells were irradiated at 5 Gray (Gy) from 137Cs source (Gamma cell 3000 Elan irradiator, Best Theratronics, Ottawa, Canada) and allowed to recover at 37°C for various amounts of time.

Cell transfection

Cells were transiently transfected using Lipofectamine-2000 reagent (DNA) and RNA-imax (RNA) (Invitrogen), according to the manufacturer's recommendations. In brief, cells were seeded in 100 mm dishes 24 h before transfection and were transiently transfected at a confluency of 40– 50%. Mock transfection, which only had the transfection reagent, was also used as a control. The transfection mixture was dissolved in Opti-MEM serum-free media (Invitrogen) and at the time of transfection cells were seeded in appropriate medium, with 10% heat-inactivated fetal bovine serum (Cambrex Corp., East Rutherford, NJ, USA) with no antibiotics. On the subsequent day the media was changed with appropriate media containing both 10% FBS and 1% antibiotic 100 units/ml penicillin, and 100mg/ml streptomycin sulfate (Invitrogen, Carlsbad, CA, USA). Cells were pelleted after 48-72 h of transfection for flow cytometry, RNA and protein extraction.

RNA extraction and Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cultured cancer cells, using TRIzol (Invitrogen) reagent. For quantitation of pre-miR-145, cDNA was synthesized using 1 μg of total RNAs, random hexamer (Promega) and M-MLV reverse transcriptase (Invitrogen). Real-time PCR analysis was performed using the SYBR green-based fluorescent method (SYBR premix Ex Taq kit, TaKaRa Bio) and the MX3000P®

qRT-PCR system (Stratagene) with specific primers. Primers used for real-time PCR are as follows: pre-miR-145 forward 5'-TCCTCACGGTCCAGTTTT-3' pre-miR-145 reverse, 5'-GTATTTCCAGGAATCCCC-3'. To quantify miRNAs, cDNA was synthesized using Mir-X^{TM} miRNA first-strand synthesis and SYBR qRT-PCR kit (Clontech) according to the manufacturer's instructions. The quantity of transcripts was calculated based on the threshold cycle (C_t) using the delta-delta C_t method that measures the relative of a target RNA between two samples by comparing them to a normalization control RNA (*gapdh* or *U6*).

Generation of stable miR-145 expression clones

The pSilencer2.1–U6 neo vector was obtained from Ambion (Austin, TX, USA The miRNA sequences are selected on the basis of an online application miRBase (http://www.mirbase.org/) Accession No. MIMAT0000437. The following sequences of oligonucleotides were used: miR-145 top 5'-GATCCgtccagttttcccaggaatccctTTCAAGAGAagggattcctgggaaaactggacTTTTTTG GAAA-3': miR-145 bottom 5'-AGCTTTTCCAAAAAAgtccagttttcccaggaatccctTCTCTTGAAagggattcctgggaaaact ggacG-3'. Vectors for expression of miRNAs were constructed by inserting corresponding pairs of annealed DNA oligonucleotides into the pSilencer 2.1–U6 vector between the BamHI and HindIII restriction sites according to the manufacturer's instructions. The resulting vector was named pSilencer/miR-145. To generate the stable miR-145 expressing clones HeLa cells and U2OS cells are

transfected with pSilencer/miR-145 vector construct. Twenty-four hours after transfection, 400mg/ml G418 was added to the culture medium for selection. After selection, stable clones were analyzed by real-time RT-PCR and western blotting to confirm overexpression of miR-145 and the down regulation of target protein (DNAPKC's).

Antibodies

The following antibodies were used for immunoblotting: mouse monoclonal anti-DNAPKC's (1:1000 Neo markers/ Thermo scientific laboratories), monoclonal anti- DNAPKC's (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal anti-Ku86 (1:1000; Santa Cruz Biotechnology), polyclonal anti-Ku70 (1:1000; Santa Cruz Biotechnology), anti-GAPDH mouse monoclonal antibody (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and monoclonal anti-α-tubulin (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). γ-H2AX foci were detected by immuno fluorescence staining using the γ-H2AX mouse monoclonal antibody (JBW301, Upstate Biotechnology, Temecula, CA, USA) at a 1:200 dilution.

Western-blot analysis

Cells were lysed in ice-cold RIPA lysis buffer 150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl

sulphate) 50 mM Tris, pH 8.0 2mM EDTA and protease inhibitor cocktail (Roche, Basel, Switzerland). Equal amounts of proteins were then resolved on 6–15% SDS-PAGE gels, followed by electrotransfer to polyvinylidene difluoride membranes (PVDF) (Millipore, Bedford, MA, USA). The membranes were blocked for 1h in TBS-T [10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20] containing 5% fat-free milk at room temperature and then incubated with the indicated primary antibodies overnight at 4°C. Membranes are washed and incubated with appropriate secondary antibodies for 1 h at room temperature and membranes are developed using enhanced chemi-luminescence detection system.

Immunostaining

To visualize γ-ray-induced foci, untreated cells or cells treated with 5Gy γ-ray were cultured on coverslips coated with poly-L-lysine (Sigma). Cells were washed twice with PBS and fixed in 100% ice cold methanol for 10 min, followed by permeabilization with 0.3% Triton X-100 for 15 min at room temperature. Next, the coverslips were washed three times with PBS, followed by blocking with 0.1% bovine serum albumin in PBS for 1 h at room temperature. The cells were immunostained using primary antibodies directed against the γ-H2AX proteins overnight at 4°C. The cells were then washed with PBS and stained with the appropriate Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (green and red fluorescence, respectively; Molecular Probes, Eugene, OR, USA). After washing, the coverslips were mounted onto slides using Vectashield

mounting medium containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). Fluorescence images were taken under a confocal microscope (Zeiss LSM 510 Meta; Carl Zeiss, Jena, Germany) and analyzed with Zeiss LSM Image Examiner software (Carl Zeiss). Percentage was calculated among at least 100 cells by dividing the number of γ-H2AX foci-positive cells by the number of DAPI-stained cells. The error bars represent standard error in three independent experiments.

Luciferase assay

Dual-Luciferase Reporter Assay System (DLR assay system, Promega, Madison, WI) was used to perform dual-reporter assays on pmir GLO based reporter systems. DLR Assay System was also used to measure luciferase activity of cells co-transfected with 3'UTR vectors and pGL4 control vector. Luciferase assay was conducted according to the manual of manufacturer (Promega, Madison, WI). After 24hr transfection, growth media were removed and cells were washed gently with phosphate buffered saline. Passive lysis buffer (Promega, Madison, WI) 100ul/well was added and with gentle rocking for 15min at room temperature cell lysates were harvested for DLR assay. 20ul of cell lysate were transferred to clean microfuge tube. Assay for Firefly and Renilla luciferase activity were performed sequentially to the cell lysate. For each luminescence reading, after injector dispensing assay reagents into each well, there would be a 2-second pre-measurement delay, followed by a 10-second measurement period, again a 2-

second pre-measurement delay for stop glow solution injecting, followed by a 10-second measurement period. Luciferase assays were analyzed based on ratio of Renilla/Firefly (pmir GLO based vectors) or Firefly/Renilla (co-transfecting 3'UTR- vectors with pGL4 vector as internal control) to normalize cell number and transfection efficiency.

Comet assay

DSB repair was assayed by alkaline single-cell agarose-gel electrophoresis as described previously. Briefly, control cells, stable miR-145 cells and transiently transfected cells were irradiated with γ-ray of 5 Gy dose followed by incubation in culture medium at 37°C for the indicated times. Cells were then harvested (~105 cells per pellet), mixed with low melting agarose, and layered onto agarose-coated glass slides. The slides were maintained in the dark for all of the remaining steps. Slides were submerged in lysis solution [10 mM Tris–HCl (pH 10), 2.5 M NaCl, 0.1 M EDTA, 1% Triton X-100, 10% dimethyl sulfoxide] for 1 h and incubated for 30 min in alkaline electrophoresis solution (300 mM NaOH, 200 mM EDTA at pH >13). After incubation slides were electrophorised (~30 min at 1 V/cm tank length), air-dried and neutralized and stained with SYBR green. Average comet tail moment was scored for 40–50 cells/slide using a computerized image analysis system (Komet 5.5; Andor Technology, South Windsor, CT, USA).

Cell survival assay

After treatment with IR, 5×102 cells were immediately seeded onto a 60-mm dish in duplicate and grown for 2–3 weeks at 37 ℃ to allow colony formation. Colonies were stained with 2% methylene blue in 50% ethanol and counted. The fraction of surviving cells was calculated as the ratio of the plating efficiencies of treated cells to untreated cells. Cell survival results are reported as the mean value ± standard deviation for three independent experiments.

Analysis of NHEJ activity

To analyze the effect of miR-145 on the proteins involved in NHEJ in vivo, we used the pEGFP-Pem1-Ad2 vector and pEND R/G vector system. The pEGFP-Pem1-Ad2 plasmid was digested with HindIII restriction endonuclease to remove Ad2 and generate staggered ends. pEND R/G vector system was digested with EcoRi and Xhoi respectively Supercoiled pEGFP-Pem1 and pEND R/G vectors was used as a positive control for standardization of transfection and analysis conditions. The pCMV-dsRed-express plasmid (Clontech) was co-transfected with either linearized pEGFP-Pem1-Ad2 or supercoiled pEGFP-Pem1 as a control for transfection efficiency. In a typical reaction, 5×102 cells were transfected with supercoiled or linearized pEGFP-Pem1-Ad2, together with pDsRed2-N1 plasmid and supercoiled or linearized pEND R/G (EcoRi and Xhoi) cut vectors, using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommended

protocol. Twenty-four hours after transfection, green (EGFP) and red (DsRed) fluorescence was measured by flow cytometry (FACS Calibur; BD Biosciences, San Jose, CA, USA).

Statistical analysis

Data are presented as means ± SD. Statistical comparisons were carried out using unpaired *t*- tests, and values of $P<0.01$ were considered to be statistically significant.

III Results

1. miR-145 suppresses DNA-PK expression by targeting 3' UTR of DNA-PKcs

DNAPKC is an important key regulatory protein in non homologous end joining repair mechanism. DNAPKC's 3' UTR as a partial complimentary sequence for microRNA 145 (fig 3) which binds to DNAPKC's UTR partially and results in translational repression of DNAPKC's gene which results in depletion of total DNAPKC's protein in the cell (fig 3 B and C). Immunoblot of HeLa, U2OS and MCF-7 cells showing depleted DNAPKC's protein level in transiently over expressed miR-145, GAPDH used as loading control and RT-PCR results showing high miR145 levels (fig 3).

2. Depletion of DNAPKC's delays DSB repair

Delayed repair of Double strand break results in accumulation of phosphorylated H2AX variant in the cells, H2ax proteins are the preliminary proteins that respond to DNA damage and phosphorylates at ser 198. Levels of γ-H2AX increases during the delay of DSB repair. Quantitative analysis of γ-H2AX foci is used to measure the efficiency of DNA repair after DSB induction by IR. We investigated the formation of γ-H2AX foci by immunoflorescence microscopy after IR treatment (fig 5). In control cells, the maximum number of γ-H2AX foci was recovered after ~12- 16h of IR treatment. Longer recovery times resulted in a

decline in the number of γ-H2AX foci. By 24 h after IR treatment, γ-H2AX foci were significantly decreased, indicating that the IR-induced DSBs were almost repaired. In contrast, stable miR145 expressing cells shows persisted γ-H2AX foci for longer period of time in compared to control cells. DNAPKC's deficient cells contained a significant amount of γ-H2AX nuclear foci even at 20 h after of IR treatment.

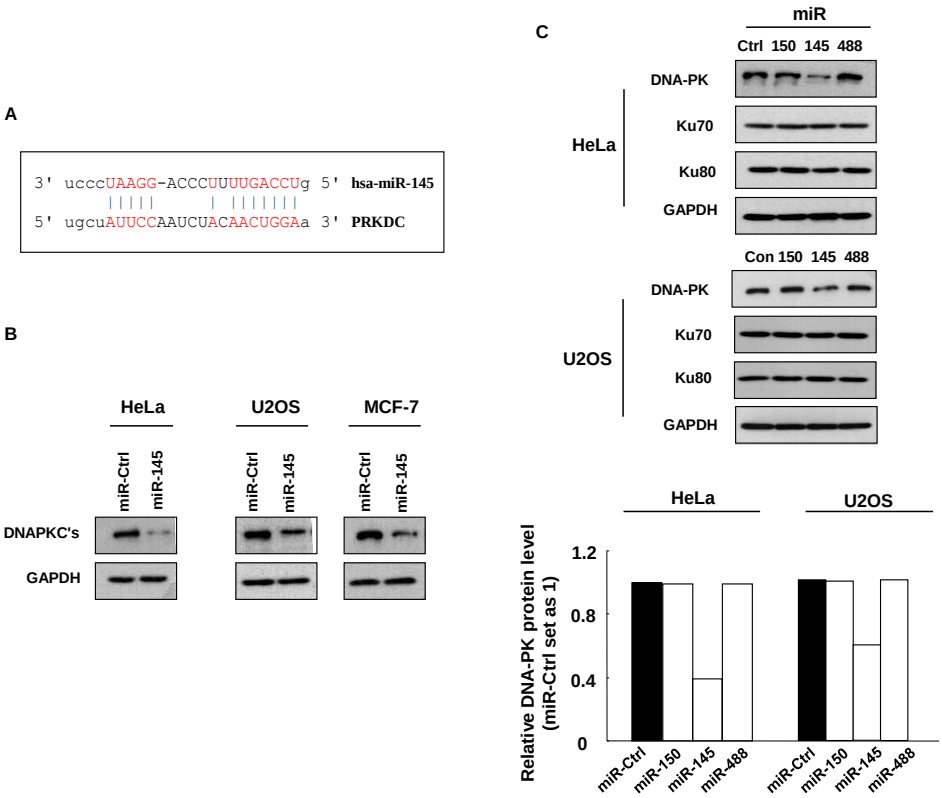


Figure 3: miR-145 binding to 3'UTR of DNAPKC's (A) partial complementary binding of miR-145 to 3'UTR of DNAPKC's (B) transient transfection of miR145

in HeLa, U2OS and MCF-7 cells respectively (C) depletion of DNAPKC's in transient transfection of HeLa cells and U2OS cells. (Lower panel) Relative DNAPKC protein levels with corresponding miRNA transfection miRctrl, miR150, miR145and miR488 miRNA respectively.

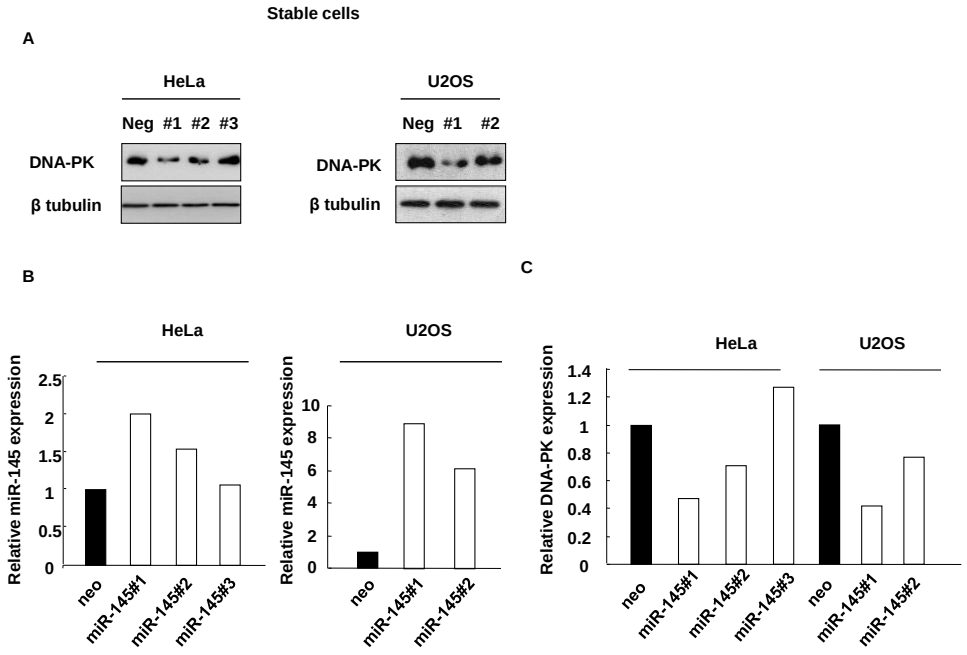


Figure 4: stable miRNA-145 expressing cells (A) HeLa cells and U2OS cell stably expressing miR-145, cells collected and immunoblotted for DNAPK, tubulin was used as loading control (B) relative miR-145 expression in stable miR-145 expressing cells (C) relative DNAPK protein expression in miR-145 stably expressing cells.

3. Persistence of DSB's in DNAPKC's depleted cells

To confirm the DSB repair delay we measured the persistence of DSB in IR treated miR-145 stably expressing HeLa cells and U2OS cells by single cell electrophoresis (comet assay). Comet is a very sensitive assay which can detect low levels of DNA breaks. IR treatment induces DSB's it appears as a comet tail in single cell electrophoresis. Unrepaired DSB's in cell were analyzed at various time after IR treatment, cells expressing stable miR-145 shows significantly lower efficiency of induced DSB compared to control cells (fig 6). Based on comet tail moment we estimate extent of DNA damage, result shows more than 3-4 fold unrepaired DSB accumulation in miR-145 overexpressed cells compared to control cells at 6h after IR induction. This results shows deficiency of DNAPKC's hinders the DSB repair after IR treatment.

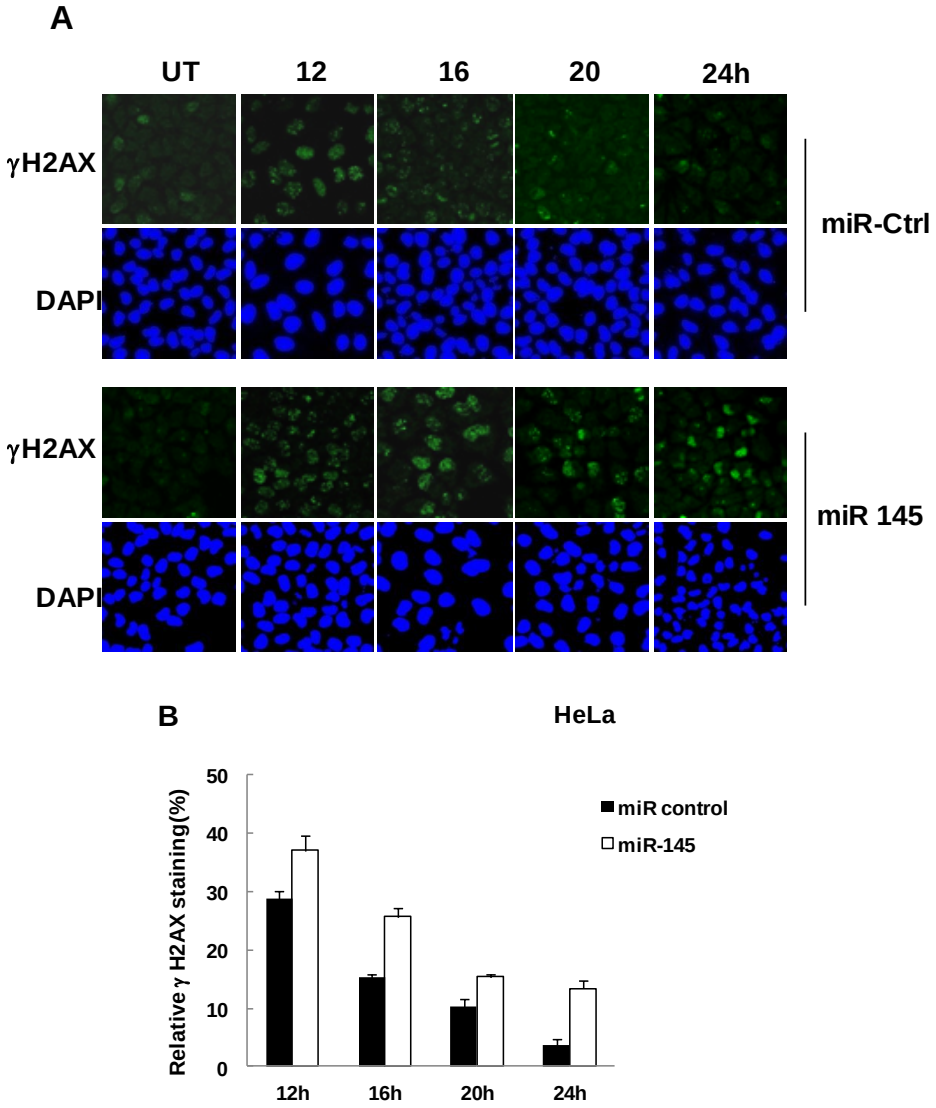


Figure 5: miR-145 stable expressing cells shows delayed repair (A) control and miR-145 expressing cells were treated with and without IR for indicated time and cells were fixed and stained with γ-H2AX antibody and nucleus was stained with DAPI.

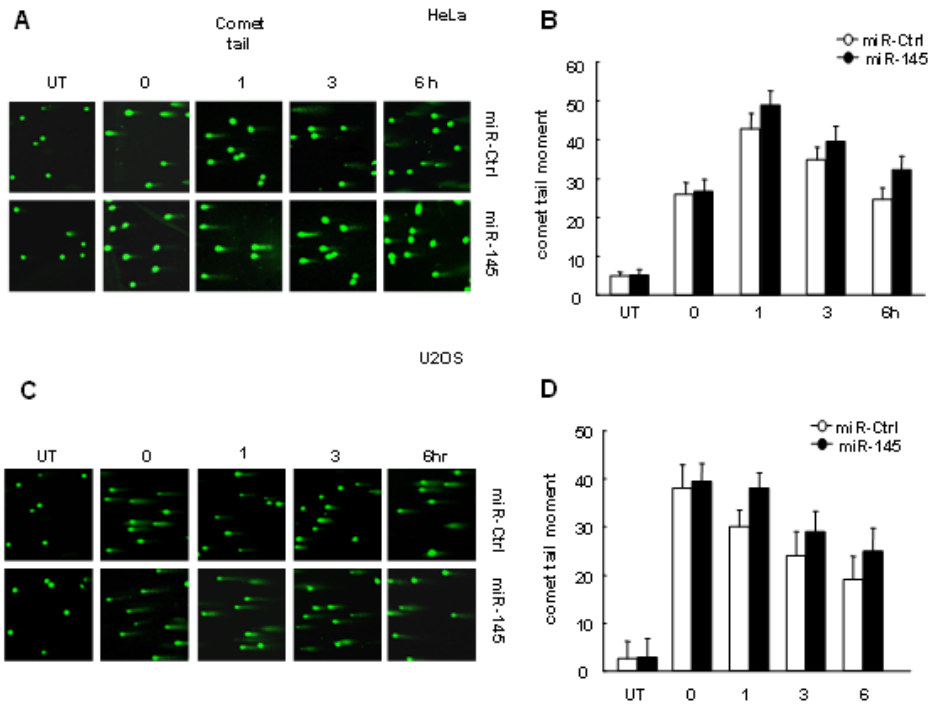


Figure 6: DNAPKC deficient cell delays DSB repair: (A) HeLa control and miR-145 stably expressing cells were treated with 5 Gy ionizing radiation and incubated for indicated time and cells were harvested and analyzed under alkaline condition and comet tails were measured under fluorescence microscope and tail moment was analyzed in 75–80 randomly chosen comets using Komet 5.5 analysis software (B and D) bar graph representing repair efficiency of control and miR-145 expressing cells (C) U2OS control cells and stable cells irradiated and analyzed under alkaline condition

4. Depletion of DNAPKC's leads to decreased NHEJ repair

DNAPKC's is an important candidate in NHEJ repair pathway, depletion of this candidate definitely alters the efficiency of DSB repair through non homologous end joining pathway, to investigate the effect of miR-145 on the proteins involved in NHEJ in vivo, we used the pEGFP-Pem1-Ad2 vector and pCMS-EGFP-dsRED vector system. The principal characteristic of the plasmid (pEGFP–Pem1–Ad2) used in this assay is the interruption of the EGFP sequence by a 2.4-kb intron derived from the rat Pem1 gene. An exon derived from adenovirus (Ad) was introduced into the middle of the intron, and it was flanked on both sides by HindIII restriction enzyme recognition sequences. In undigested or partially digested plasmids, GFP is not expressed because the Ad exon is efficiently incorporated into the GFP mRNA. However, when the plasmid is linearized by HindIII digestion, the Ad2 exon is removed, enabling expression of EGFP upon successful intracellular recircularization. EGFP expression can then be easily detected and quantified by flow cytometry. Transfection with the supercoiled pEGFP– Pem1 plasmid is used to evaluate EGFP-signal expression without the requirement for rejoining, and the pCMVdsRed-expression plasmid is used as a control to determine transfection efficiency for this assay. The pEGFP-Pem1-Ad2 plasmid was digested with HindIII restriction endonuclease to remove Ad2 and generate staggered ends. To investigate the efficiency of NHEJ activity, control cells and miR-145 stably expressing cells are transfected with either linearized pEGFP–Pem1–Ad2 or supercoiled pEGFP–Pem1 together with pDsRed2–N1.The

cells were then incubated for 24 h to allow expression of EGFP (green) and DsRed (red), followed by flowcytometry analysis (fig 7). To control for the efficiency of transfection, the ratio of GFP+ cells to DsRed+ cells was used as the normalized measure of NHEJ efficiency. pCMS-EGFP-dsRED vector system was digested with EcoRI and XhoI to cut multi restriction enzyme site which having red fluoresemce and generate leniarized vectors. Supercoiled pCMS-EGFP-dsRED vectors were used as a positive control for standardization of transfection and analysis conditions. The pCMV-sport6-expression plasmid (Clontech) and pEGFP-N3 supercoiled plasmids used as control for transfection efficiency.control and miR-145 stably expressing cells were transfected with linearized pCMS-EGFP-dsRED or supercoiled pCMS-EGFP-dsRED together with pCMV-sport6 and pEGFP-N3 plasmid, using lipofectamine 2000 (Invitrogen) according to manufactures recommended protocol. Twenty-four hours after transfection, green (EGFP) and red (DsRed) fluorescence was measured by flow cytometry (FACS Calibur; BD Biosciences, San Jose, CA, USA). NHEJ activity was significantly decreased in miR-145 staby expressing cell compard to control cells in both the vector indicating micro RNA interfered NHEJ activity by targeting DNAPKC's.

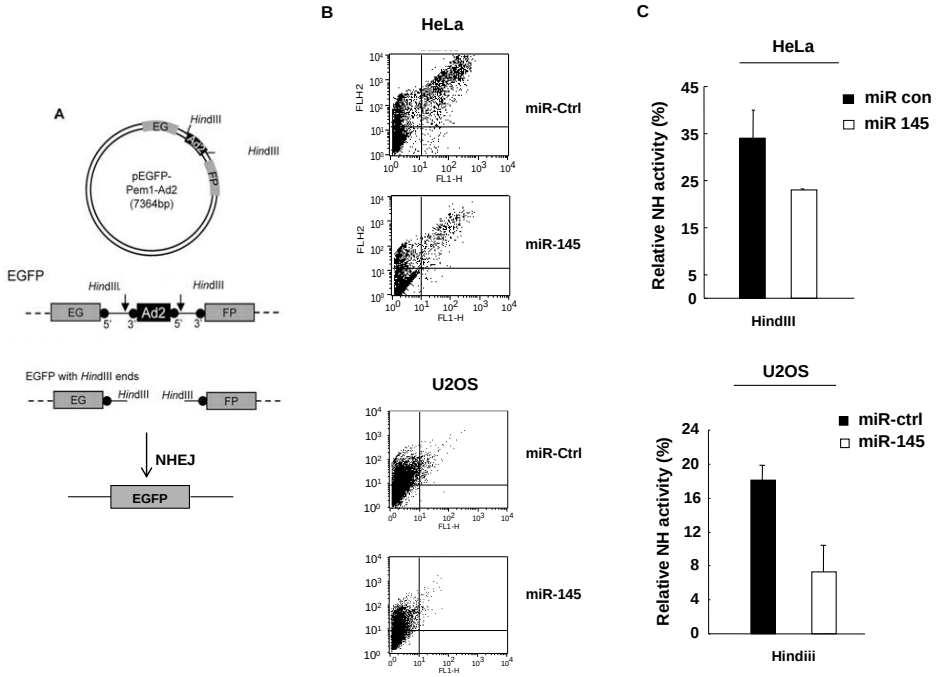


Figure 7: Effect of miR-145 on NHEJ repair activity. (A) Map of the pEGFP–Pem1–Ad2 vector. The reporter substrate consists of the GFP gene under control of the CMV promoter. The GFP gene contains an engineered intron from the rat Pem1 gene, which is interrupted by an adenoviral exon (Ad) that is flanked byHindIII recognition sites. In this construct, the GFP gene is inactive; however, after digestion with HindIII and successful NHEJ, the construct expresses GFP (B) Control and miR-145 overexpressed HeLa cells (DNAPKC's deficient cell) were transfected with supercoiled pEGF–Pem1 or HindIII-linearized pEGFP-Pem–Ad2 together with pCMV-dsRed-express. To quantify NHEJ events, the cells were analyzed by flow cytometry 24 h after transfection. DsRed expression was used to

normalize transfection efficiency. The ratio of GFP-positive (GFP+) to DsRed-positive (DsRed+) cells was used as a measure of NHEJ (C) Relative levels of plasmid rejoining compared to control miRNA-transfected cells were calculated by dividing the GFP+/DsRed+ ratios of the samples and plotted. Means are representative of at least three independent experiments. Error bars indicate the SD.

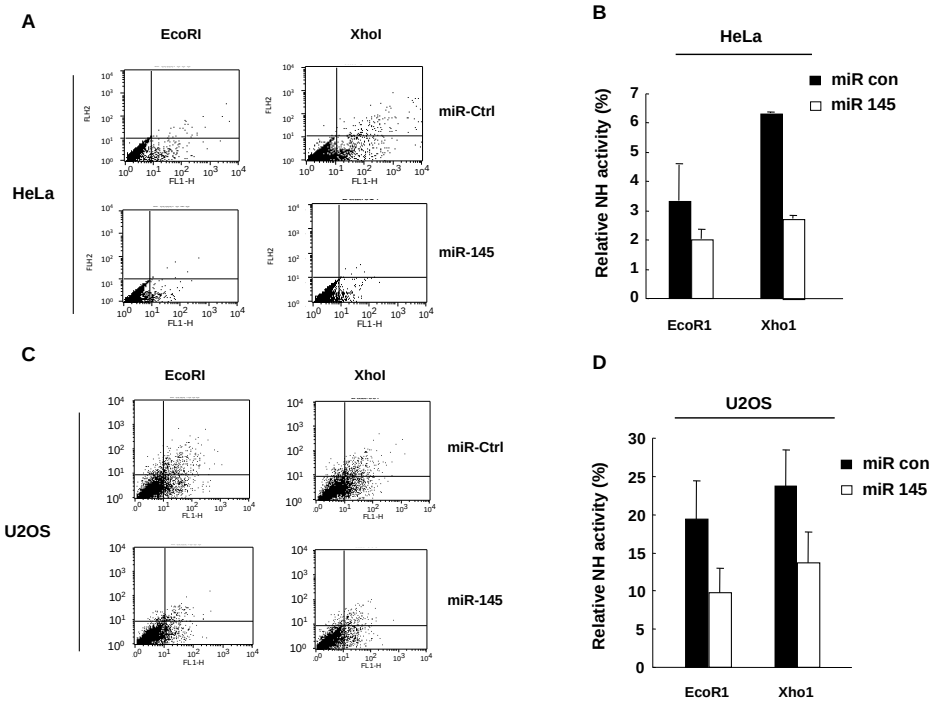


Figure 8: Effect of miR-145 on NHEJ. (A) Control and miR-145 overexpressed HeLa cells (DNAPKC's deficient cell) were transfected with linearized pCMS-EGFP-dsRED or supercoiled pCMS-EGFP-dsRED. To quantify NHEJ events, the cells were analyzed by flow cytometry 24 h after transfection. The ratio of GFP-positive (GFP+) to DsRed-positive (DsRed+) cells was used as a measure of NHEJ (C) Relative levels of plasmid rejoining compared to control miRNA-transfected cells were calculated by dividing the GFP+/DsRed+ ratios of the samples and plotted. Means are representative of at least three independent experiments. Error bars indicate the SD.

5. Dual luciferase assay in miR-145 expressing cells.

Dual-Luciferase Reporter Assay System (DLR assay system), was used to perform dual-reporter assays on pmir GLO based reporter systems. The priniciple of DLR luciferase assay system is the luciferase gene is inserted to vector after that a MCS region for inserting target 3'UTR, suitable miRNA interacts with the target 3'UTR and resluts in reduced lucifearse gene expression. pmir GLO vector was constructed with the insertion of 3' UTR of WT (wild type) DNAPKC's and 3' UTR of mut (mutant) DNAPKC's. Vector expressing WT and mut DNAPKC's 3' UTR was transected to the cells expressing miR-145 and incubated for 24 hrs and cells were collected for assay (fig 9). The cells expressing DNAPKC's WT 3' UTR showed less luciferase activity compared to cells expressing DNAPKC's mut 3' UTR.

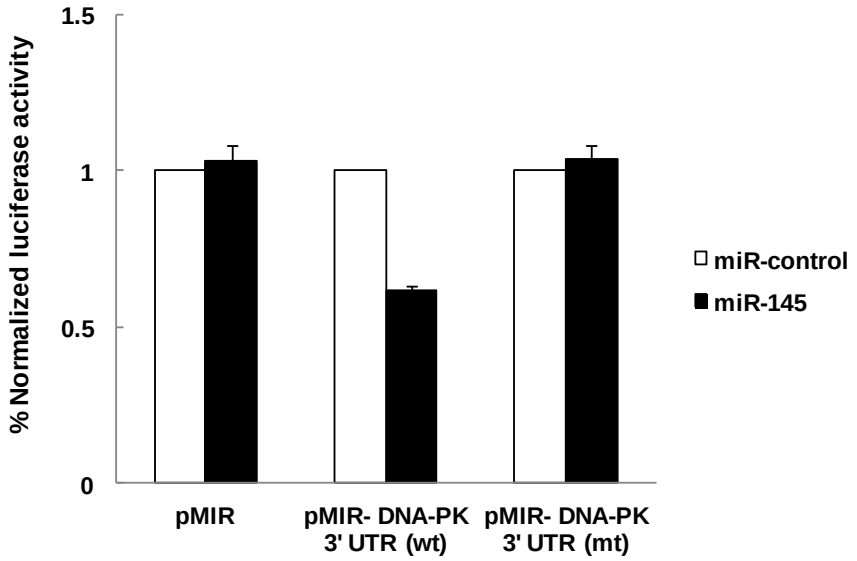
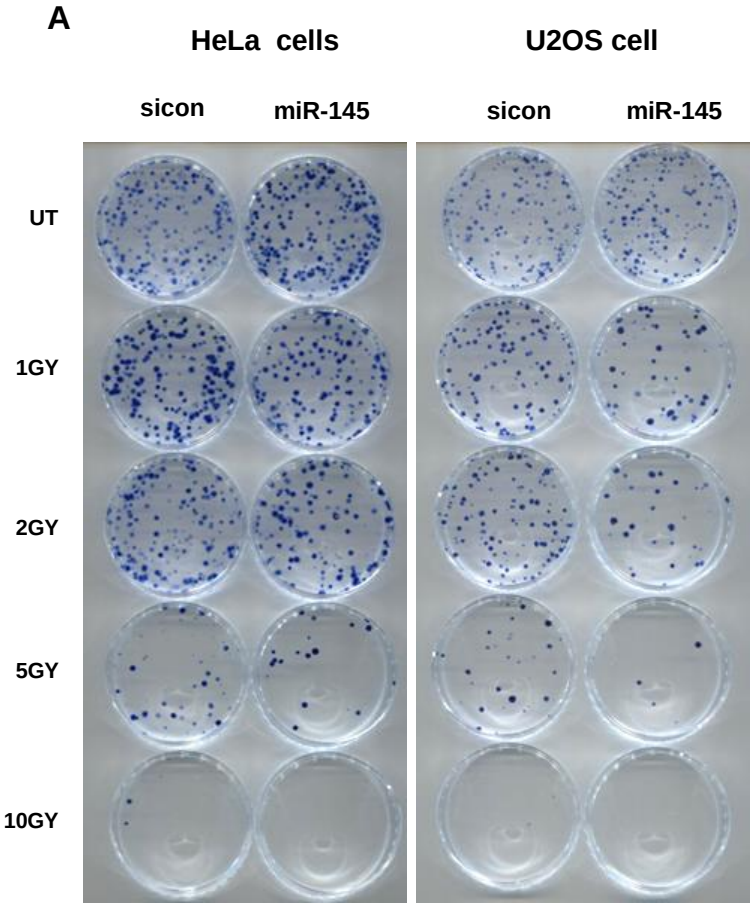


Figure 9: Lcuiferase assay: HeLa cells expressing miR-145 were transfected with pmiR control vector, pmiR DNAPK WT 3'UTR and DNAPK mut 3' UTR and incubated for 24 hrs and subjected for luciferase assay. Data represent the mean ±SD of three independent experiments.

6. DNAPKC's depleted cells are radio sensitive to IR irradiation.

Cells lacking or depletion of any DNA repair proteins are generally sensitive to IR. DNAPKC's depleted cells will be under immense stress due to lack of important candidate in non homologous end joining repair mechanism of induced double strand breaks. To examine the radio sensitivity of DNAPKC's depleted cells, cells were exposed to IR and examined for colony formation and cell survival. miR-145 stably expressing cells were irradiated and plated for colony formation. The cells expressing miR-145 stably (DNAPKC's depleted cells) shows less colonies or survival ability than the control miRNA expressing cells.



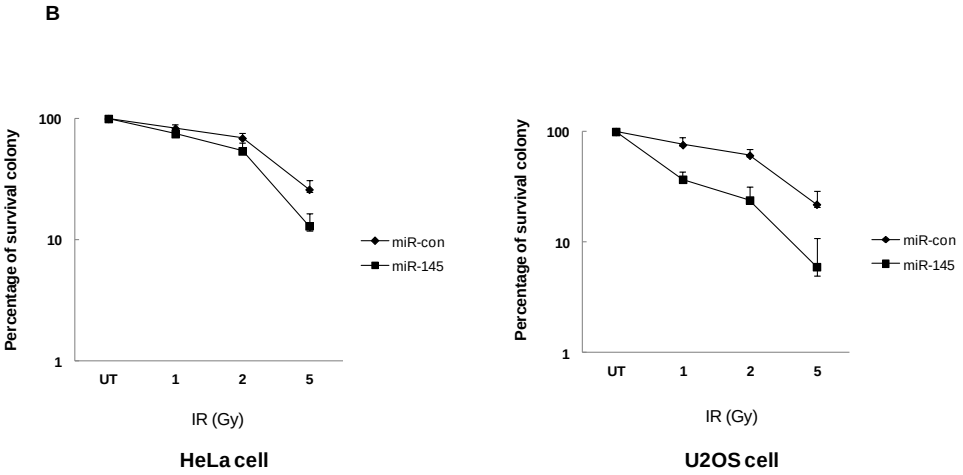


Figure 10: Radio sensitivity of miR-145 stably expressing cells (DNAPKC depleted cells). (A) miRcontrol and miR-145 stably expressing HeLa cells and U2OS cells were untreated or treated with the indicated doses of ionizing radiation, after 2 weeks cells were fixed and stained with methylene blue and cell survival was assessed using a clonogenic assay (B) graph representing percentage of survival cells. Data represent the mean ±SD of three independent experiments.

7. DNAPKC's recovery by antagonizing miR-145 effect by transient transfection of ASO-145 and DNAPKC's overexpression

Micro RNA's target 3'UTR of the target proteins and results in protein regulation. These micro RNA's can be antagonized with ASO (Anti Sense Oligo nucleotides), they have exact complementary sequence to corresponding miRNA. They compete with target proteins and bind strongly to miRNA and neutralize its action. Transiently transfected cells with miR-145 and ASO-145 showing depleted and recovered levels of DNAPKC's protein (fig 10). Transient overexpression of plasmid containing DNAPKC's cDNA, recovers the DNAPKC's protein level in depleted cells (fig 11).

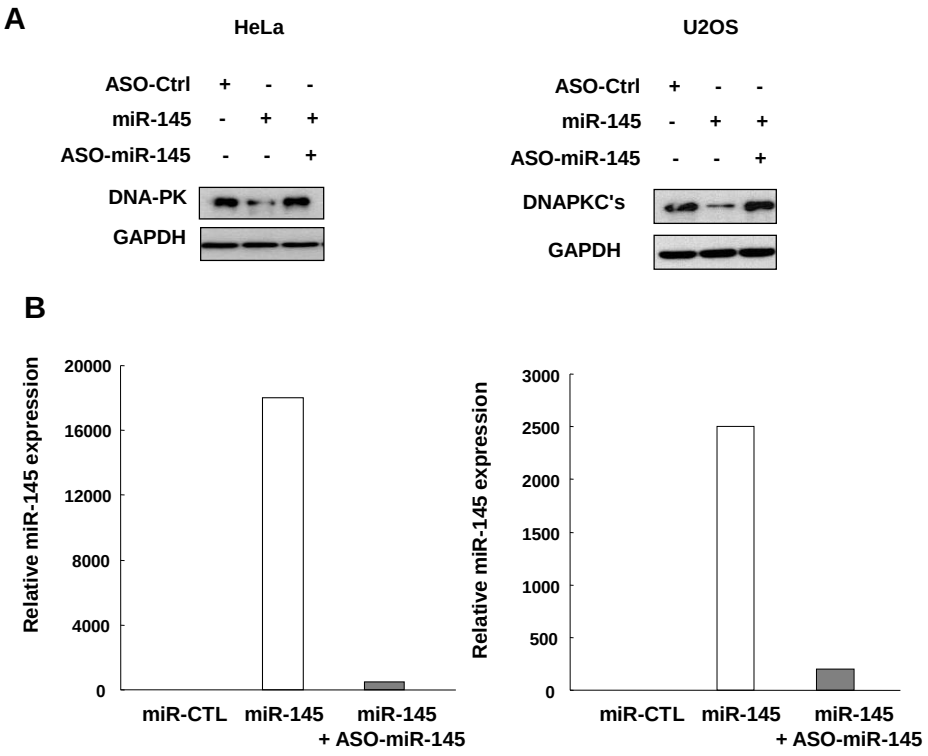


Figure 11: Recovery of DNAPKC's levels with ASO-145. (A) HeLa cells and U2OS cells were transiently transfected with control, miR-145 and ASO-145 and incubated for 24 hours and harvested for immuno blot (B) bar graph showing relative miR-145 levels in corresponding transfected cells.

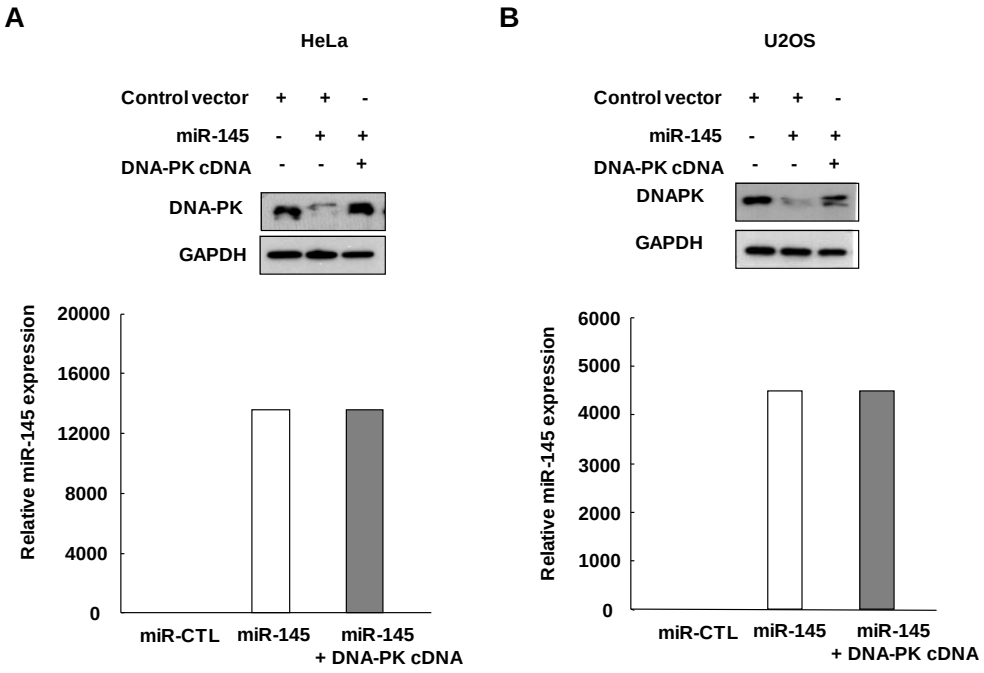


Figure 12: Recovery of DNAPKC's levels by over expressing DNAPKC's. (A and B) HeLa cells and U2OS cells were transiently transfected with control vector, miR-145 and miR-145+DNAPKC cDNA and incubated for 24 hours and harvested for immuno blot GAPDH was used as loading control in the experiments. Bar graph showing relative miR-145 levels in corresponding transfected cells.

8. Recovery of delayed DSB repair by ASO-145 transfection and DNAPKC's over expression transiently.

DNAPKC's depleted cells(miR-145 expressing cells) showing delayed repair was confirmed in comet assay to recover the delayed DSB repair, HeLa and U2OS cells expressing miR-145 were antagonized with anti ASO-145 and the other set of cells were transiently over-expressed with the plasmid containing DNAPKC's cDNA and irradiated with IR and subjected to single cell gel electrophoresis (comet assay). IR treatment induces DSB's it appears as a comet tail in single cell gel electrophoresis. Unrepaired DSB's in cell were analyzed at various time after IR treatment, cells expressing stable miR-145 shows significantly lower repair efficiency compared to control miR cells and cells expressing ASO-145 and overexpressed DNAPKC's (fig 12). The cells antagonized with ASO-145 and DNAPKC's over-expressing cells recoverd the repair ability and showed less tail in single cell gel electrophoresis. Based on comet tail moment we estimate extent of DNA damage. Result shows accumulated unrepaired DSB's in miR-145 overexpressed cells were reduced after antagonizing with the ASO-145 and in DNAPKC's overexpressed cells. This results shows deficiency of DNAPKC's hinders the DSB repair after IR treatment and the anti miR145 and overexpression of DNAPKC's can restores the miR-145 effect.

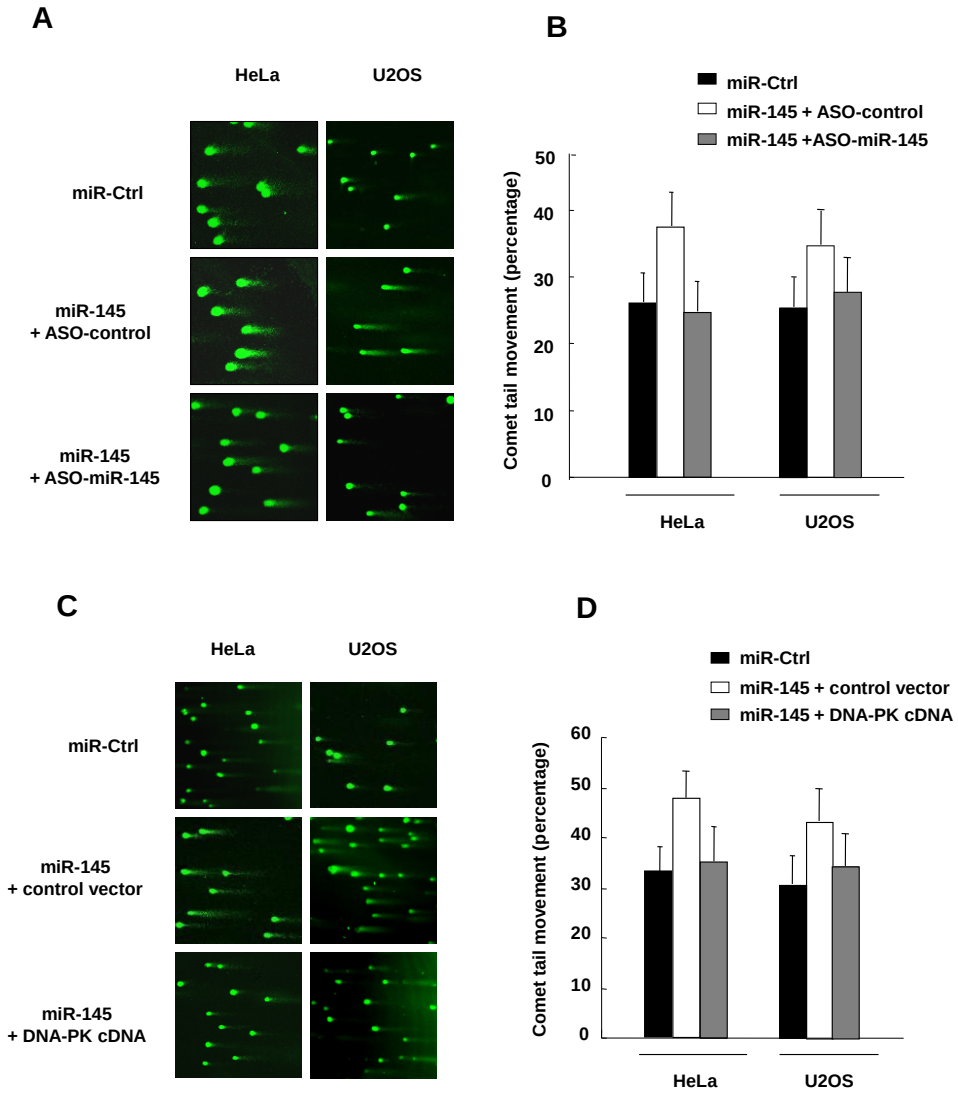


Figure 13: Recovery of delayed DSB repair by ASO-145 transfection and DNAPKC's overexpression. (A) HeLa cells U2OS cells were transiently transfected with miR-con, miR-145 and ASO-145 combined with miR-145 (C) HeLa cells U2OS cells were transiently transfected with miR-con, miR-145 and DNAPKC's combined with miR-145 and exposed to 5 Gy of ionizing radiation and incubated

for 3h and cells were harvested and analyzed under alkaline condition and comet tails were measured under fluorescence microscope and tail moment was analyzed in 75–80 randomly chosen comets using Komet 5.5 analysis software (B and D) bar graph representing repair efficiency of corresponding transfected cells. Data represent the mean ±SD of three independent experiments.

9. NHEJ repair recovered of by transiently trasfected ASO-145 and DNAPKC's over-expression.

Depletion of DNAPKC's in cells results in reduced NHEJ repair because DNAPKC's is an important candidate in NHEJ repair mechanism. miR-145 over-expressed cells shows reduced DNAPKC's levels which results in reduced NHEJ repair in the cells, reduced repair efficiency in DNAPKC's depleted cells were recovered by antagonizing with ASO-145 and over-expressing DNAPKC’s (DNAPKC's cDNA plasmid) in miR-145 expressing cells due to exact sequence complimentary nature of ASO-145 to miR145, ASO-145 competes with the target protein and recovers the NHEJ repair activity in the cells. Over-expression of DNAPKC's with DNAPKC's cDNA plasmid in DNAPKC's depleted cells also recovers the DNAPKC's level and its activity. To investigate the repair efficiency, we used the pEGFP-Pem1-Ad2 vector and pCMS-EGFP-dsRED vector system. The pEGFP-Pem1-Ad2 plasmid was digested with HindIII restriction endonuclease to remove Ad2 and

generate staggered ends. To investigate the efficiency of NHEJ activity, cells expressing control miR, miR-145 and miR-145 + ASO-145 are transfected with either linearized pEGFP–Pem1–Ad2 or supercoiled pEGFP–Pem1 together with pDsRed2–N1.The cells were then incubated for 24 h to allow expression of EGFP (green) and DsRed (red), followed by flowcytometry analysis (fig 13). To control for the efficiency of transfection, the ratio of GFP+ cells to DsRed+ cells was used as the normalized measure of NHEJ efficiency. pCMS-EGFP-dsRED vector system was digested with EcoRI and XhoI to cut multi restriction enzyme site which having red fluoresemce and generate leniarized vectors. Supercoiled pCMS-EGFP-dsRED vectors were used as a positive control for standardization of transfection and analysis conditions. The pCMV-sport6-expression plasmid (Clontech) and pEGFP-N3 supercoiled plasmids used as control to check transfection efficiency. the cells expressing control miR, miR-145 and miR-145 + ASO-145 expressing cells were transfected with linearized pCMS-EGFP-dsRED or supercoiled pCMS-EGFP-dsRED together with pCMV-sport6 and pEGFP-N3 plasmid, using lipofectamine 2000 (Invitrogen) according to manufactures recommended protocol. Twenty-four hours after transfection, green (EGFP) and red (DsRed) fluorescence was measured by flow cytometry (FACS Calibur; BD Biosciences, San Jose, CA, USA). NHEJ activity was significantly decreased in miR-145 staby expressing cell compard to control cells in both the vector indicating micro RNA interfered NHEJ activity by targeting DNAPKC's.

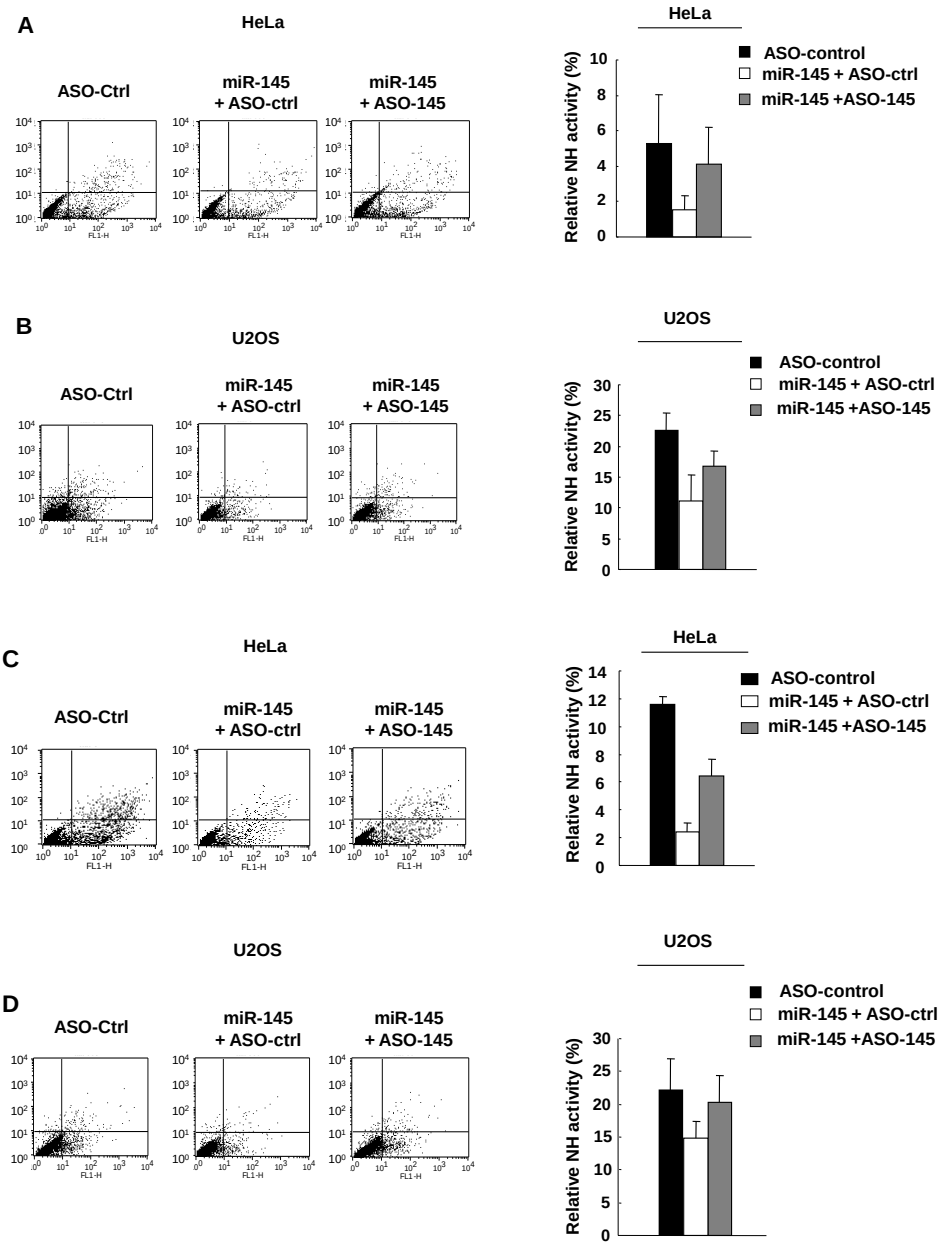


Figure 14: Effect of miR-145 antagonised by ASO-145 expression pend vectors.

(A) HeLa cells (B) U2OS cells were transiently transfected with miRControl, miR-

145 and ASO-145 combined with miR145 were coexpressed with EcoRI linearized pend-RED-GFP or supercoiled pend-RED-GFP, (C and D) with XhoI linearized pend-RED-GFP or supercoiled pend-RED-GFP and incubated for 24h. To quantify NHEJ events, the cells were analyzed by flow cytometry 24 h after transfection. The ratio of GFP-positive (GFP+) to Red-positive (Red+) cells was used as a measure of NHEJ. Relative levels of plasmid rejoining compared to control miRNA-transfected cells were calculated by dividing the GFP+/DsRed+ ratios of the samples and plotted. Means are representative of at least three independent experiments. Error bars indicate the SD.

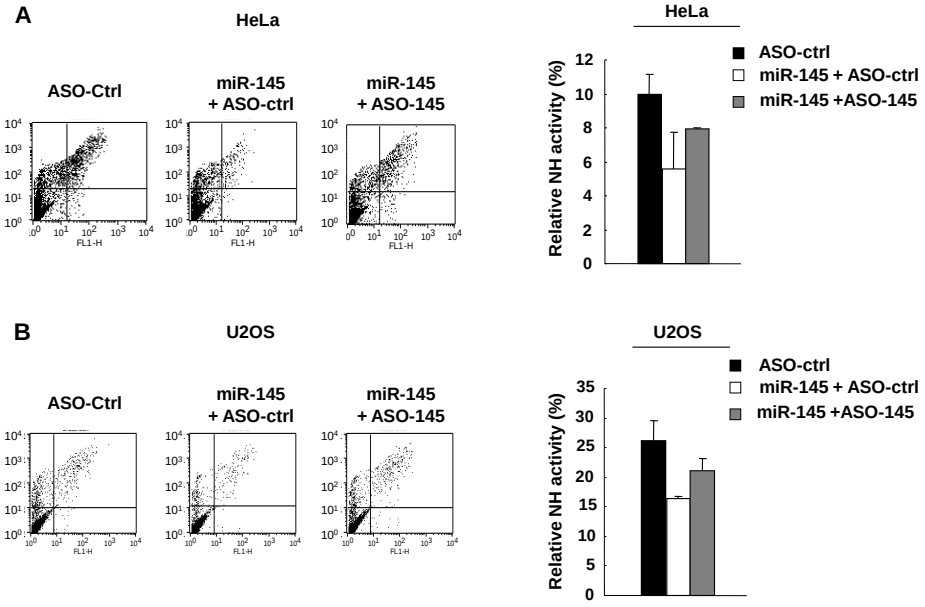


Figure 15: Effect of miR-145 antagonised by ASO-145 expression. Pem vectors (A) HeLa cells (B) U2OS cells were transiently transfected with miR-Control, miR-145 and ASO-145 combined with miR145 were coexpressed with supercoiled pEGF–Pem1–Ad2 or HindIII-linearized pEGFP-Pem1–Ad2 together with pCMV-dsRed-express. To quantify NHEJ events, the cells were analyzed by flow cytometry 24 h after transfection. DsRed expression was used to normalize transfection efficiency. The ratio of GFP-positive (GFP+) to DsRed-positive (DsRed+) cells was used as a measure of NHEJ. Relative levels of plasmid rejoining compared to control miRNA-transfected cells were calculated by dividing the GFP+/DsRed+ ratios of the samples and plotted. Means are representative of at least three independent experiments. Error bars indicate the SD.

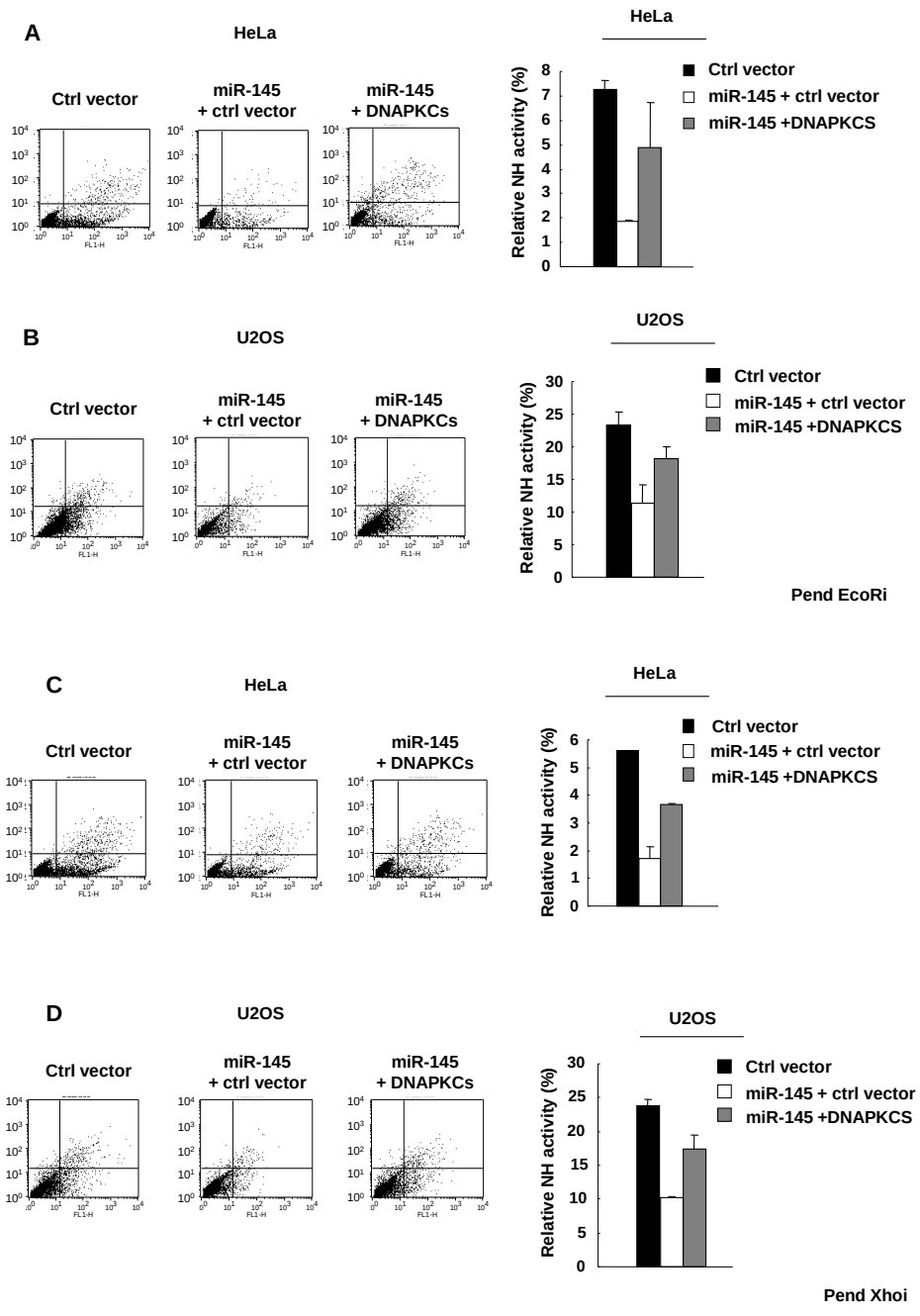


Figure 16: Effect of miR-145 recovered by DNAPKC's overexpression pend vector.

(A) HeLa cells (B) U2OS cells were transiently transfected with miRControl, miR-

145 and DNAPKC's combined with miR145 were coexpressed with EcoRI linearized pend-RED-GFP or supercoiled pend-RED-GFP, (C and D) with XhoI linearized pend-RED-GFP or supercoiled pend-RED-GFP and incubated for 24h. To quantify NHEJ events, the cells were analyzed by flow cytometry 24 h after transfection. The ratio of GFP-positive (GFP+) to Red-positive (Red+) cells was used as a measure of NHEJ. Relative levels of plasmid rejoining compared to control miRNA-transfected cells were calculated by dividing the GFP+/DsRed+ ratios of the samples and plotted. Means are representative of at least three independent experiments. Error bars indicate the SD.

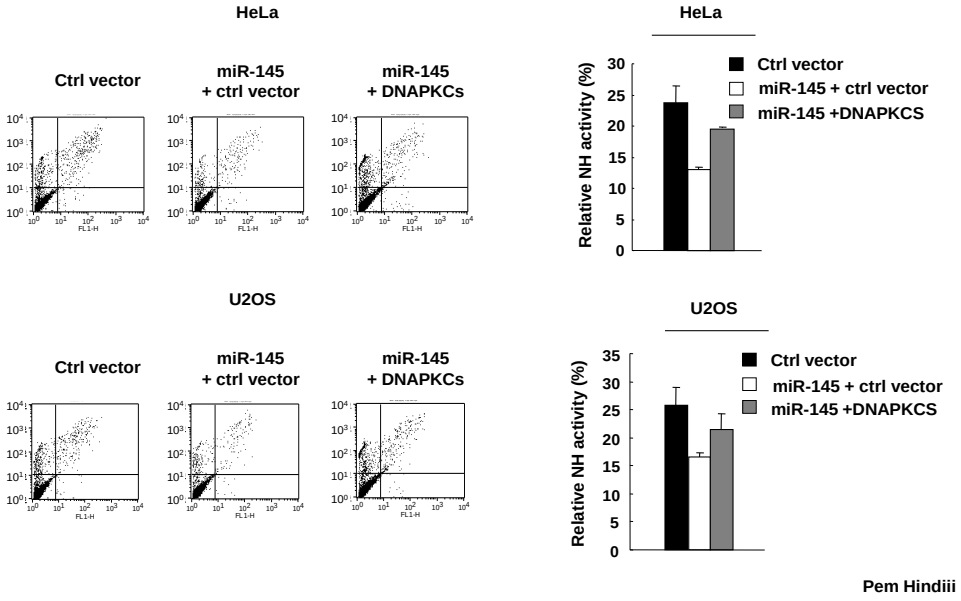


Figure 17: Effect of miR-145 recovered by DNAPKC's overexpression. (A) HeLa cells (B) U2OS cells were transiently transfected with miR-Control, miR-145 and DNAPKC's combined with miR145 were coexpressed with supercoiled pEGF–Pem1–Ad2 or HindIII-linearized pEGFP-Pem1–Ad2 together with pCMV-dsRed-express. To quantify NHEJ events, the cells were analyzed by flow cytometry 24 h after transfection. DsRed expression was used to normalize transfection efficiency. The ratio of GFP-positive (GFP+) to DsRed-positive (DsRed+) cells was used as a measure of NHEJ. Relative levels of plasmid rejoining compared to control miRNA-transfected cells were calculated by dividing the GFP+/DsRed+ ratios of the samples and plotted. Means are representative of at least three independent experiments. Error bars indicate the SD.

10. Antagonizing ASO-145 and over-expressing DNAPKC's in miR-145 expressing cells results in radio resistance.

Cells lacking or depletion of any DNA repair proteins are generally sensitive to IR. DNAPKC's depleted cells will be under immense stress due to lack of important candidate in non homologous end joining repair mechanism of induced double strand breaks. The depleted cells are restored by antagonizing the effect of miR-145 by ASO-145 and over expressing DNAPKC's. To examine the radio sensitivity of DNAPKC's depleted cells and restored cells, cells were exposed to IR and examined for colony formation ability and cell survival. miR-145 expressing cells shows less colony or survival ability than the control cells the cells antagonized with ASO-145 and DNAPKC's overexpressing cells recovered cell death and formed more colony compared to miR-145 expressing cells.

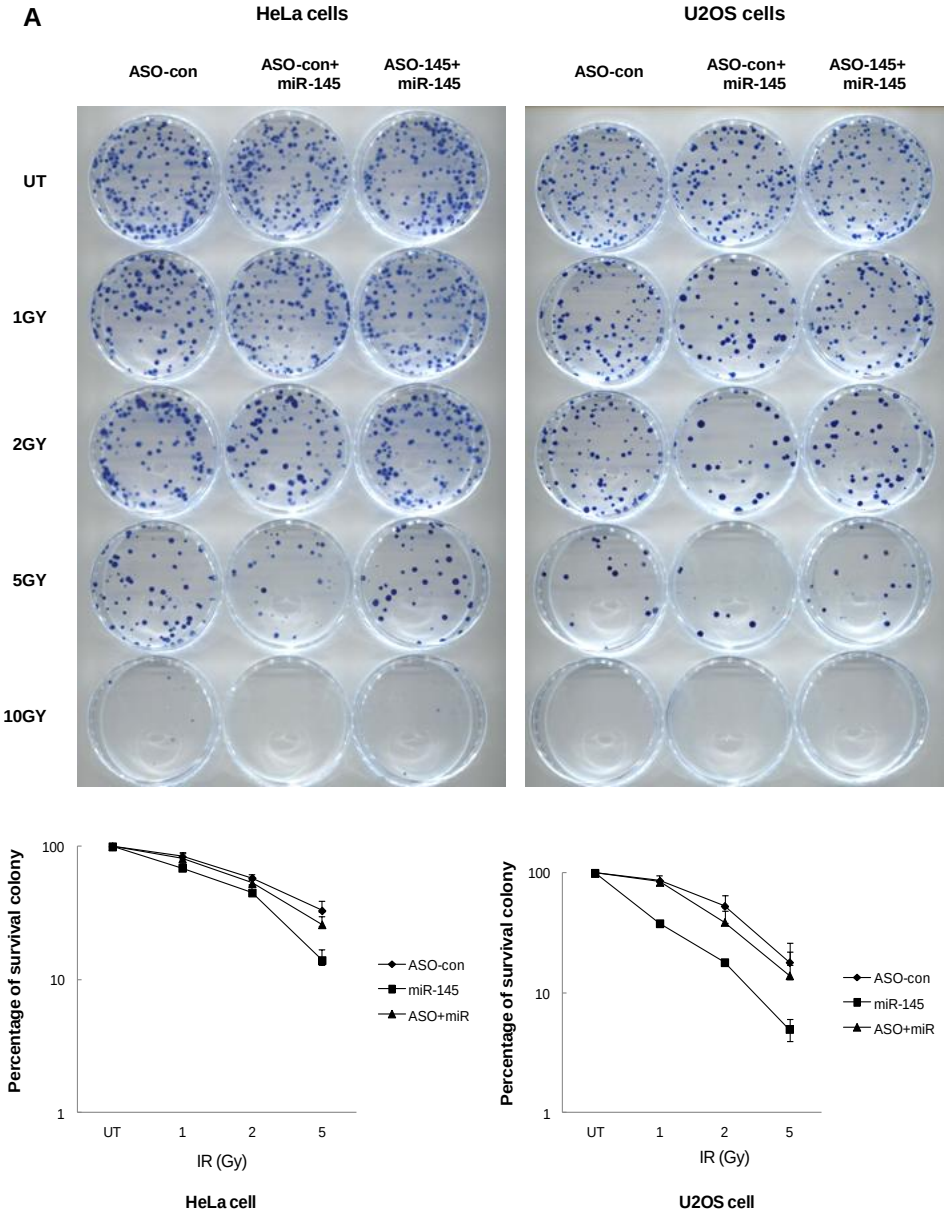


Figure 18: Radio sensitivity of miR-145 and ASO-145 transiently expressed cells (A) Control and miR-145 and miR-145 + ASO-145 expressing HeLa cells and U2OS cells were untreated or treated with the indicated doses of ionizing radiation,

after 2 weeks cells were fixed and stained with methylene blue and cell survival was assessed using a clonogenic assay (B) graph representing percentage of survival cells. Data represent the mean ±SD of three independent experiments.

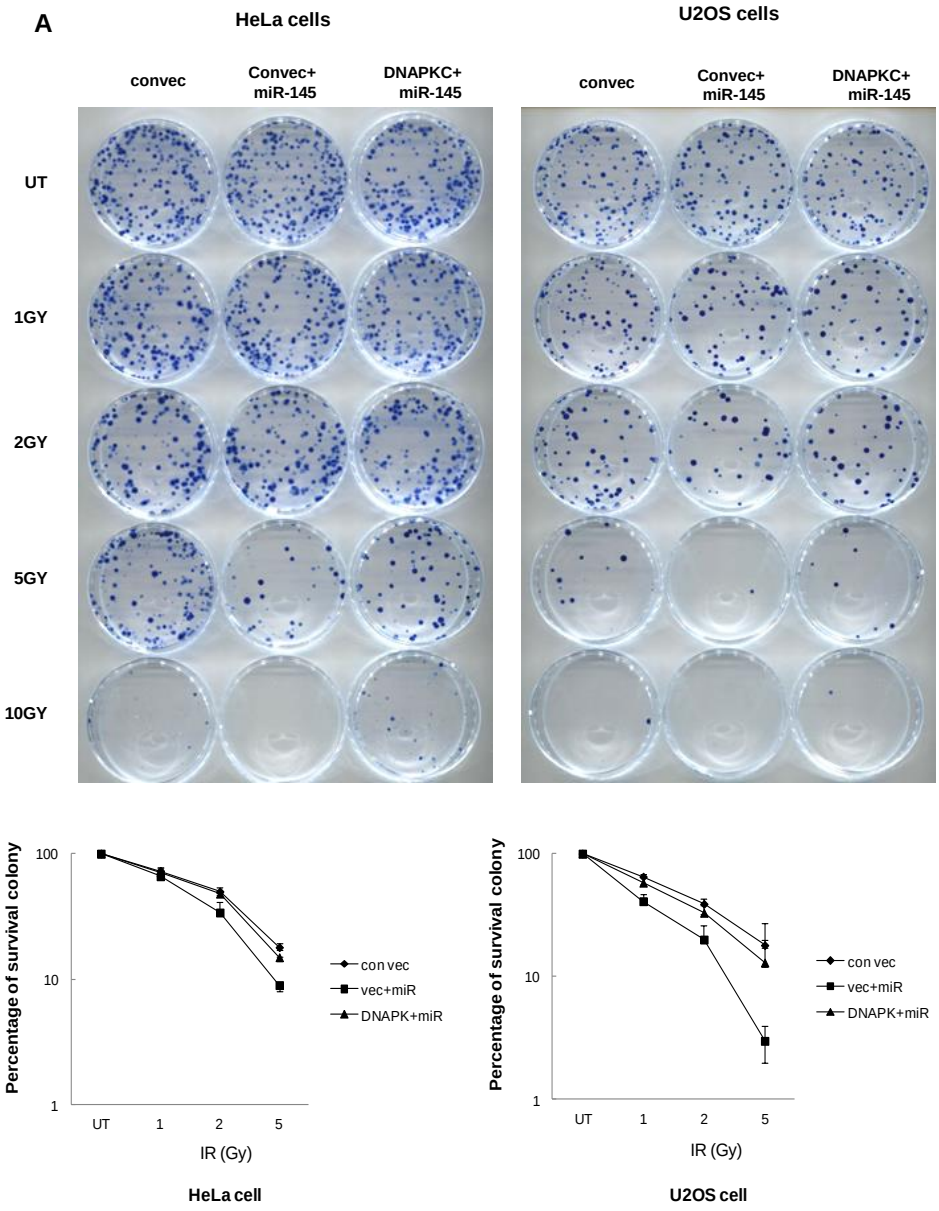


Figure 19: Radio sensitivity of miR-145 and DNAPKC's transiently overexpressed cells (A) Control ASO, miR-145 and miR-145 + DNAPKC's expressing HeLa cells

and U2OS cells were untreated or treated with the indicated doses of ionizing radiation, after 2 weeks cells were fixed and stained with methylene blue and cell survival was assessed using a clonogenic assay (B) graph representing percentage of survival cells. Data represent the mean ±SD of three independent experiments.

IV DISCUSSION

NHEJ (Non Homologous End Joining) is one of the main repair mechanisms that repairs DNA double strand break (DSB's) by joining broken DNA ends. Repair is initiated by binding of KU proteins, XRCC and ligase IV with DNAPKC's in the DNA break site. MiRNA's (Micro RNA's) are important regulators in the cell which regulates cells function by regulating various proteins at its translation level by translational repression or mRNA decay. During the stress condition cells expresses micro RNA's to regulate the cell function, thousands of micro RNA's were expressed in cells that targets more than thousands of proteins inside the cells, one such micro RNA (miR-145) expressed during stress condition (Sachdeva, M. *et al.2009)* that targets DNAPKC's protein. mRNA's that translates into protein, contains untranslated regions (UTR's) in both 5' and 3' regions which contains sequence which are complimentary to some micro RNA, miR-145 that binds to 3' UTR region in the DNAPKC's mRNA. Binding of miR-145 to 3' UTR of DNAPKC's mRNA results in translational repression or mRNA degradation which results in protein depletion that affects NHEJ activity of the cells. To further explore miR-145 effect on DNAPKC's we prepared miR-145 stably expressing cells in HeLa and U2OS cells and used to

investigate weather over expression of miR-145 affects DSB repair by using many techniques and assays. Phosphorylation of H2ax variant to γ-H2AX is the early response during the DSB, number of γ-H2AX foci in the cell corresponds to level of DNA damage and single cell gel electrophoresis also measures the damage level in the cell. γ-H2AX foci were analyzed in both control and miR-145 stably over expressing cells after treating IR for stipulated time and the result showed that miR-145 stably over expressing cells have significant repair defect in repairing damaged DNA and it shows more γ-H2AX foci than control cells that indicates delayed repair activity in miR-145 stable cells. Comet assay results also confirm the delayed repair activity by forming long comet tail compared to control after electrophoresis in stably expressing cells. This findings shows that the overexpressed miR-145 targets DNAPKC's and alters cells Non Homologous End Joining repair activity in the cell. Previously many papers also reported cells deficient in DNAPKC's proteins or other proteins involved in NHEJ mechanism like KU, ligase IV shows delayed repair activity. These two findings were supported by another in vivo assay which requires a plasmid linearized with any restriction enzyme (Hindiii, EcoRi and Xhoi) to generate complimentary ends, the transfected linearized plasmids were rejoined by cells repair protein. Number of plasmids rejoined by cells repair protein machinery is

corresponds to efficiency of repairing the DSB in DNA. The recircularized plasmids expresses green fluorescent protein which can be measured by Fluorescence assisted cell sorter (FACS) which also gives similar results comparable with immuno fluorescence and single cell gel electrophoresis. The above results confirm that the cells deficient in DNAPKC's are highly radi sensitive and exhibits delayed repair NHEJ activity. We further confirmed overexpression of Anti sense oligonucleotides (ASO) and DNAPKC's results in rescue of lost NHEJ activity, ASO interferes with miR-145 by its sequence complimentary and makes unavailable to interact with DNAPKC's 3'UTR. Overexpression of the DNAPKC's compensates the suppressed DNAPKC's level by miR-145 and restores cells NHEJ activity.

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ABSTRACT

miR-145 regulates non-homologous end joining by targeting DNA-PKcs

Muddenahalli Srinivasa Sudhanva

Advisor: Prof. Ho Jin You, MD., Ph.D.

Department of Bio-materials

Graduate School of Chosun University

The DNA double-strand breaks (DSB) are the most lethal lesions which are caused by ionizing radiation and radio-mimetic chemicals, but can also be caused by mechanical stress on chromosomes. The cells have many repair pathways for repairing the damage, the two main pathways are homologous recombination (HR) and non homologous end joining (NHEJ). NHEJ pathway is the one pathway which is used by the cells to repair the damaged DNA at any stage of the cell because unlike HR, NHEJ doesn't

require homologous sister chromatid for repairing the damaged DNA. DNAPKC's is an important paralog of NHEJ repair mechanism.

MicroRNAs (miRNAs) are short non-coding RNAs, which are viewed as fundamental regulators of cell function. MiRNAs are approximately 20–25 nucleotides (nt) long which are involved in regulation of cellular mechanism at translational level by mRNA decay or translation repression. MiRNAs regulates various proteins in cell which are involved in various mechanisms it also regulates some proteins which are involved in DNA repair. During the cell stress condition, cells express various miRNA to regulate protein expression and vary the repair mechanism. MiR-145 is a tumor suppressor micro RNA and its expression will be suppressed in many cancer cell types. MiR-145 plays major role in NHEJ (Non Homologous End joining mechanism) by targeting most fundamental protein DNAPKC's. The 3' UTR of DNAPKC's protein has partial sequence complimentary to miR-145 miRNA. Over-expression of miR-145 results in depletion of DNAPKC's which results in suppression of NHEJ activity in cells and DNAPKC's depleted cells show hypersensitivity to IR radiation. We show that the cell expresses miR-145 during stress condition and regulates the cells. This study also provides a new molecular mechanism for controlling the DNA damage repair by micro RNA.

ACKNOLEDGEMENT

With deep immense of gratitude, I would like to thank everyone who has been helpful in the successful completion of my Ph.D. study

It is great pleasure to express my hearty gratitude to my esteemed supervisor, Prof. You, Ho Jin for giving me an opportunity, guidance, suggestions, constant support, and care throughout this study. It has been a great opportunity to be part of his team and to work on an exciting topic with a lot of scientific freedom which helped me to complete this Ph.D. study successfully.

It is an honour for me to thank my co-supervisor Prof. Jung Hee Lee for her guidance, suggestions, constant support, and care throughout this study which helped me to complete this study. She has always been nice to me, with her calm nature. This work would not have been possible without her guidance and support.

I would like to thank cometee member's prof. In-youb Chang, Prof. Jung-Hee Lee, Prof. Young-Jin Jeon, and Prof. Yoon-Sang Pil.

I express my deepest gratitude to Dr. Lalitha R. gowda Senior Scientist, Dept. of PCT who gave me a basic platform for the entry to research field without her I would not have been in this place. I would like to thank my lab members in that lab Dr. Ganesh, Dr.Jimsheena V.K., Dr. Vijay, Dr. Devavratha Rao, Dr. Vinodh, and Dr. Sriranga for their support and guidance.

I would like to thank my senior Dr. Ankita for teaching me all techniques and for her support and Dr. Hari Hara Sudhan who bought me here and for his help and support throughout my Ph.D.

I would like to thank my colleagues Dr. Hari Hara Sudhan, Dr. Dev, Mr Kamalakannan and Mr, Trin Duc Ahn we had good scientific discussions as well as personal sharing's with good mutual understanding.

Especially I need to thank my best buddies Mr. Kamalakannan, Dr. Hari Hara Sudhan and Mr. Yogeendh for their great help during my difficult times.

I would like to thank my lab mates Kim Minji, Sung Mi, Ting Ting, Chin Young, Ohkva, Semo, Son Hyo Jin, Eun Jung, Kim Yuri, Dr. Hyun Seo Kim Hye Rim Kim Jiho, for their support during my Ph.D.

I like to thank my friends Mr. Inbarasan, Dr. Gugan, Dr. Ramesh, Dr. Kathiravan, Mr. Balaji, Mr. Aravindh, Dr. Vijay Dr. Chang Dev Dr. Bijoy, Dr Thiru moorthy, Dr. Palani, Dr. Rajesh and Gwanagju Cricket Club members who made my days in Chosun University I really enjoyed a lot shared lot of happy moments in this past 4 years thank you very much guys for that.

I would like to thank my best best buddies Dr. C.D. Mohan, Mr. Chandrakanth .R, Mr. Ragi Jadi Murthy, Mr. sunil, Mr. Sadashiv and Mr. Shobith for their help, support, encouragement, and for bearing me as one of their best friend.

I would like to thank my M.Sc. friends Mr Chandrakanth .R, Mr Ragi Jadi Murthy, Mr Sayeed Dawood Ahamed Mujtaba, Miss chinmayi .R. Koundinya

And Mrs. Roopa Rani for their support and help please forgive me I have hurted you guys in many circumstance and thank you very much for being a good buddies.

I like to thank to Dr. N.S. Devaki and Dr jagadeesh, Dept. of Molecular Biology Yuvarajas College for their help, guidance and support throughout my studies. I thank my all Molecular Biology buddies for their support and kindness.

My humble thanks to my almighty my parents for everything without them nothing, a special thank to my wife and my family members for their love and support.

Mudenahalli Srinivasa Sudhanva

Chosun University.