**MicroRNA-22 Suppresses DNA Repair and Promotes Genomic Instability through Targeting of MDC1**

**Supplementary information**

**Supplementary Materials and Methods**

**Cell culture and treatment**

HeLa, Si-Ha, U2OS, HEK293T, HCT116, MCF7, MDA-MB-231, MRC-5, IMR-90 and GM00637 cells were obtained from the American Type Culture Collection (ATCC) and maintained as recommended. MRC-5 and IMR-90 cells at early passages (10-20 passages) were used in all experiments because two human primary fibroblasts cells have a mean lifespan of approximately 50-60 population doublings. To induce DNA double strand breaks, exponentially growing cells were irradiated at 10 Gy from 137Cs source (Grammacell 3000 Elan irradiator, Best Theratronics) and were allowed to recover at 37°C for the indicated times. For induction of senescence, cells at approximately 70-80% confluence were treated with 150 μM H2O2 (Sigma) or 120 μM Busulfan (Sigma) for 2 hr or 24 hr, respectively. The cells were washed with PBS to remove reagents and placed in fresh media for 6-7 days.

**miRNA and plasmid transfection**

Has-miR-22 duplex and negative control miRNA were purchased from Bioneer. Cells were transfected with 50 nM miRNA using lipofectamine RNAiMax (Invitrogen) according to the manufacturer’s instructions. For rescue experiments, the pcDNA-HA-MDC1 construct (a gift from Zhenkun Lou, Mayo Clinic, Rochester, MN), CA-Akt1 (Millipore, Billerica, MA), and miR-22 inhibitor (anti-miR-22, miR-22 antisense-oligonucleotide (ASO), Panagene) were used. pcDNA-HA empty vector and scrambled oligonucleotide were used as a negative control. To analyze Akt1-mediated miR-22 regulation, a CA-Akt1 expression plasmid was used. Cells were first transfected with 50 nM miR-22 for 4-6 hr using lipofectamine RNAiMax, and sequentially transfected with 1 μg of pcDNA-HA-MDC1, CA-Akt1 vector or 50 nM miR-22 inhibitor using lipofectamine 2000 reagent (Invitrogen).

**Western blot analysis**

Cells or mouse tissues were lysed in RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml aprotinin]. Equal amounts of cell or tissue extracts were separated by 6-12% SDS-PAGE followed by electrotransfer onto a PVDF membrane (Pall Life Sciences). Western blots were performed by using the appropriate primary and secondary antibodies. The amount of MDC1 protein was quantified using Scion Image software (Scion Corp.).

**Immunofluorescence staining**

Cells cultured on cover slips were irradiated at 10 Gy and allowed to recover for adequate times and then fixed with 4% paraformaldehyde and 98% methanol, followed by permeabilization with 0.3% Triton X-100. After permeabilization, coverslips were blocked with 5% BSA in PBS and then immunostained with primary antibodies and Alexa Fluor 488- (green, Molecular Probe) or Alexa Fluor 594- (red, Molecular Probe) conjugated secondary antibodies. After washing, the coverslips were mounted onto slides using Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Fluorescence images were taken using a confocal microscope (Zeiss LSM 510 Meta; Carl Zeiss) and analyzed with Zeiss microscope image software ZEN (Carl Zeiss).

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

Total RNA was isolated from cultured cells, mouse tissues, and prostate cancer samples using TRIzol (Invitrogen). For quantitation of *mdc1* mRNA and pre-miR-22, 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: *mdc1* forward, 5`-tgctcttcacaggagtggtg-3` and *mdc1* reverse, 5`-gggcacacaggaacttgact-3`; pre-miR-22 forward, 5`-ctgagccgcactagttcttc-3` and pre-miR-22 reverse, 5`-ggcagagggcaacagttctt-3`; *gapdh* forward, 5`- ttcaccaccatggagaaggc-3` and *gapdh* reverse, 5`-ggcatggactgtggtcatga-3`. To quantify miRNAs, cDNA was synthesized using Mir-XTM 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 (Ct) using the delta-delta Ct method that measures the relative of a target RNA between two samples by comparing them to a normalization control RNA (*gapdh* or *U6*).

**Luciferase assay**

A segment of the 3′-UTR of MDC1 (a 658 bp fragment starting after the TGA stop codon) containing the putative miR-22 binding site was cloned into pMIR-REPORT *firefly* luciferase vector (Applied Biosystems). A deletion mutant of the miR-22 binding site was made using the GENEART Site-Directed Mutagenesis kit (Invitrogen). For the luciferase activity assay, the pMIR-REPORT luciferase vector containing 3′-UTR of MDC1 wt (wild type) or mt (mutant) and pRL-TK vector containing *Renilla* luciferase as a control were co-transfected into cells using Lipofectamine 2000 (Invitrogen), and sequentially transfected with miR-22, anti-miR-22 or CA-Akt1 vector. After 24 hr of transfection, the luciferase assay was performed using the dual luciferase reporter assay system (Promega) according to the manufacturer’s instructions. Luciferase activity was quantified using a luminometer (Glomax, Promega).

**Extraction of RNA and protein from mouse tissues**

The mouse lung (n=5) and colon tissues (n=5) were obtained from the Aging Tissue Bank (ATB), a member of the National Biobank of Korea. Tissues were homogenized in TRIzol (Invitrogen) and RIPA buffer according to the manufacturer’s protocol for extraction of RNA and protein, respectively.

**Immunohistochemistry**

The tissue microarray slides include human prostate cancer tissues taken form Super Bio-Chips (SuperBioChips Laboratories, Seoul, Republic of Korea) and the fresh prostate tumor tissues from Chosun university Hospital. The young and senescent colon tissues were obtained from the Chosun University Department of Pathology tissue bank. Slides were stained with rabbit anti-pAkt1 or anti-MDC1 polyclonal antibodies. The primary antibody binding was visualized using a biotinylated goat anti-rabbit antibody and horseradish peroxidase-conjugated streptavidin (Vector Laboratories). After immunolabeling, the specimens were briefly counterstained with hematoxylin. Immuno-labeled images were captured using an Olympus C-4040Z digital camera and an Olympus BX-50 microscope (Olympus Corp.).

**Isolation of mononuclear cells from human blood**

Blood samples were provided by the Kwang-San Health Center (Gwangju, Republic of Korea). Blood donors were healthy men or women in their twenties (n=7) or sixties (n=11). The protocol for human studies was approved by the Institutional Review Board of Chosun University School of Medicine. Human blood samples were collected in a tube containing heparin as an anticoagulant. The peripheral blood mononuclear cells (PBMCs) were extracted from the blood using LymphoprepTM (Axis-Shield), and total RNA was purified using TRIzol (Life Technologies). Briefly, in the Lymphoprep protocol, blood was diluted with 0.9% NaCl (1:1), and isolated by density gradient centrifugation at 800g for 20 min using Lymphoprep. The collected mononuclear cells were washed twice with PBS and then used for experiments.

**BrdU incorporation assay**

For the analysis of irradiation-induced S-phase cell cycle checkpoint, the incorporation of bromodeoxyuridine (BrdU) was monitored as a parameter for DNA synthesis (Roche Diagnostic Corp.). Briefly, cells were treated with the indicated gray of γ-ray. After 2 hr, 10 μM BrdU was added to the culture medium for 2 hr at 37°C for incorporation into freshly synthesized DNA. After fixation of the cells, cellular DNA was partially digested by nuclease treatment. A peroxidase-labeled antibody against BrdU and a peroxidase substrate were added sequentially to yield colored reaction products, which are proportional to the level of BrdU incorporation into the cellular DNA. Colored products were measured using a microplate reader at 405 nm with a reference wavelength at approximately 490 nm. The relative DNA synthesis was calculated as the percentage of absorbance of cells treated with irradiation from the absorbance of untreated cells.

**CGH array and data analysis**

Array CGH analysis was performed using the Nimblegen Human CGH 12×135K whole-genome tiling v3.1 Array (Agilent Technologies). Human genomic DNA (1 μg) from miR-22-tranfected cells and reference DNA samples from control cells were independently labeled with fluorescent dyes (Cy3/Cy5), co-hybridized at 65°C for 24 hr, and then subjected to the array. The hybridized array was scanned using NimbleGen MS200 scanner (NimbleGen Systems Inc.) with 2 μm resolution. Log2-ratio values of the probe signal intensities were calculated and plotted versus genomic position using Roche NimbleGen NimbleScan v2.5 software. Data are displayed and analyzed in Roche NimbleGen SignalMap software and CGH-explorer v2.55.

**SA-β-gal (Senescence Associated-β galactosidase) assay**

SA-β-gal activity was determined using the Senescence Cells Histochemical Staining Kit (Sigma) according to the manufacturer’s protocols. Briefly, cells were washed with 1X PBS, fixed with 1X fixation buffer and incubated in staining solution containing X-gal at 37°C without CO2 until the cells stained blue. Senescent cells were defined as those that appeared blue when viewed under a light microscope. A minimum of 1000 cells in randomly chosen fields were used to calculate the percentage of SA-β-gal positive cells.

**Chromosomal aberration analysis**

Indicated transfected-U2OS cells were treated with 1 Gy of γ-ray for 24 hr and then incubated with 300 ng/ml colcemid (Sigma) for 4 hr before cell harvest. Cell were collected, gently resuspended in 40% of culture media for 10 min at 37°C, and then fixed in equivalent volume of a freshly prepared fixative solution (3:1 mixture of methanol/acetic acids, Carnoy’s solution). After removal of supernatant, pellets were resuspended in fixative solution, dropped onto a cleaned glass slide and air-dried overnight. The slide was mounted in Vectashield with DAPI (Vector Laboratories). Metaphase images were captured using a confocal microscope and analyzed with the Zeiss microscope image software ZEN.

**Single cell gel electrophoresis (Comet) assay**

Cells were harvested (20 μl, 1×105 cells per pellet), mixed with 200 μl low-melting agarose and layered onto agarose-coated glass slides. The slides were immersed in lysis solution [2.5 mol/L NaCl, 100 mmol/L Na2EDTA, 10 mmol/L Tris-HCl (pH 10), and then placed into a horizontal electrophoresis apparatus filled with fresh alkaline electrophoresis buffer [1 mmol/L Na2EDTA, 300 mmol/L NaOH (pH >13)]. After electrophoresis (30 min at 1V/cm tank length), air-dried and neutralized slides were stained with ethidium bromide (20 μg/ml) overnight, and kept in a moist chamber in the dark at 4°C until needed. The slides were analyzed at 400X magnification using a fluorescence microscope (Nikon). The microscope images revealed circular shapes, indicating undamaged DNA, or comet-like shapes, indicating the DNA had migrated out from the head to form a tail (damaged DNA). Average comet tail moment was scored for 40-50 cells/slide using a computerized image analysis system (Komet5.5, Andor Technology).

**Statistical analysis**

Data in all experiments are represented as mean ± s.d. Statistical comparisons were carried out using two-tailed paired *t*-test. We considered *p* <0.01(\*\*) as significant. Analyses were carried out with Prism software (GraphPad) and Excel (Microsoft). Negative correlation of MDC1 expression with pAkt1 levels was assessed using the Pearson correlation test with *p* value. *p* values less than or equal to 0.01 were considered statistically significant.

**Supplementary Figure Legends**

**Supplementary Figure S1**. miR-22 negatively regulates MDC1 expression.

Western blot to examine endogenous levels of MDC1 protein in HEK293T cells after 48 hr of transfection with various amounts of miR-22 (the left panel) or pre-miR-22 (the right panel). Control miR (miR-Ctrl) or control pre-miR (pre-miR-Ctrl) was used to maintain the total amount of miRNAs equal in each transfection.

**Supplementary Figure S2.** The effect of miR-22 on the IR-induced nuclear foci of MDC1, 53BP1, and BRCA1 in U2OS cells.

A, B and C, Control miRNA or miR-22-transfected U2OS cells were irradiated with 10 Gy of IR and fixed at the indicated time points. Left panel; immunostaining experiments were performed using indicated antibodies. Nuclei were counterstained with DAPI. Right panel; quantification of the number of IR-induced MDC1 (A), 53BP1 (B), and BRCA1 (C) foci. At least 100 cells were counted for MDC1, 53BP1, and BRCA1 foci at each time point. The experiment was repeated three times and error bars indicate standard deviation. Data are reported as mean ± s.d., as representative values of three independent experiments. \*\**p* < 0.01.

**Supplementary Figure S3.** Reintroduction of MDC1 rescues miR-22-mediated inhibition of DSB repair in U2OS cells.

A, B, miR-22-expressing U2OS cells were transfected with miR-22-insensitive MDC1 and irradiated with 10 Gy of IR. Cells were then analyzed by γ-H2AX staining (A) and by comet assay (B) at the indicated time points after IR. Representative images (the left panel) and quantification (the right panel) of unrepaired DSBs are shown. DAPI was used for nuclear staining. Results are shown as means ± SD (*n* = 3). \*\* *P* < 0.01.

**Supplementary Figure S4.** Genome-wide DNA copy number variation in the miR-22-overexpressing GM00637 cells.

Using the copy number of control miRNA-transfected GM00637 cells as the control, the relative changes in the copy number of the miR-22-overexpressing GM00637 cells were measured. The chromosomal regions indicating amplification or deletion are marked with a blue line or a red line, respectively. A chromosome number and the schematic of it are shown. Array comparative genomic hybridization revealed alterations of the clonal DNA copy number, including deletion in Chr4, Chr6, Chr8, Chr11, Chr14, and Chr15, and amplification in Chr2, Chr5, Chr6, Chr7, Chr8, Chr10, Chr14, and Chr21 in miR-22-overexpressing GM00637 cells.

**Supplementary Figure S5.** miR-22 levels frequently increased in prostate cancer tissues with high pAkt and low MDC1 expression.

A, Representative images from immunohistochemistry of pAKt and MDC1 protein levels in serial frozen section of the primary prostate cancer tissues from Chosun University Hospital.

B, Analysis of miR-22, pAkt, and MDC1 expression in prostate cancer samples by real-time qPCR. The lowest PCR value of miR-22 levels examined was set at 1.

**Supplementary Figure S6.** Busufan-induced premature senescence leads to suppression of MDC1 expression.

A, Senescence in MRC-5 cells was induced by busufan (BU). Representative images for SA-β-gal activity in young and BU-induced premature senescent cells are shown. The lower histograms show the percentage of SA-β-gal-positive cells (left) and miR-22 levels (right). Results are shown as means ± SD (*n* = 3). \*\* *P* < 0.01.

B, Representative western blot analysis of MDC1 in young (Y) and BU-premature senescent (BU-S) cells.

C, Expression of MDC1 mRNA in young (Y) and BU-premature senescent (BU-S) cells was quantitated using real-time qPCR. Results are shown as means ± SD (*n* = 3). \*\* *P* < 0.01.

**Supplementary Figure S7.** Impairment of IR-induced MDC1 foci formation in senescent cells.

A and B, Young, H2O2-induced premature senescent, and replicative senescent MRC-5 (A) and IMR-90 (B) cells were irradiated with 10 Gy and fixed at the indicated time points. Immunofluorescence was performed using anti-MDC1antibody. Nuclei were counterstained with DAPI. The lower histogram shows the percentage of cells containing > five distinct MDC1 foci per cells. More than 100 cells were counted for MDC1 foci at each time point. Results are shown as means ± SD (*n* = 3). \*\* *P* < 0.01.

**Supplementary Figure S8.** Decline of DNA double-strand break (DSB) repair efficiency in senescent cells.

Young (Y), replicative senescent (R-S), and H2O2-induced premature senescent (H-S) MRC-5 and IMR-90 cells were irradiated with 10 Gy and were then allowed to repair for 6 hr. The pictures show representative comet images. The length and intensity of a comet tail relative to the head is shown as % of the relative tail moment (n = 100). Results are shown as means ± SD (*n* = 3). \*\* *P* < 0.01.

**Supplementary Figure S9.** Rescue of MDC1 expression in senescent MRC-5 and IMR-90 cells by transfection of miR-22-insensitive MDC1.

The level of MDC1 protein was measured in young and H2O2-induced premature senescent MRC-5 and IMR-90 cells transfected with control vector or miR-22-insensitive HA-MDC1 expression vector.

**Supplementary Figure S10.** Overexpression of miR-22 induces senescent-like phenotypes in human cancer cells.

Senescence-associated β-galactosidase (SA-β-gal) activity were analyzed using phase-contrast microscopy at day 6 after transfection with miR-22 into MCF-7, MDA-MB-231, and si-Ha cells. The lower histograms show the percentages of SA-β-gal-positive cells. Results are shown as means ± SD (*n* = 3). \*\* *P* < 0.01.