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P65/RelA is involved in translational repression of Fanconi Anemia protein FANCM via up-regulation of MiR-146a

Graduate School of Chosun University

Department of Bio-Materials

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miR-146a에 의한 FANCM 단백질 활성 조절기전 연구

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Advisor: Prof. Ho-Jin You

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국문초록

miR-146a에 의한 FANCM 단백질 활성 조절기전 연구

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FANCM 단백질은 판코니 빈혈을 활성화 시키고, DNA 복제포크 안정성을 유지하는데 관여하는 Fanconi Anemia(FA) 복합체를 이루는 단백질중의 하나이다. FANCM의 기능소실은 유전체 불안정성을 통한 세포의 악성암을 야기한다. 그러나 FNACM 단백질 자체 발현을 조절하는 기전에 대한 연구는 미흡한 실정이다. 본 연구를 통해 miR-146a의 새로운 타깃으로 FANCM을 동정하였다. miR-146a는 FANCM의 3`UTR에 결합하여 FANCM을

발현을 억제하고, 복제 스트레스에 따른 FANCM의 활성을 저해함을 확인하였다. 또한 p65/RELA 과발현에 의해 세포내 miR-146a가 증가되고, 그에 따라 FANCM 단백질의 발현 및 활성이 저해됨을 관찰하였다. 이상의 결과를 토대로 miR-146a는 FANCM 단백질 발현을 조절함으로써 ICL repair에 관여함을 증명하였다. 또한 본 연구는 NF-kB 단백질이 염색체 불안정을 야기함으로써 악성암을 유도하는 분자적 기전을 제시한다.

I. Introduction

Maintenance of genomic stability is essential for dividing cells to face genomic stress. Fanconi Anemia (FA) is a rare genetic disorder which is characterized by genomic instability, bone marrow failure, developmental abnormalities, microcephaly and premature ageing [1]. Individuals affected by this disorder are predisposed to cancer [2, 3]. FA genes including FANCD1, FANCN and FANCJ were found to be breast cancer susceptibility genes, BRCA2, PALB2 and BRIP1 [4-12]. Fanconi anemia genes involving FANC-A,B,C,E,F,G,L,M,D1,D2,J and FAAP-20, 24, 100 [13-15], associate together to form the FA core complex. Mutation in any one of these genes results in defective interstrand cross-link repair (ICL) activity. FA protein interaction with DNA is mediated by FANCM and FAAP24 which have DNA-interacting domains. In which FANCM has helicase and ERCC-like endonuclease domain [16, 17]. FANCM is directly involved in DNA repair and is required for monoubiquitination of FANCD2 [18, 19]. FANCM is an ortholog of archaeal DNA repair protein HEF which contains DEAD helicase domain and endonuclease domain in N-terminal and C-terminal regions respectively [16, 17]. The helicase domain corresponds to yeast orthologs MPH1 (*Saccharomyces cerevisiae*) and FML1 (*Schizosaccharomyces pombe*). They play a regulatory role in homologous recombination repair by replication fork

reversal and D-loop disruption [20-22]. Also the ATPase activity of FANCM is found to be essential for cross-linker tolerance [19].

Negative regulation of FANCM by microRNA has not been reported so far. MicroRNA belongs to a class of non-coding RNA molecule usually consisting of 20-22 nucleotides. They are endogenous and play an important role as gene regulatory elements [23]. Their unique functions in transcript degradation, sequestering and translational suppression have made them recognized as one of the best molecular tool in understanding the defects in mammalian biological pathway leading to several diseases including cancer. Among several different miRNA's, miR146a is vastly studied in regard to its role in adaptive immune response and innate immunity [24, 25]. LPS upregulates miR146a in NFkB dependent manner and that it down-regulates it target genes TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1) [25]. Silencing of miR146a in Langerhans cells (LC) enhanced Toll-like receptor-2 (TLR-2) dependent NFkB signaling [26]. MiR146a is also upregulated during overexpression of p65 in Huntington's disease model [27]. However the negative interplay between p65 and FANCM via up-regulation of miR146a is not reported. Here we report a novel mechanism of miR146a-mediated down-regulation of FANCM in NFKB dependent manner.

II. Materials and Methods

Cell culture and treatment

Human cervix adenocarcinoma cell line HeLa, human fetal gastric epithelial cell line GES-1, human gastric carcinoma cell line HGC-27 and human osteosarcoma bone morphogenetic cell line U2OS was cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY, USA). In all cases, the media was supplemented with 10% heat-inactivated fetal bovine serum (Cambrex Corp., East Rutherford, NJ, USA), 100 units/ml penicillin, and 100 mg/ml streptomycin sulfate (Invitrogen, Carlsbad, CA, USA). All cells were maintained in a humidified incubator containing 5% CO_2 at 37^{o}C. HeLa, GES-1, HGC-27 and U2OS cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). To induce ICL damage, exponentially growing cells were exposed to 5mM Hydroxyurea (HU) and 1uM Cisplatin and allowed to recover at 37^{o}C for 5 hours.

Antibodies

The following antibodies were used for immunoblotting: rabbit polyclonal anti-FANCM (1:1000; (H-300) sc-98710; Santa Cruz

Biotechnology, Santa Cruz, CA, USA). Mouse monoclonal anti-FANCD2 (1:1000; (FI17): sc-20022; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Rabbit-polyclonal BRCA1 (1:1000; (C-20): sc-642; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Rabbit-polyclonal BRCA2 (1:2000; A303-434A; Bethyl Laboratories, USA). Mouse monoclonal anti-b actin (1:10,000; (C4): sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA, USA). FANCD2 foci was detected by immunofluorescence staining using FANCD2 antibody (AB2187; Abcam, Cambridge, UK) at 1:500 dilution. G-H2AX foci were detected by immunofluorescence staining using g-H2AX mouse monoclonal antibody (JBW301), Upstate Biotechnology, Temecula, CA, USA) at the dilution of 1:200. RAD51 foci were detected by immunofluorescence staining using mouse monoclonal anti-RAD51 (14B4) (ab213; Abcam, Cambridge, UK).

DR-GFP assay (homologous recombination assay)

U2OS-DR-GFP cells were transfected with control or miR146a using lipofectamine 2000, and then infected with I-SceI–carrying adenovirus at an estimated multiplicity of infection of 10. After 72 hours, GFP-positive cells were measured by FACS (FACSCalibur; BD Biosciences). The acquired data were analyzed using Cell- Quest Pro software (BD Biosciences).

Luciferase assay

A segment of the 3′-UTR of FANCM, BRCA1 and BRCA2 containing putative miR-146a binding site was cloned into pMIR-REPORT *firefly* luciferase vector (Applied Biosystems). A deletion mutant of the miR-146a 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 FANCM , BRCA1 and BRCA2 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-146a, anti-miR-146a or RELA/P65 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).

miRNA and plasmid transfection

Has-miR-146a 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, miR-146a inhibitor (anti-miR-146a, miR-146a antisense-

oligonucleotide (ASO), Panagene) were used. pcDNA-HA empty vector and scrambled oligonucleotide were used as a negative control.

Western blot analysis

Cells 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 FANCM, FANCD2, BRCA1 and BRCA2 protein was quantified using Scion Image software (Scion Corp.).

Immunofluorescence staining

Cells cultured on cover slips were treated with hydroxyurea 5mM and cisplatin 1uM 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 m icroscope image software ZEN (Carl Zeiss).

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-146a 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.

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 *FANCM* mRNA and pre-miR-146a, 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: *fancm* forward, 5`-tgctcttcacaggagtggtg-3` and *fancm* reverse, 5`-gggcacacaggaacttgact-3`; pre-miR-146a forward, 5`-ctgagccgcactagttcttc-3` and pre-miR-146a reverse, 5`-ggcagagggcaacagttctt-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*).

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.

III. Results

MiR146a is a direct target for Fanconi Anemia, Complementation Group M

To search for miRNAs that regulate FANCM expression, we carried out TargetScan (MIT; release 6.2) analysis to generate selective miRNA library that could be used for screening. From this analysis, a total of 6 miRNAs were identified as candidates. Each miRNA was reversely screened for the effect on FANCM expression by using luciferase assay. The results showed that overexpression of mi146a led to remarkably lower luciferase activity when compared to scrambled control miRNA (Figure 1A). Consistent with these results, when overexpressed only miR146a significantly reduced endogenous FANCM protein expression in HELA cells (Figure 1B). We next analyzed putative miR146a target site using TargetScan algorithm and found out that bases 704 to 711 and 755 to 761 in the FANCM 3′-UTR, bases 500 to 507 in BRCA1 3′-UTR and bases 584-590 on BRCA2 3″-UTR are complementary to the target sites if miR-146a (Figure 2A). To determine whether miR-146a

binds to these sites to repress FANCM, BRCA1 and BRCA2 expression, we constructed 3 mutant FANCM 3′-UTR and 1 BRCA1 and BRCA2 mutant 3′-

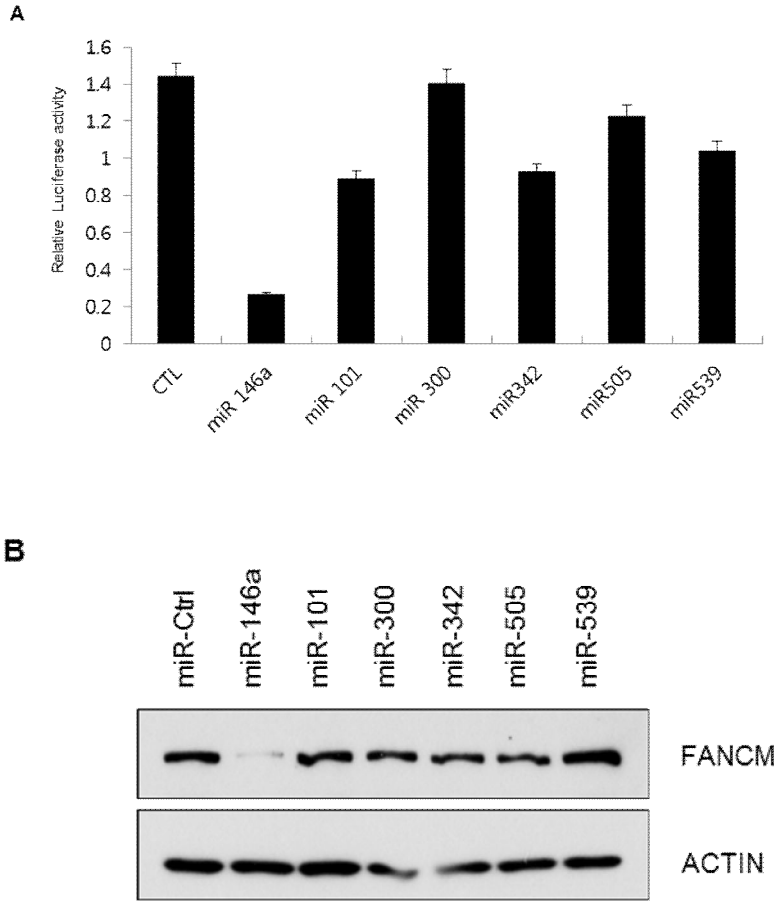
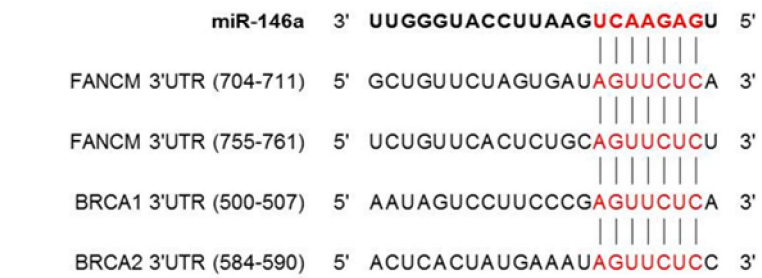


Figure 1. Mir-146a is a direct target for FANCM

A.HeLa cells were cotransfected with the FANCM 3'-UTR luciferase reporter vector along with the candidate miRNAs, which were predicted by TARGETSCAN v6.2, or the miRNA-negative control (miR-Ctrl). Results are

shown as mean ± SD (n = 3). B. The levels of FANCM protein were measured using Western blotting in HeLa cells transfected with the indicated miRNAs.

A



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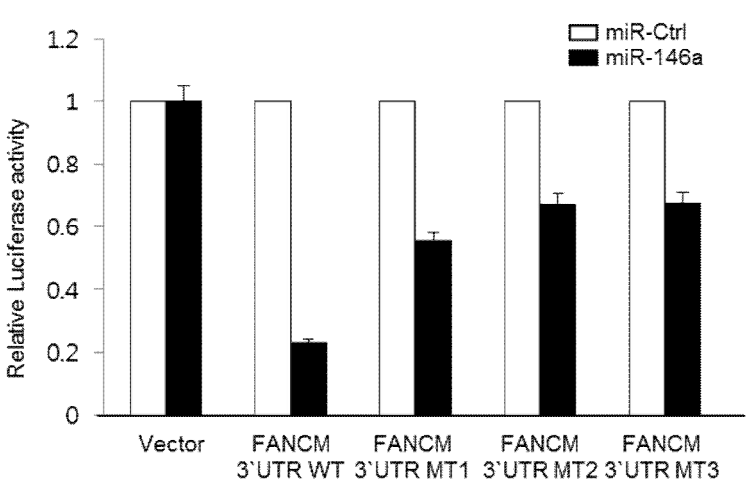
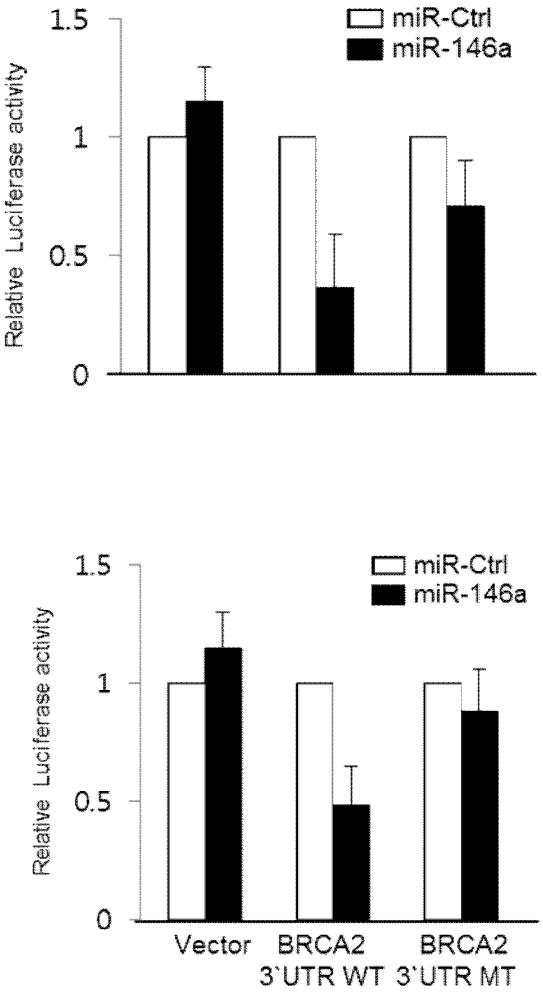


B

C



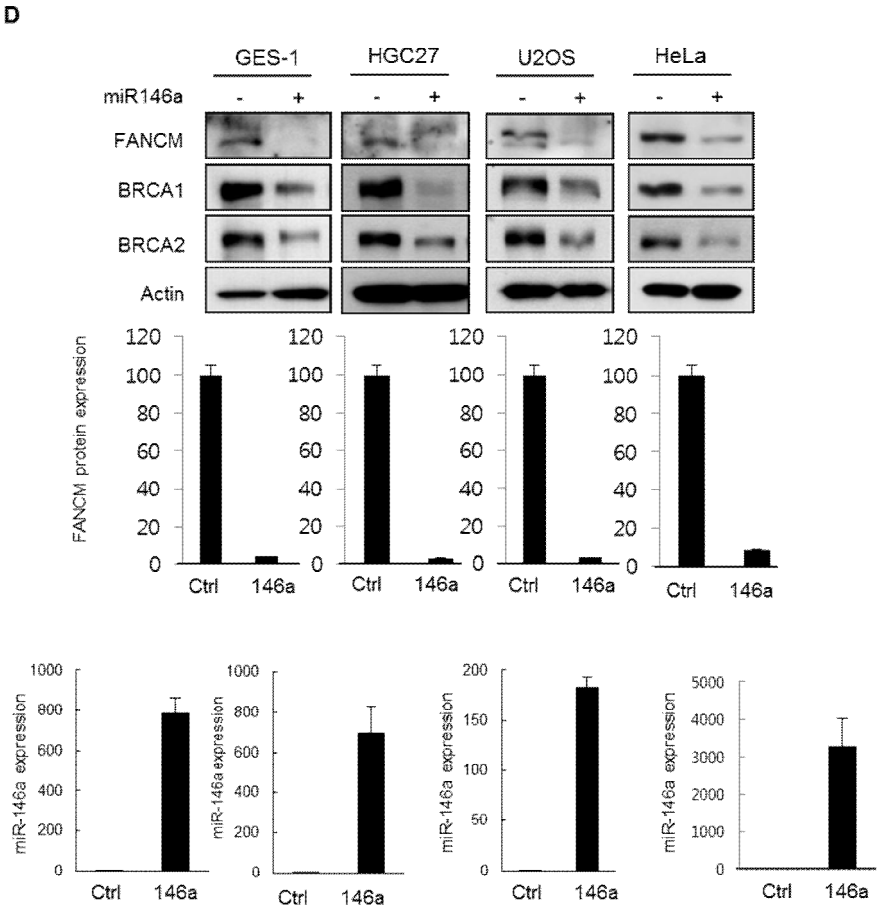


Figure 2 Mir-146a mediated down-regulation of FANCM, BRCA1 and BRCA2

A. A schematic representation of FANCM, BRCA1 and BRCA2 3'-UTR. Red, the seed sequence of miR-146a. B. FANCM 3'-UTR-wt and FANCM 3'-UTR-mt (1-3) were cotransfected with miR-146a in HeLa cells. Luciferase activity was measured 24 hours after the transfection. Data represent mean $\pm$ SD (n = 3); **, $P < 0.01$. C. BRCA1, BRCA2 3'-UTR-wt and BRCA1,

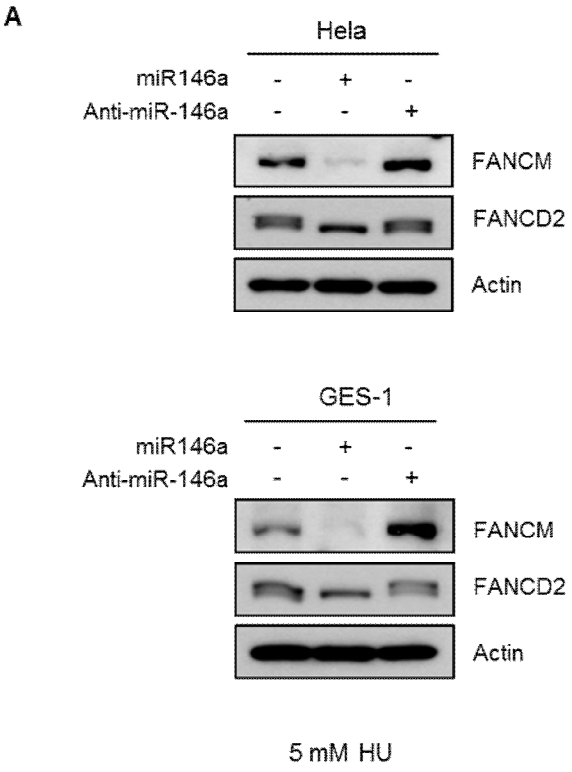
BRCA2 3'-UTR-mt (1-3) were cotransfected with miR-146a in HeLa cells. Luciferase activity was measured 24 hours after the transfection. Data represent mean ± SD (n = 3); **, $P < 0.01$. D. Indicated cells were transfected with control miRNA or miR-146a. The levels of indicated proteins were determined using Western blotting (top) and and quantitative densitometry of FANCM protein expressions + SD (n = 3); **, $P < 0.01$. MiR-146a levels in the indicated cells were determined using real-time qPCR analysis (bottom). Results are shown as mean ± SD (n = 3); **, $P < 0.01$.

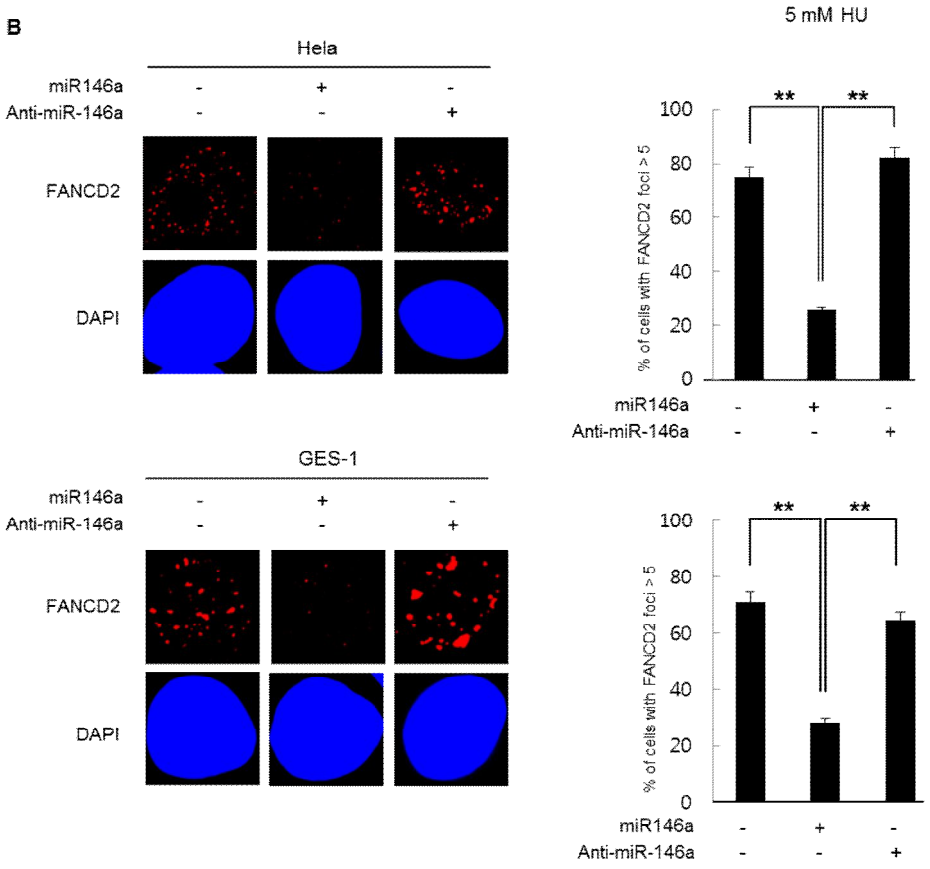
UTR luciferase reporter that was lacking the predicted seed region for the miR-146a. As sown in Figure 2B, deletion of the miR146a binding site in the seed region of FANCM, BRCA1 and BRCA2 3′UTR abrogated the suppressive ability of miR-146a. These results together demonstrate that miR-146a represses FANCM along with BRCA1 and BRCA2 by directly targeting its 3-UTR region.

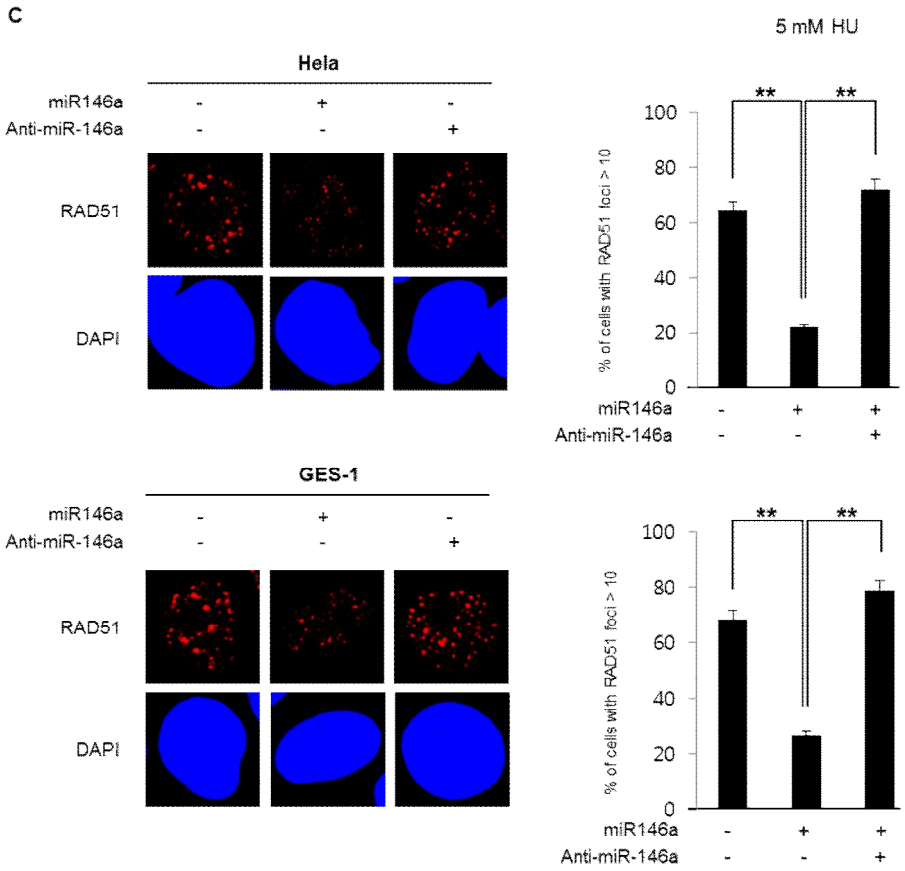
We also found that overexpression of miR-146a not only reduced endogenous FANCM protein expression but also reduced BRCA1 and BRCA2 protein levels. This was not restricted to HELA cells, as we also observed specific repression in GES-1, HGC-27 and U2OS cells (Figure 2C). A simultaneous quantitative PCR (qPCR) analysis revealed several hundreds of fold increase in miR146a expression in these with an exception of HeLa cells, which showed fold increase in thousands. qPCR analysis also showed that FANCM, BRCA1 and BRCA2 mRNA was reduced so a significant level, when miR-146a was overexpressed in HeLa and GES-1 cells (Supplementary Figure S1). These data indicates that miR-146a post transcriptionally down-regulates FANCM, BRCA1 and BRCA2, probably by promoting both mRNA decay and inhibiting translation.

Over-expression of miR146a leads to defective FANCD2 monoubiquitination

FANCM is a critical protein in FA core complex which regulate FANCD2 monoubiquitination (2). Since miR146a acts as a direct suppressor for endogenous FANCM protein expression we sought to find out if over-expression of miR146a resulted in defective monoubiquitination of FANCD2 protein. We transfected GES-1 and HeLa cells with miR control, miR146a and Anti-miR146a respectively. The transfected plates were subjected to interstrand cross-link repair agent, hydroxyurea (HU) 5mM. After required time period cells were harvested and protein samples were subjected to western blot analysis. The results revealed defective FANCD2 monoubiquitination in miR146a transfected cells along with FANCM down-regulation, compared to control and anti-miR146a transfected cells (Figure 3A). Further we checked if FANCD2 foci assembly is affected due to over-expression if miR146a in HeLa and GES-1 cells. As expected miR146a transfected cells exhibited defective foci assembly compared to control and anti-miR146a treated cells (Figure 3B). Next we wanted to find out if down-regulation of FANCM by miR146a affected DNA damage induced RAD51 foci formation. We observed a marginal decrease of RAD51 foci formation in miR146a treated HeLa and GES-1 cells after damage induced by hydroxyl urea when compared to control. However anti-miR146a transfected cells







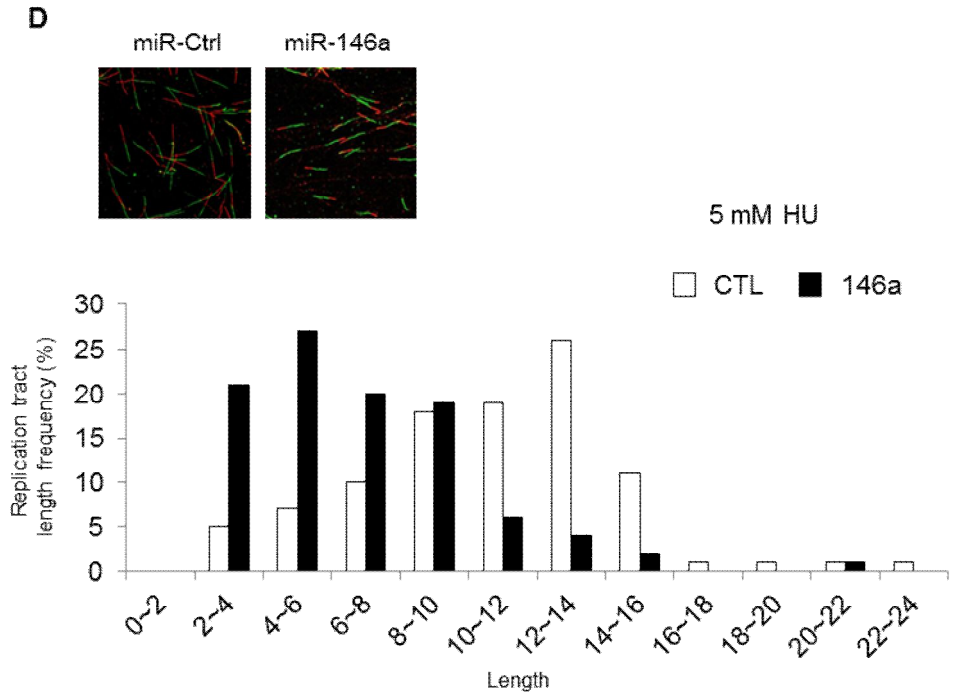


Figure 3 Effect of miR146a on FA pathway

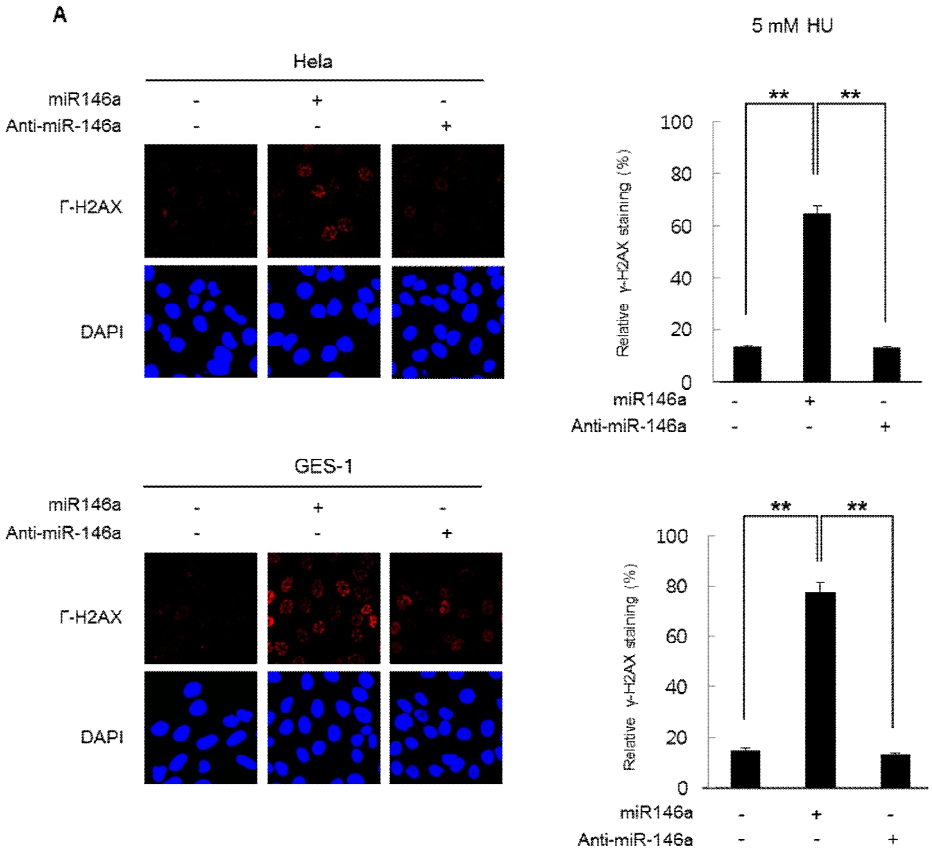
A.Indicated cells were treated with miR146a and miR146a + Anti-miR-146a. 48hrs later the cells were treated with 5mM HU for 5 hours. FANCM protein level and FANCD2 ubiquitination were measured using Western blotting. Actin was used as loading control. B. Indicated cells were treated with miR146a and miR146a + Anti-miR-146a. 48hrs later the cells were treated with 5mM HU for 5 hours. Cells were analysed for FANCD2 foci. Results are shown as mean ± SD (n = 3). **. C. Indicated cells were treated with

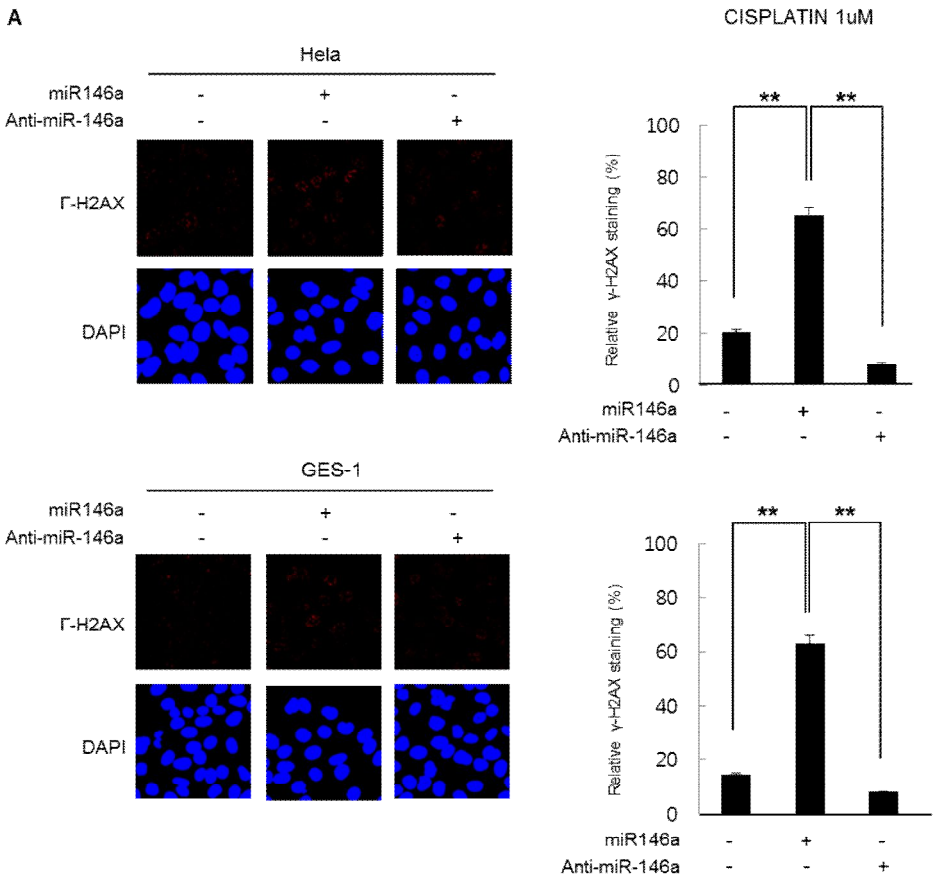
miR146a and miR146a + Anti-miR-146a. 48hrs later the cells were treated with 5mM HU for 5 hours. Cells were analysed for RAD51 foci. Results are shown as mean ± SD (n = 3). ** D. Representative images of DNA fibres obtained from Control and miR146a overexpressing HeLa cells. Distribution curves of the ratios between CldU and IdU tract lengths in WT miR-146a overexpressing cells.

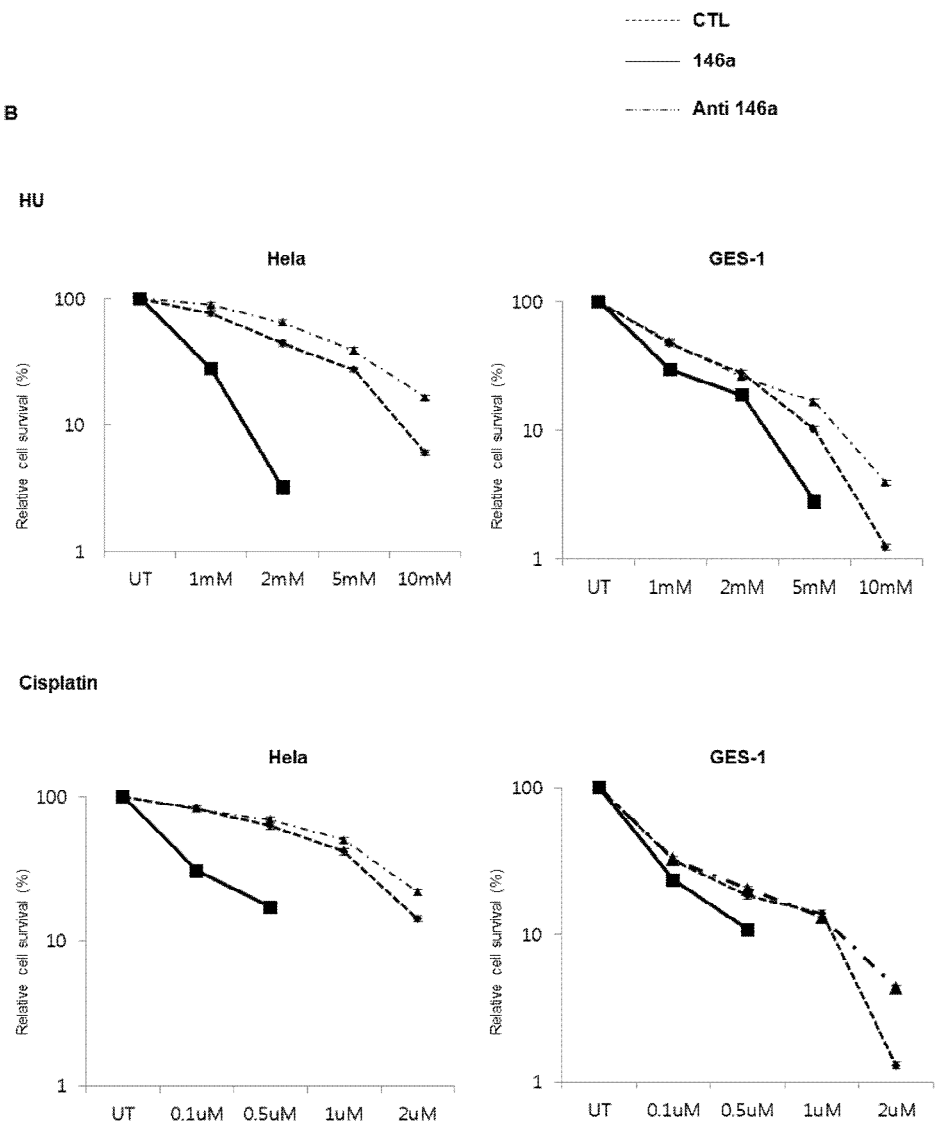
showed a marginal increase in RAD51 foci formation when compared to control. This further adds to the critical role of miR146a in affecting homologous recombination repair (Figure 3C). Next we checked if down-regulation of FANCM by miR146a leads to defective replication fork restart. Previously FANCM was known to be involved in maintenance of genome integrity during S phase and that FANCM-deficient cells results in defective replication fork restart (3). We observed that in miR146a transfected cells, replication stalling results in shortening of median IdU tract length compared to control (Figure 3D). These results obtained suggest that FANCM down-regulation by miR146a affects fork stabilization and maintenance of genome stability.

MiR146a mediated down-regulation of FANCM affects cell survival and homologous recombination.

After confirming the effects of miR146a on Interstrand cross-link repair activity we sought to find out if miR146a causes replication stress. We used hydroxyurea, a ribonucleotide reductase inhibitor and Cisplatin, a DNA-damaging agent, to check if miR146a affected DNA synthesis. We transfected HeLa and GES-1 cells with miR146a and after required time of incubation we treated the cells with hydroxyurea and cisplatin in separate experiments. The cells were then stained with g-h2ax, a marker for detecting replication stress. As expected miR146a transfected cells, when compare to control and anti-miR146a, exhibited large number of cells with g-h2ax foci (Figure 4A). This showed that miR146a affects DNA synthesis by causing replication stress. Next we checked if hydroxyurea and cisplatin affected clonal cell survivability of cells over-expressing miR146a. We transfected HeLa and GES-1 cells with control miRNA, miR146a and anti-miR146a. The cells were later subjected to increasing dose of hydroxyurea and cisplatin in two different experiments and clonal cell survival was assessed after 2 weeks. The results revealed that the clonogenic survivability of cells transfected with miR146a was greatly reduced when compared to control and anti-miR146a transfected cells after hydroxyurea and cisplatin treatment (Figure 4B).

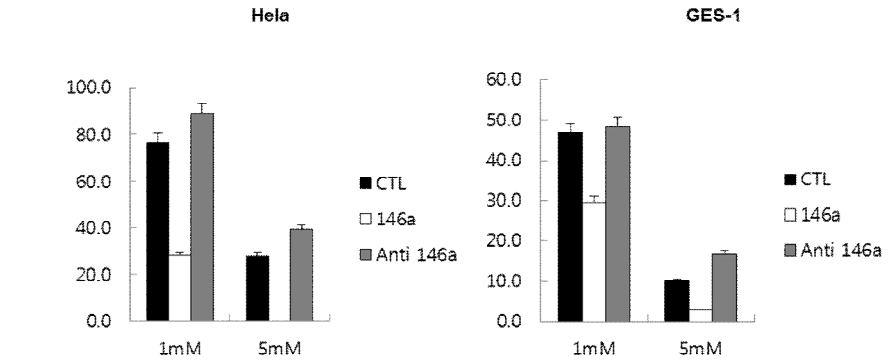






B

HU



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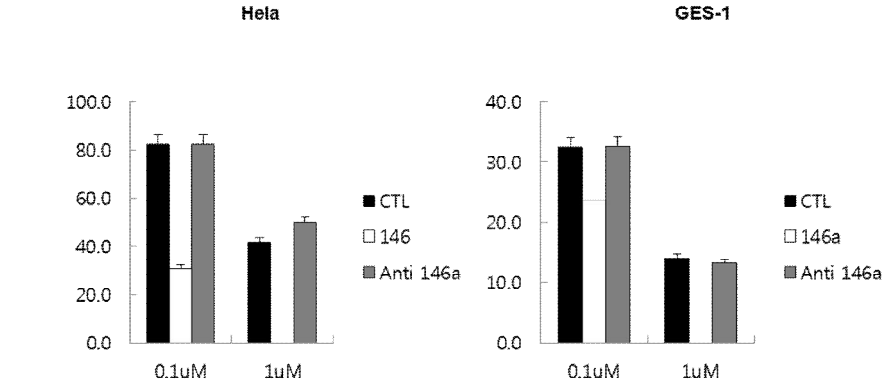


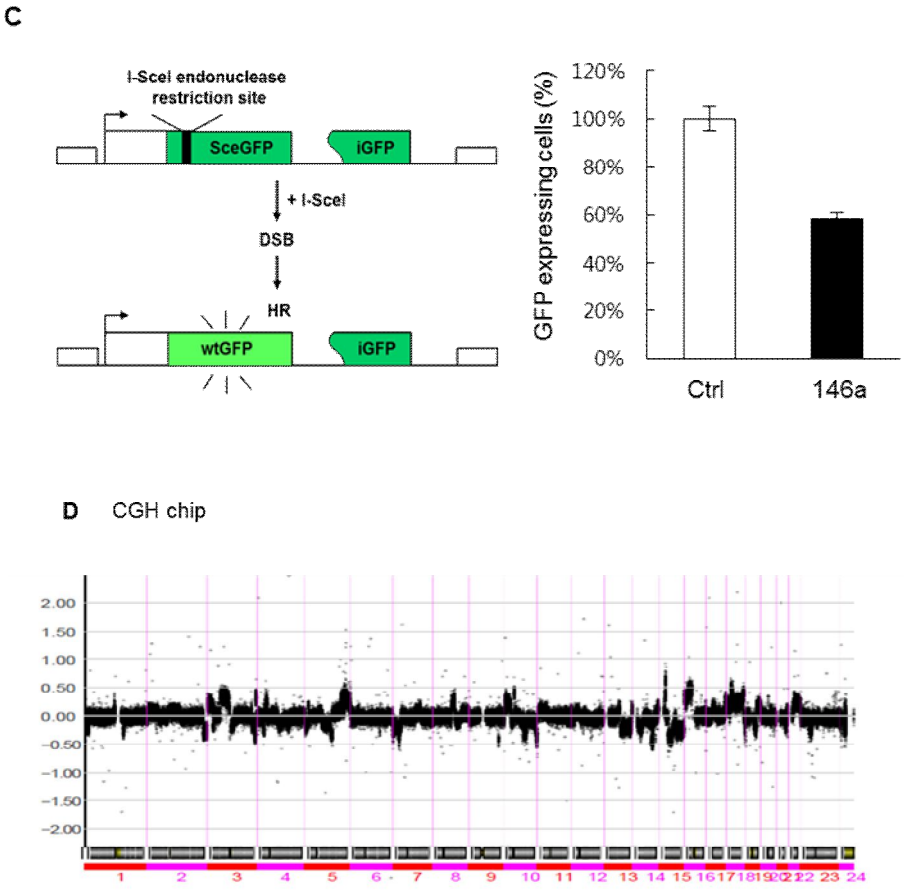


Figure 4 Effect of miR146a on ICL repair

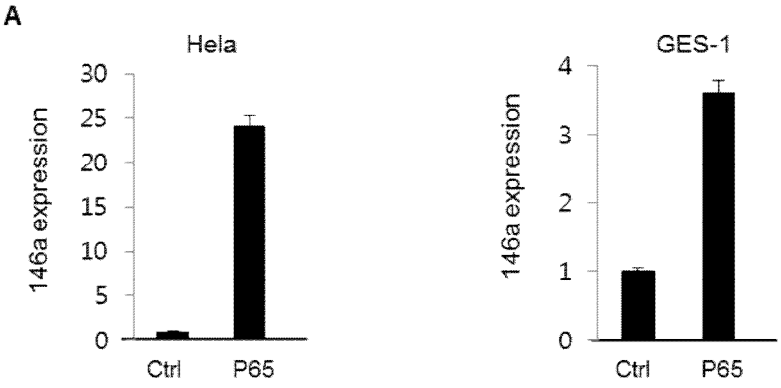
A.Indicated cells were treated with miR146a and miR146a + Anti-miR-146a. 48hrs later the cells were treated with 5mM HU and 1uM Cisplatin. Cells were then analyzed by γ-H2AX staining 16 hours after HU and Cisplatin treatment. Results are shown as mean ± SD (*n* = 3). ** B. HeLa and GES-1 cells were transfected with Control, miR146a and miR146a + Anti-miR-146a

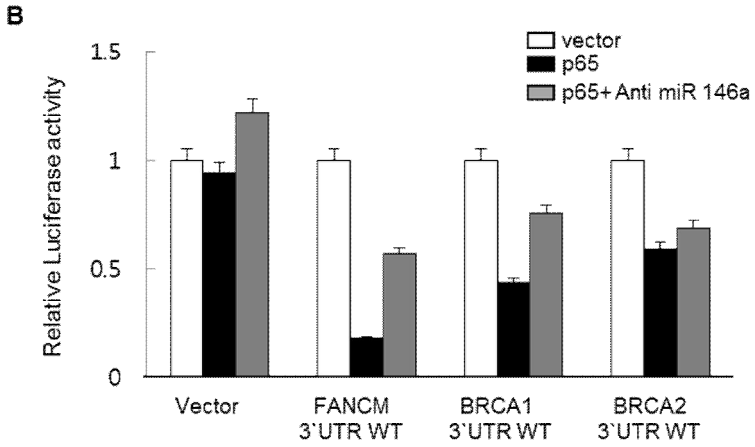
and subjected to increasing dosage of HU and Cisplatin as indicated. Viabilities of cells were examined by clonogenic survival assay. Results are shown as mean ± SD (n = 3). **, $P < 0.01$. C. A schematic showing the assay for the fluorescence-based measurement of HR-mediated DSB repair. D. CGH profiles of clones derived from GM00637 cells transfected with control miRNA or miR-146a. Chromosomal regions above or below the red dotted line indicate amplifications or deletions of genomic positions, respectively.

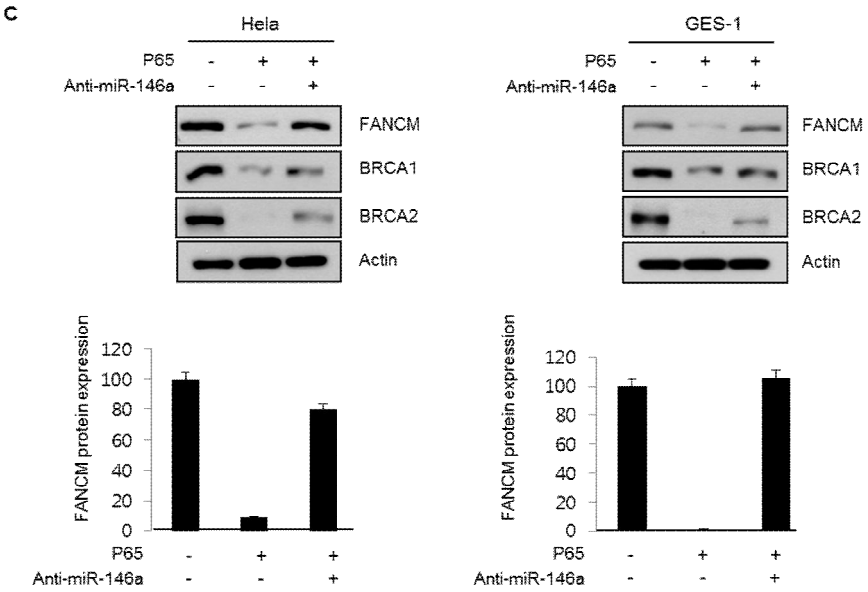
Next we wanted to see if down-regulation of FANCM by miR146a inhibited homologous recombination (HR). We assayed for HR-mediated repair of I-SceI–induced DSBs, in U2OS cells, using a recombination substrate DR-GFP. When DSBs are repaired by HR, GFP is expressed and levels can be quantitated using flow cytometry. We found that cells overexpressing miR-146a had significantly reduced HR efficiency (Figure 4C). This result suggests that induction of miR-146a and subsequent down-regulation of FANCM along with BRCA1 and BRCA2 are responsible for the repression of HR. Further To assess the subsequent genomic effects resulting from the numerous chromosomal breaks in miR-146a–expressing cells, we performed array comparative genomic hybridization (arrayCGH) using human fibroblast GM00637 cells. From this analysis, we conclude that there was a high frequency of chromosomal abnormalities in miR-146a–expressing cells, including clonal amplifications and deletions in discrete regions (Figure 4D). Taken all together, these results provide evidence that miR-146a–mediated down regulation of FANCM results in defects in DSB repair and allows cells to bypass an intra–S-phase checkpoint causing a decrease in chromosome integrity.

RELA/p65 affects down-regulates FANCM via up-regulation of miR146a

NFKB mediated induction of miR146a was previously reported (4). So we wanted to find out if overexpression of p65 subunit of NFKB resulted in down-regulation of FANCM via up-regulation of miR146a. We overexpressed HeLa and GES-1 cells with p65 subunit and did RT-PCR analysis to evaluate fold increase of miR146a. Compared to control cells p65 transfected cells showed increased miR146a expression (Figure 5A). Since over-expression of p65 alone increased miR146a expression, we wanted to confirm if p65 mediated miR146a targeted 3′ UTR of FANCM along with BRCA1 and BRCA2. To analysis this we performed luciferase assay by transfecting HeLa cells with luciferase reporter construct containing wild type FANCM, BRCA1 and BRCA2 3′ UTR. The relative luciferase activity of the wild type construct was reduced upon overexpression of p65 when compare to control and that anti-miR146a recovered p65 mediated reduced luciferase activity to a considerable extent (Figure 5B). Thus it was evident that miR146a was up-regulated during p65 over-expression and targeted 3′ UTR region of FANCM, BRCA1 and BRCA2. Further we wanted to check if p65 overexpression resulted in down-regulation of FANCM along with BRCA1 and BRCA2. We transfected HeLa and GES-1 cells with REL/p65 subunit



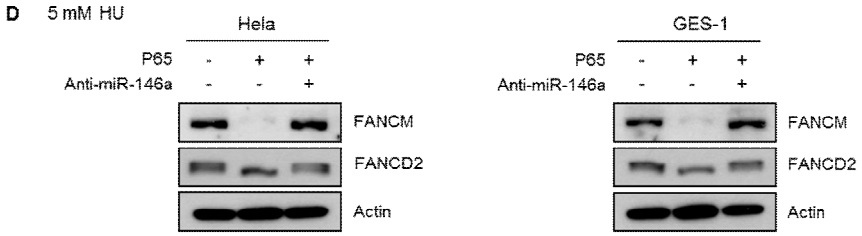


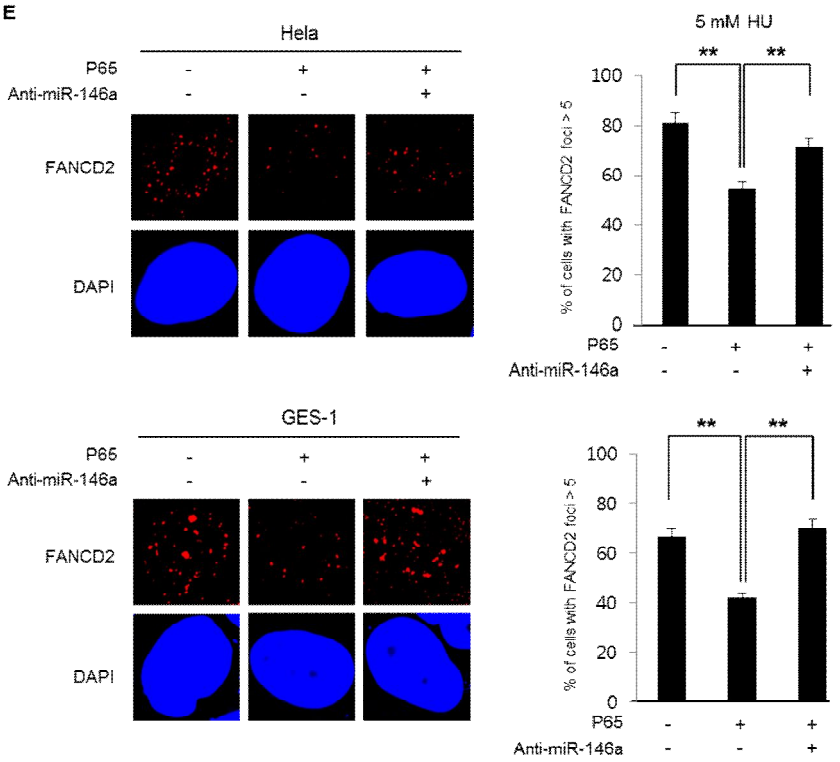


and anti-miR146a co-transfected with REL/p65 separately. After required time of incubation samples were subjected to western blot analysis. The results were as follows. P65 over-expressing cells showed marked down-regulation of FANCM, BRCA1 and BRCA2 and the recovery using miR146a antisense oligonucleotides completely recovered negative-regulation of FANCM and a considerable recovery was observed in BRCA1 and BRCA2 when compared to control (Figure 5C). These preliminary results thus proved that p65 over-expression resulted in negative-regulation of FANCM via up-regulation of miR146a.

P65 overexpression impairs FANCD2 monoubiquitination and RAD51 foci assembly

After confirming that p65 negatively regulates FANCM via miR146a regulation, we sought to find out if this affected FANCD2 ubiquitination and RAD51 foci assembly. HeLa and GES-1 cells were transfected with control, p65 and anti-miR146a plus p65 in separate sets and after 24 hours the cells were treated with 5mM HU for 5 hours. After required time of incubation the cells were harvested and western blot analysis was performed. As we expected, the results showed that in p65 transfected cells, FANCD2 ubiquitination was defective whereas this effect was recovered by anti-miR146a (Figure 5D). FANCD2 foci in p65 overexpressing HELA and GES-1 cells were less when compared to control and that the same effect was recovered by anti-miR146a (Figure 5E), however this reduction in foci was less in comparison with reduction in foci obtained from direct miR146a transfection (Figure 3B). We further elaborated this study by checking RAD51 foci assembly after p65 transfection. HeLa and GES-1 cells were again subjected to p65 and anti-miR146a transfection like before and treated with 5mM HU for required time period. After incubation we performed immunocytochemistry technique to observe RAD51 foci formation. We observed that RAD51 foci assembly was significantly affected in RELA/p65 overexpressing cells when compared to control.





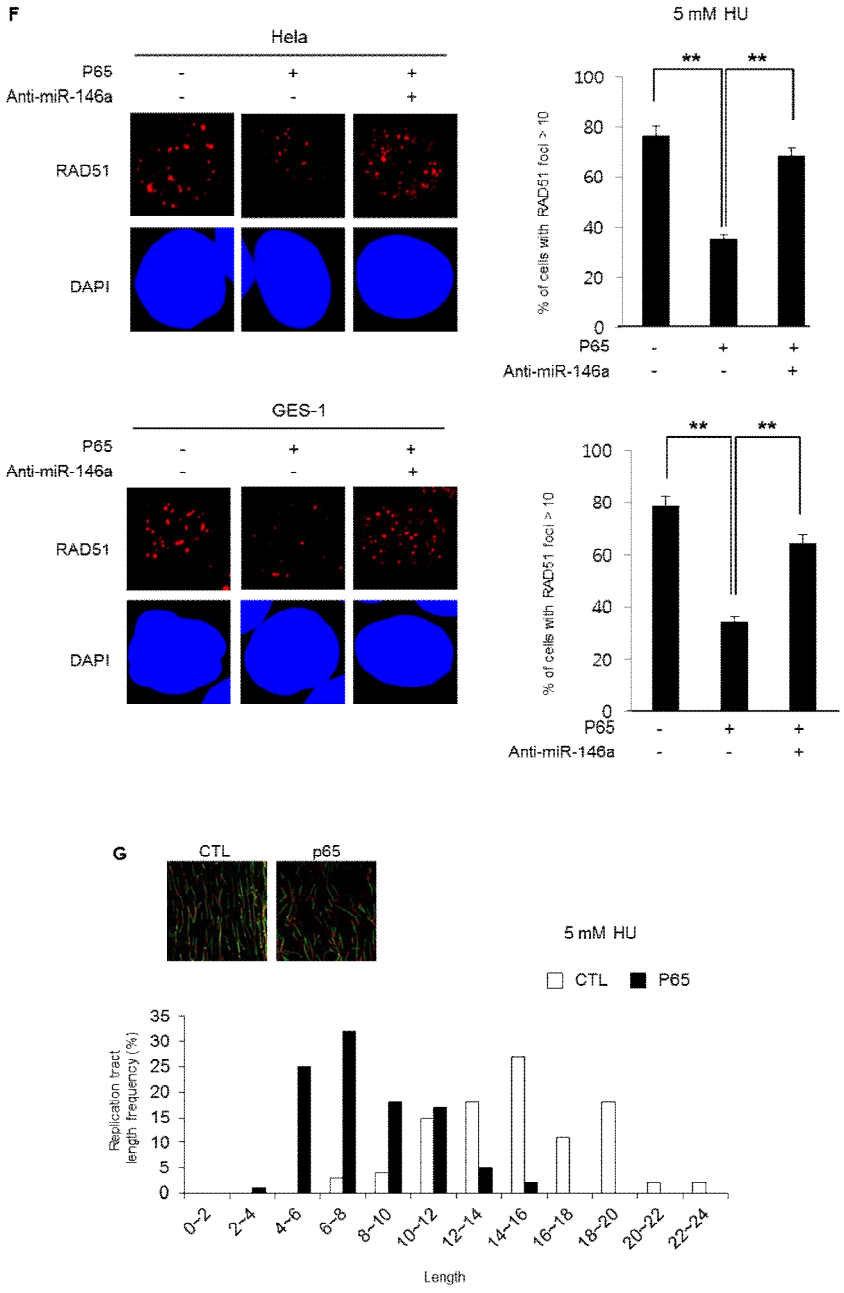


Figure 5 The role of NF-kB in miR-146a-related Fanconi anemia and ICL-repair

A. Indicated cells were transfected with control miRNA or p65. MiR-146a levels in the indicated cells were determined using real-time qPCR analysis. B. FANCM, BRCA1, BRCA2 3'-UTR-wt were cotransfected with p65 and p65+Anti miR146a in HeLa cells. Luciferase activity was measured 24 hours after the transfection. Data represent mean ± SD (n = 3); **, $P < 0.01$. C. Indicated cells were transfected with control miRNA, p65 and p65+Anti-miR-146a. The levels of indicated proteins were determined using Western blotting (top) and quantitative densitometry of FANCM protein expressions + SD (n = 3); **, $P < 0.01$. D. Indicated cells were treated with p65 and p65 + Anti-miR-146a. 48hrs later the cells were treated with 5mM HU for 5 hours. FANCM protein level and FANCD2 ubiquitination were measured using Western blotting. Actin was used as loading control. E. Indicated cells were treated with p65 and p65 + Anti-miR-146a. 48hrs later the cells were treated with 5mM HU for 5 hours. Cells were analysed for FANCD2 foci. Results are shown as mean ± SD (n = 3). ** F. Indicated cells were treated with p65 and p65 + Anti-miR-146a. 48hrs later the cells were treated with 5mM HU for 5 hours. Cells were analysed for RAD51 foci. Results are shown as mean ± SD (n = 3). ** G. Representative images of DNA fibres obtained from Control and p65 overexpressing HeLa cells. Distribution curves of the ratios between CldU and IdU tract lengths in p65 overexpressing cells.

This defective foci assembly was restored in anti-miR146a transfected cells (Figure 5F). Thus this data showed that p65 subunit of NFKB is distinctively involved in affecting FANCD2 ubiquitination and RAD51 foci assembly via up-regulation of miR146a and negative regulation of FANCM.

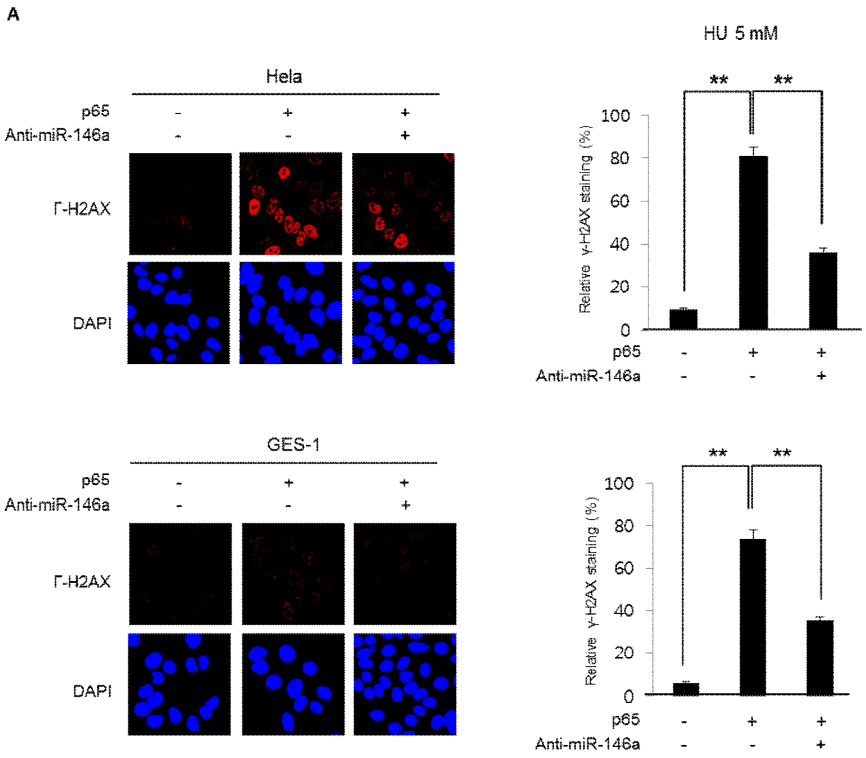
Overexpressing P65 subunit of NFKB causes replication stress and affects clonal cell survivability

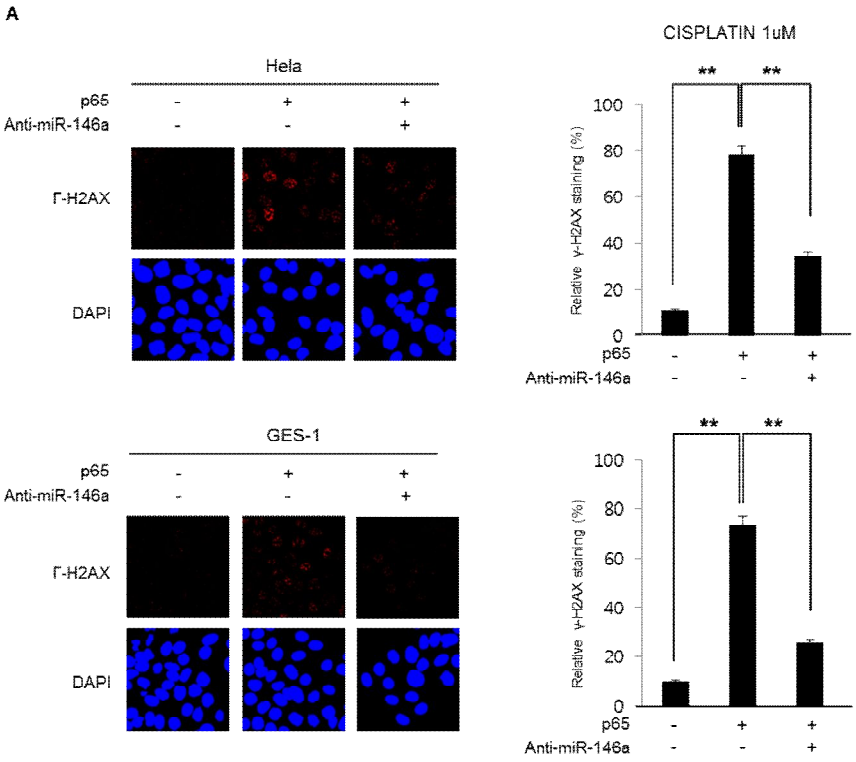
Previously in this study we observed that direct miR146a transfection affected replication fork stalling and recovery. We wanted to see if this

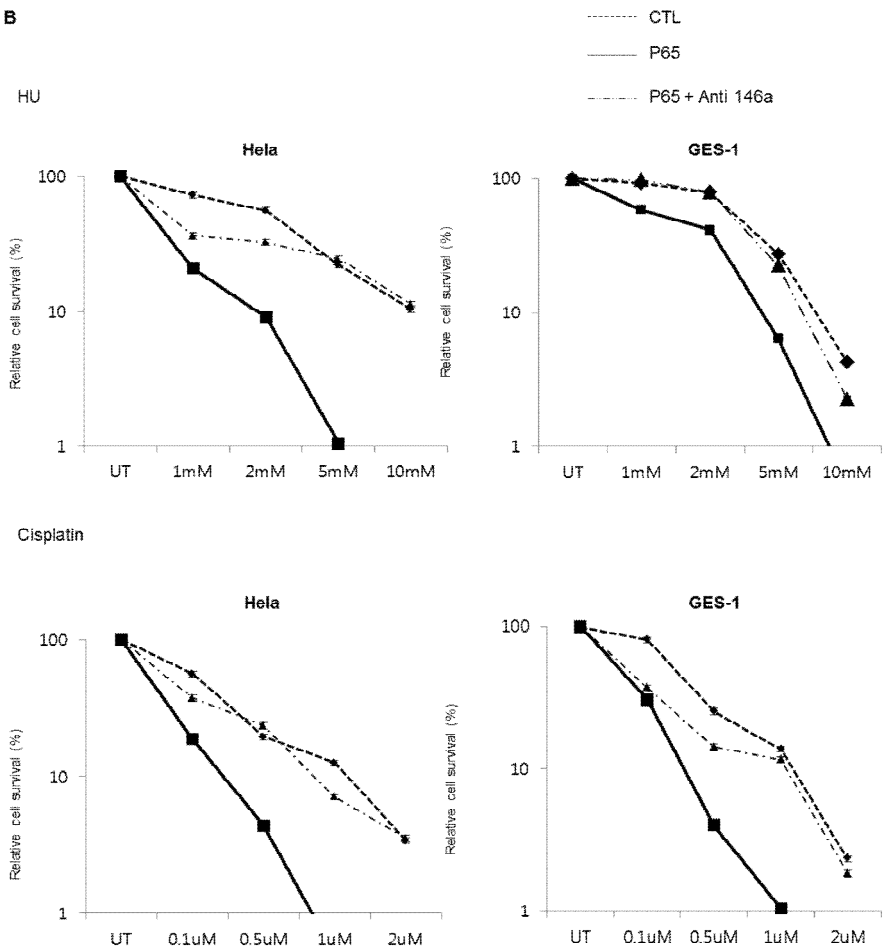
phenomenon was observed in p65 overexpressing cells. We analyzed DNA synthesis in populations of cells treated with scrambled control and RELA/p65. Treatment with 5mM HU resulted in reduction of average fork velocity in p65 transfected cells when compared to control, visualized by a leftward shift of the distribution of CldU/IdU tract length ratio (Figure 5G). This result shows that overexpression of p65 results in reduction in rate of DNA synthesis due to repression of FANCM.

Next we used immunocytochemistry technique to visualize the ongoing replication using g-H2ax as a stress marker. We treated p65 and anti-miR146a transfected HeLa and GES-1 cells with 5mM hydroxyurea and cisplatin respectively. After 16hrs we stained the cells with g-H2ax antibody. We observed replication stress in p65 overexpressing cells when compared to control and that this effect was considerably reversed in cells transfected with anti-miR146a (Figure 6A).

Next we investigated if p65 affected the clonogenic cell survival. HeLa and GES-1 cells were transfected with control, p65 and p65+anti-miR146a







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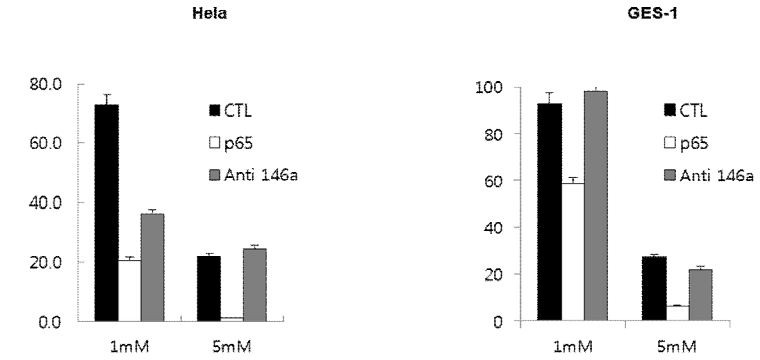

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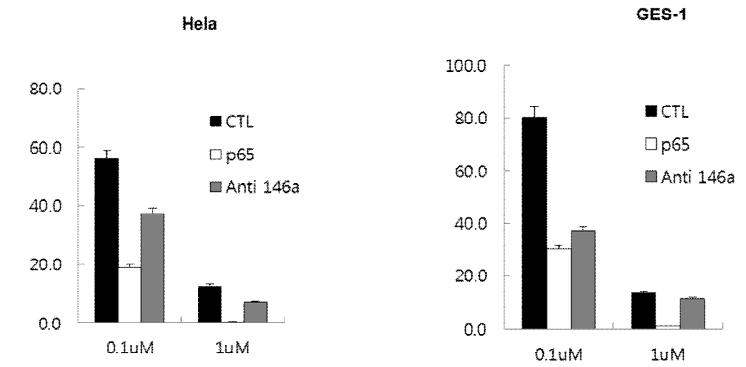

Figure 6 Role of NF-kB in Replication Stress and Clonal Survival.

A. Indicated cells were treated with p65 and p65 + Anti-miR-146a. 48hrs later the cells were treated with 5mM HU and 1uM Cisplatin. Cells were then analyzed by γ-H2AX staining 16 hours after HU and Cisplatin treatment. Results are shown as mean ± SD (n = 3). ** B. HeLa and GES-1 cells were transfected with Control, p65 and p65 + Anti-miR-146a and subjected to increasing dosage of HU and Cisplatin as indicated. Viabilities of cells were examined by clonogenic survival assay. Results are shown as mean ± SD (n = 3). **, $P < 0.01$.

plasmids. After required time of incubation the cells were treated with increasing concentration of hydroxyurea and cisplatin. After 2-3 weeks the cells were stained and the number of colonies formed were counted and plotted. We found that the survival fractions of colonies in p65 overexpressing cells following HU and Cisplatin treatment were significantly reduced to those of control and the clonal survival was recovered in anti-miR146a transfected cells (Figure 6B). Taken all together, these results provide evidence that p65 mediated negative regulation of FANCM via up-regulation of miR146a leads to genomic instability and modulates DSB repair.

Helicobacter pylori mediated down-regulation of FANCM

NFKB is extensively activated during inflammatory response triggered during Helicobacter pylori infection. We wanted to find out if this event affected FANCM protein level via up-regulation of miR146a. We infected GES-1 cells with H.pylori. After 48 hours of incubation we harvested the cells and subjected to western blot analysis. Surprisingly FANCM protein level was significantly down-regulated in H.pylori infected cells when compared to control. A parallel RT-PCR analysis was performed which revealed that miR146a was up-regulated in H.pylori infected cells (Figure 7A). To confirm that miR146a was indeed involved in down-regulation of FANCM we transfected anti-miR146a in H.pylori infected cells and found that it recovered the FANCM protein when compared to H.pylori infected cells (Figure 7B). However BRCA1 and BRCA2 protein level remained unaltered after H.pylori infection when compared to control and remained to be unaffected in anti-miR146a transfected cells.

Next we wanted to see if H.pylori infection affected FANCD2 monoubiquitination. We infected GES-1 cells with H.pyroli and H.pyroli + anti-miR146a. After required time of incubation the cells were treated with 5mM hydroxyurea and after 5 hours the cells were harvested and western blot analysis was performed. The results were as follows. H.pylori infected cells showed defective monoubiquitination of FANCD2 protein corresponding to

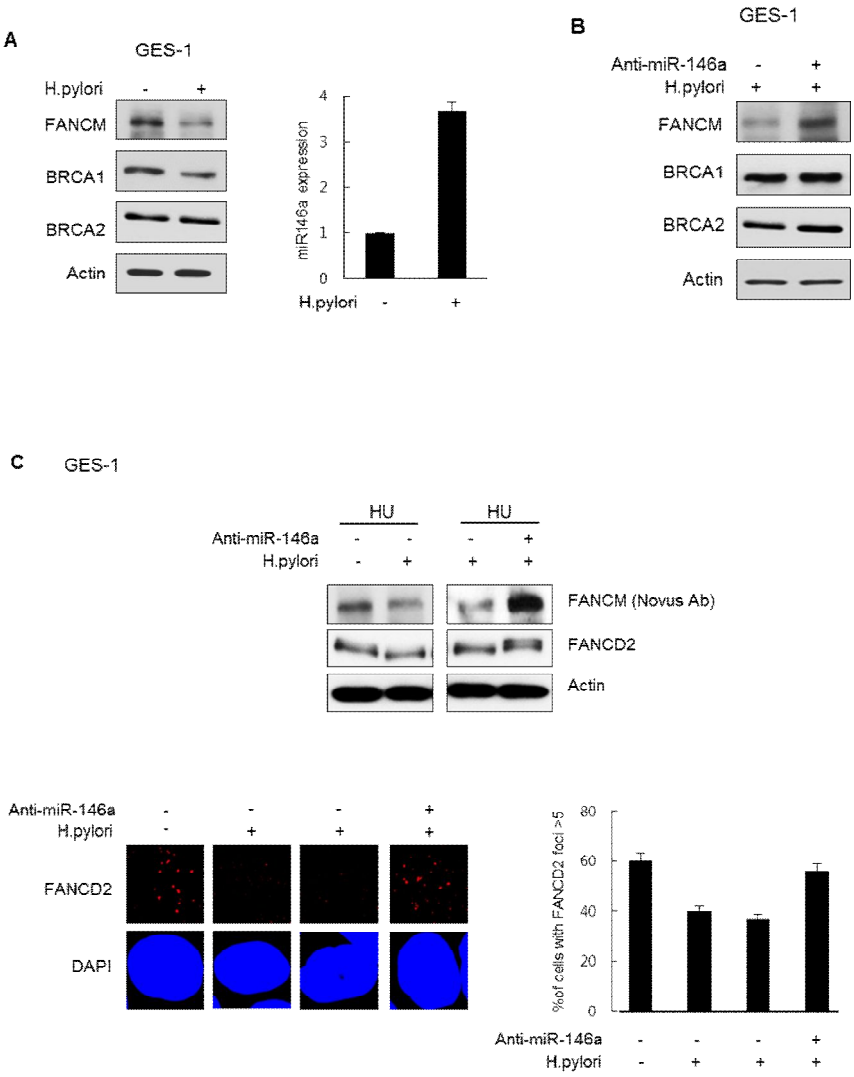

Figure 7 Helicobacter pylori mediated FANCM regulation

A. GES-1 cells were infected with Helicobacter pylori. The levels of indicated proteins were determined using Western blotting. MiR-146a levels in the

indicated cells were determined using real-time qPCR analysis. B. GES-1 cells were infected with Helicobacter pylori and co-transfected with Anti-miR-146a. The levels of indicated proteins were determined using Western blotting. C. GES-1 cells were infected with Helicobacter pylori and co-transfected with Anti-miR-146a as indicated. FANCM protein level and FANCD2 ubiquitination were measured using Western blotting. Actin was used as loading control (top). Cells were analyzed for FANCD2 foci (bottom).

down-regulation of FANCM; however this was reversed in anti-miR146a transfected cells. This effect was simultaneously observed after performing FANCD2 immunostaining, which showed reduced foci formation in H.pylori infected GES-1 cells when compared to control (Figure 7C). All these data proved that inflammatory response triggered during H.pylori down-regulated FANCM protein via up-regulation of miR146a.

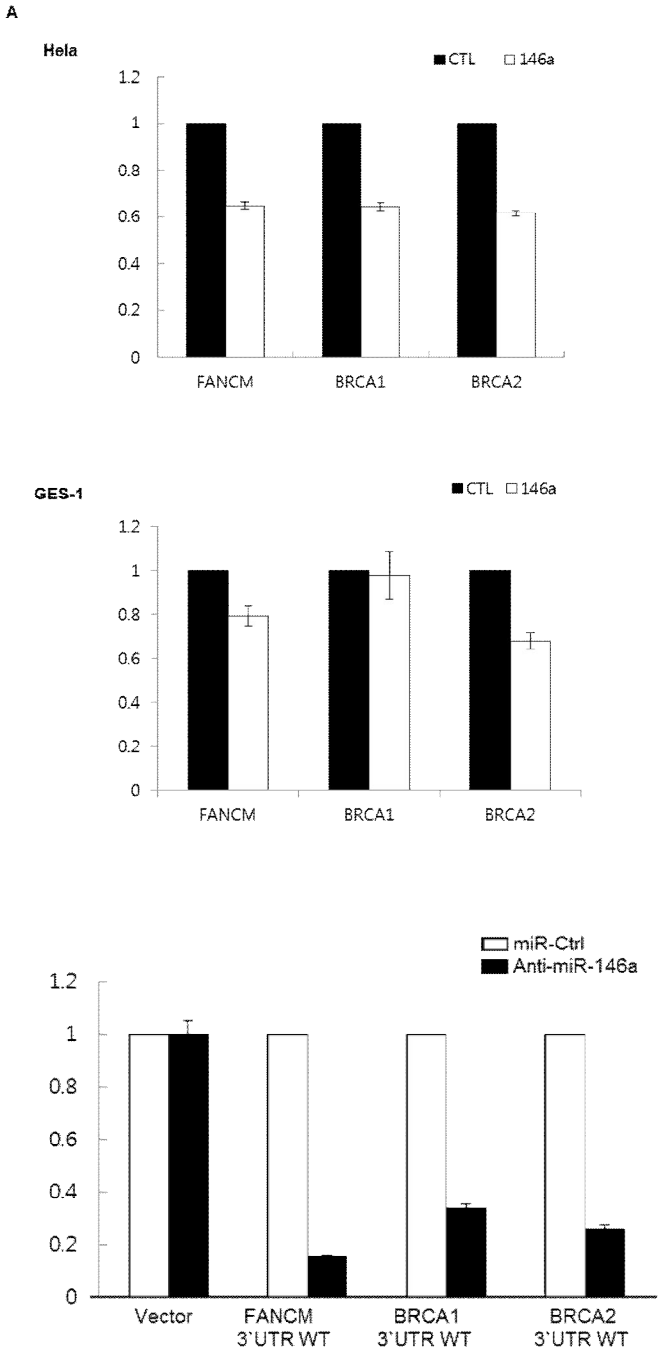

Figure 8 Supplementary

A. Indicated cells were cotransfected with FANCM, BRCA1 and BRCA2 3'-UTR-wt. Luciferase activity was measured 24 hours after the transfection. B. FANCM, BRCA1, BRCA2 3'-UTR-wt were cotransfected with miR-146a and miR146a+Anti miR146a in HeLa cells. Luciferase activity was measured 24 hours after the transfection. Data represent mean $\pm$ SD (n = 3); **, $P < 0.01$.

IV. Discussion

Mir146a – A key regulatory element of FANCM

Among several microRNAs that have been discovered so far, miR146a has been implicated as one of the key regulator of cellular process especially as a posttranscriptional repressor. This is achieved by microRNA binding to the 3`UTR region of target proteins mRNA and repressing their translation. This specific property of microRNA has been exploited in various cellular processes to maintain the integrity of the cell. Mis-regulated microRNAs thus become an evident marker for several disease pathways. Carefully monitoring such events has provided a gateway to formulate a therapeutic option. In line with that our present research focuses on regulation of FANCM by miR146a. FANCM belongs to FA group of proteins and along with its associated protein FAAP24 it is involved in maintaining the integrity of the genome [28]. Thus we set out to find if miR146a can repress FANCM at post transcriptional level. Our initial confirmatory results showed that miR146a effectively down-regulates FANCM at the mRNA level along with its established targets such as BRCA1 and BRCA2. BRCA1 and BRCA2 has been included in the study so as to reiterate the link between Fanconi Anemia and BRCA (FA/BRCA) pathway and to establish miR146a as a common bystander to a major regulatory

element between two diverse disease pathways that has been recently studied widely in terms of cellular DNA damage and repair response.

MiR146a affects inter-strand cross link repair activity and stalls replication forks.

After thoroughly making sure that miR146 is a regulatory element of FANCM we analyzed if down-regulation of FANCM by miR146a leads to cell sensitivity. According to Yocai Wang et al., the translocase activity of FANCM is necessary for Inter-strand crosslink repair and thus loss of FANCM will lead to impaired recombination-independent ICL removal. This will naturally make FANCM deficient cells vulnerable towards ICL agents like hydroxyurea and mitomycin C leading to cell death. We found that cells that exogenously express miR146a showed a marked decrease in clonogenic survival rate compared to normal cells when treated with ICL agent′s hydroxyurea and cisplatin at various concentrations (Figure 4B). Earlier in an independent study FANCM deficient cells ΔFANCM showed decreased cell survival rate when treated with hydroxyurea, camptothecin and aphidicolin [29]. Further we also observed that down-regulation of FANCM by miR146a increased γH2AX foci indicating an ongoing DNA replication which in this case correlates to a stalled or collapsed replication fork. This effect was

reversed when anti-miR-146a was introduced in cells over-expressing miR-146a. This result is again in relation to work done by RA Schwab et al. with cells devoid of FANCM. In another study Andrew N Blackford et.al described that the translocase activity protects stalled replication forks and cells with defective FANCM accumulate DNA damage due to replication fork collapse [30]. To understand if miR146a indeed involved in replication fork stalling we did a DNA fiber analysis in miR146a treated cells. As expected we found that cells with down-regulated FANCM showed reduction in replication velocity when compared to control cells indicating stalled or collapsed fork (Figure 3D). This was a key finding in this research as it raised a question against the significance of microRNA mediated replication fork stalling and defective cell survival, altogether making the cells susceptible to ICL agents.

Defective FANCD2 monoubiquitination

Out of all FA core proteins FANCM has shown a significant role in FANCD2 monoubiquitination. Cells devoid of FANCM when treated with ICL agents show defective FANCD2 monoubiquitination. Transiently down-regulated FANCM leads to defective FANCD2 monoubiquitination because the helicase domain of FANCM is required for the ubiquitination [18]. In another study by Thiyam Ramsing singh et al, they have made a similar study in EUFA867 lymbhoblasts (FANCM and FANCA defective) cell line

showing defective FANCD2 monoubiquitination; however correction of FANCA only partially recovered the monoubiquitination [31]. In our present study translational repression of FANCM by miR146a resulted in defective FANCD2 monoubiquitination as well when treated with hydroxyurea. Immunocytochemistry results revealed that FANCD2 foci formation is reduced in miR146a treated cells. When antisense nucleotide was used to inhibit miR146a it greatly recovered FANCD2 foci formation along with elevated FANCM protein level. The recovery experiment thus revealed the impact of miR146a over FANCM regulation that it affects its downstream cellular process in a substantial manner.

NFKB – FA/BRCA pathway

MiR146a has been identified to be an anti-inflammatory agent involved in regulation of innate and adaptive immune response. This is primarily due to its regulation by NFkB. Konstantin D. Taganov et.al in their research work showed that miR146a is a NFkB dependent gene via promotor analysis [32]. Also they have found out that LPS mediates miR146a up-regulation via NFkB. We in our study tried to find out if activation of NFkB could repress FANCM translation via up-regulation of miR146a. Over-expression of RelA/p65 in cells up-regulated miR146a which in turn down-regulated FANCM expression significantly. Previously overexpression of p65 was

found to increase NFkB transcriptional activity in two independent research work done by Gavin P.Collett et.al and Ling Wang et.al [33, 34]. We also found out that p65 over expressing cells also show decreased BRCA1 and BRCA2 protein level. Previously Benezra. M et.al reported that p65/RelA subunit of NFkB binds to BRCA1 and that absence of BRCA1 greatly influence NFkB activation [35]. Our study which involves overexpression of p65/RelA subunit decreased the protein level of BRCA1 via miR146a up-regulation whilst keeping NFkB active in the process. This correlates to the fact that NFkB is constitutively kept active in breast cancer cell lines. Kangjijan Wu et.al in their study earlier mentioned that NFkB induces BRCA2 expression and that specifically overexpression of p65 induced BRCA2 promotor activity [36]. However our research clearly yielded an opposite effect that BRCA2 was down-regulated upon p65 over-expression. Further when we observed that the clonogenic survival rate of cells transfected with p65/RelA subunit was greatly decreased when compared to control cells. When treated with interstrand cross linkers such as hydroxyurea, the cell survival deteriorated at an exponential rate. Our previous understanding of FANCM down-regulation and lack of cell survival thus became evident again when the same was replicated with p65 overexpression. This factor is primarily due to up-regulation of miR146a which led to translational repression of FANCM. Another important factor which needed attention was that, NFkB and miR146 are involved in intricate negative-

feedback loop mechanism. Jin Ho Paik et al in their research showed that miR146a down-regulates NFkB by targeting TRAF6 and it acts as a tumor suppressor [37]. This research work along with previous research on NFKB mediated down-regulation of miR146 by Konstantin D. Taganov et.al, led to our understanding that for a normal cell to perform its basic cellular function a balance between NFkB and mimR146 a level is needed, which is achieved by a sophisticated negative-loop mechanism between NFkB and miR146a. A mis-regulation that occurs in either of these components will lead to cellular abnormality resulting in tumorigenesis. NFkB abnormal expression leading to cancer is a well-studied phenomenon. However miR146a up-regulation and its effects related to tumor and metastasis suppressor but not as a role in promoting tumorigenesis which happens otherwise. Thus we propose that miR146 potentially acts as a missing link to this process where it is used as a regulatory tool by NFkB which under abnormal condition gets mis-regulated and results in cancer.

Inflammation and Fanconi Anemia

In our research we have tried to understand if a regulatory molecule such as miR146 can be used in a way that leads to Fanconi anemia via down-regulation of FANCM. Also if inflammation as such can play an indirect role in causing Fanconi anemia via NFkB mediated miR146a up-regulation. Du.W

et.al described that deregulation of Notch signaling skews hematopoietic stem cell differentiation which leads to bone-marrow failure, resulting in leukemia and cancer progression [38]. Also there is a functional cross talk between NFkB pathway and notch signaling in FA associated HSC differentiation. In another research done by Xiaoling Zhang et.al, inflammatory reactive oxygen species associate with Fanconi anemia and cause DNA-damage induced premature senescence in HSC cells [39]. Several of these research talk about the relation between inflammation and Fanconi Anemia. Also numerous other research works have been done to establish the role of miR146a as an anti-inflammatory agent and mediating inflammatory response however its direct role in anemia has not been studied so far. We in our research thus try to bring out the fact that inflammation is closely related with Fanconi anemia. We found that inflammatory cytokines such as TNF alpha and interleukin-1 up-regulates miR146a via NFkB, which in-turn down-regulated FANCM. This is the first time such data is reported. Guo.F et.al proposed that mammalian target of rapamycin (mTOR) regulates DNA damage response and genomic stability which involves NFkB-mediated FANCD2 expression [40]. We also found that FANCD2 foci formation was also affected to a significant level upon treatment with TNF alpha and IL-1 beta. In a parallel research done by Nobuko Matsushita et.al, they have shown that FANCD2 deficiency promotes transcriptional activity of TNF alpha [41]. Our results thus reveal the fact that

inflammatory response is closely related with Fanconi Anemia via miR146a regulation.

Gastric cancer and Fanconi Anemia

After understanding the relation between inflammation and cancer we sought out to find if Gastric cancer, an inflammatory related disease could lead to Fanconi anemia predisposition. Several studies have pointed out the relation between miR146a and Gastric cancer. Elevated level of miR146a in gastric cancer has been shown to target SMAD and that it modulates cell proliferation and apoptosis [42]. When we subjected gastric cancer cells and other cell lines to Helicobacter pylori infection we found out that FANCM along with BRCA1 and BRCA2 was down-regulated. Also miR146a level was elevated when compared to control. But this was not a consistent and that the translation repression of FANCM varied upon cell number, time and several other factors. However our data suggest a possible relationship between H.pyroli infection and Fanconi anemia which needs to be explored further. Earlier a clinical study reported an association between H.pyroli and anemia [43]. Another independent clinical research revealed a similar relationship [44]. Moreover NFkB activation by H.pyroli is a well-studied phenomenon. The cag pathogenicity island of H.pyroli utilizes the activation of NFkB for H.pyroli induced inflammatory response [45]. In another study

NFkB was found to be activated by AKT-mediated phosphorylation of p65 by H.pyroli. All these and other similar works reveal the importance of NFkB activation by H.pyroli to cause inflammatory response. These findings along with our research which focuses on down-regulation of FANCM by miR146a (a critical NFkB regulatory molecule) bridges a possible connection between Helicobacter pylori infection and Fanconi anemia disposition.

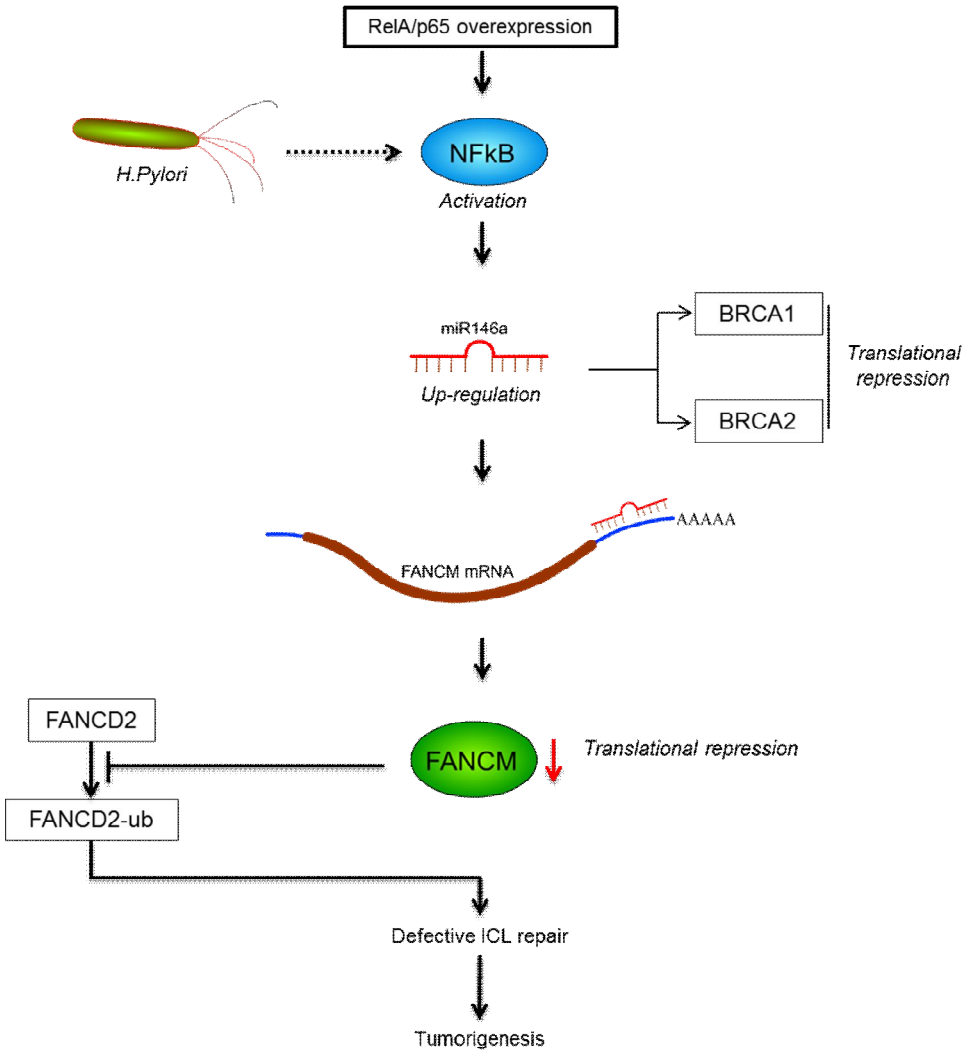

Figure 8. NFkB-FANCM schematics

Conclusion

In summary our study unveiled two important outcomes. Firstly, we found that miR146a is a key regulator that leads to translational repression of FANCM protein and it plays a vital role in regulation of FANCD2 monoubiquitination. Mis-regulated miR146a leads to loss of recombination-independent ICL repair through down-regulation of FANCM, an important component which maintains cellular resistance to ICL agents.

Secondly p65 mediated translational repression of FANCM via up-regulation of miR146a is a new hypothesis which is ambiguous and falls under debating zone in FA/BRCA research. Our results confirmed that direct overexpression of p65 leads BRCA1 and BRCA2 down-regulation; however previous studies have showed that p65 subunit enhances the promotor activity of BRCA1 and BRCA2 and increases the protein level. Also in our study direct H.pylori infection only down-regulated FANCM but not BRCA1 and BRCA2. Thus a detailed understanding of the complex negative-loop mechanism occurring between NFKB and miR146a is needed, to comprehend this part of the research. Nevertheless, our study shows in first-hand that p65/RELA subunit of NFKB is involved in translation repression of FANCM via up-regulation of miR146a. MiR146a therefore acts as a conduit between inflammation, Fanconi anemia and cancer progression and it stands as a viable target for therapeutic intervention.

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ABSTRACT

P65/RelA is involved in translational repression of Fanconi Anemia protein FANCM via up-regulation of MiR-146a

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FANCM is a DNA remodeling enzyme associated with Fanconi Anemia (FA) core complex, involved in activation of Fanconi Anemia (FA) and maintains DNA replication fork stability. Loss of function of FANCM leads to genomic instability and failure in replication restart which attributes to tumorigenicity. However, microRNA mediated regulation of FANCM its physiological effects remains elusive. Here we show that miR146a inhibits FANCM translation via direct binding to its 3' untranslated region, leading to replication stress and defective monoubiquitination of FANCD2. We have also demonstrated that overexpression of p65/RELA subunit of NFkB negatively represses FANCM protein by up-regulation of miR146a. Our results point out a central role of miR146a in the physiologic regulation of

FANCM-dependent ICL repair and suggest a molecular mechanism of how aberrant activation of NFkB leads to increased genomic instability which leads to tumorigenesis.

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"This PhD is a dedication to family"

Devakumar Sundaravinayagam
Chosun University