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Original Article

RECK impedes DNA repair by inhibiting the erbB/JAB1/Rad51 signaling axis and enhances chemosensitivity of breast cancer cells

Kun-Jing Hong[1], Ming-Chuan Hsu[2], Wen-Chun Hung[1,2]

[1]*Institute of Biomedical Sciences, National Sun Yet-Sen University, Kaohsiung 804, Taiwan, Republic of China;* [2]*National Institute of Cancer Research, National Health Research Institutes, Tainan 704, Taiwan, Republic of China*

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Abstract: The reversion-inducing cysteine-rich protein with kazal motif (RECK) is an endogenous matrix metalloproteinase (MMP) inhibitor and a tumor suppressor. Its expression is dramatically down-regulated in human cancers. Our recent results suggest a novel MMP-independent anti-cancer activity of RECK by inhibiting the erbB signaling. Activation of the erbB signaling is associated with chemotherapeutic resistance, however, whether RECK could modulate drug sensitivity is still unknown. Here we demonstrated that expression of RECK induced the activation of ATM and ATR pathways, and the formation of γ-H2AX foci in breast cancer cells. RECK inhibited the erbB signaling and attenuated the expression of the downstream molecules Jun activation domain-binding protein 1 (JAB1) and the DNA repair protein RAD51 to impede DNA repair and to increase drug sensitivity. Treatment of epidermal growth factor or over-expression of HER-2 effectively reversed the inhibitory effect of RECK. In addition, ectopic expression of JAB1 counteracted RECK-induced RAD51 reduction and drug sensitization. Our results elucidate a novel function of RECK to modulate DNA damage response and drug resistance by inhibiting the erbB/Jab1/RAD51 signaling axis. Restoration of RECK expression in breast cancer cells may increase sensitivity to chemotherapeutic agents.

Keywords: RECK, HER-2, JAB1, RAD51, γ-H2AX

Introduction

The human RECK gene is located at the 9p13.3 region of chromosome 9 and encodes a membrane-anchored glycoprotein with different biological functions [1]. Knockout of RECK gene causes embryonic lethality with destruction of extracellular matrix integrity and angiogenesis indicating this gene plays a critical role in developmental stage [2]. In addition, RECK is essential for skeletal muscle development and cartilage differentialtion [3, 4].

Lines of evidence support the notion that RECK is a tumor suppressor. First, expression of RECK is significantly down-regulated in most of human cancers studied and is frequently associated with poor prognosis and increased metastasis. Second, overexpression of RECK in cancer cells induces growth inhibition by suppressing proliferation and angiogenesis. Third, re-activation of RECK expression by natural or synthetic compounds attenuates migration and invasion in vitro and metastasis in vivo. We have previously elucidated the molecular mechanism by which various oncogenes including HER-2, RAS and latent membrane protein 1 repressed RECK transcription by epigenetic inhibition [5-7]. In addition, we identified non-steroidal anti-inflammatory drugs and histone deacetylase (HDAC) inhibitors as potential compounds to re-activate RECK in cancer cells [8, 9].

Because RECK is an endogenous matrix metalloproteinase (MMP) inhibitor which effectively suppresses MMP-2, MMP-9 and MT1-MMP activity in vitro, the tumor-suppressive effect of RECK is considered to be mediated via inhibition of MMPs. However, recent results of our and other groups elucidate a MMP-independent anti-cancer mechanism of RECK. We find that

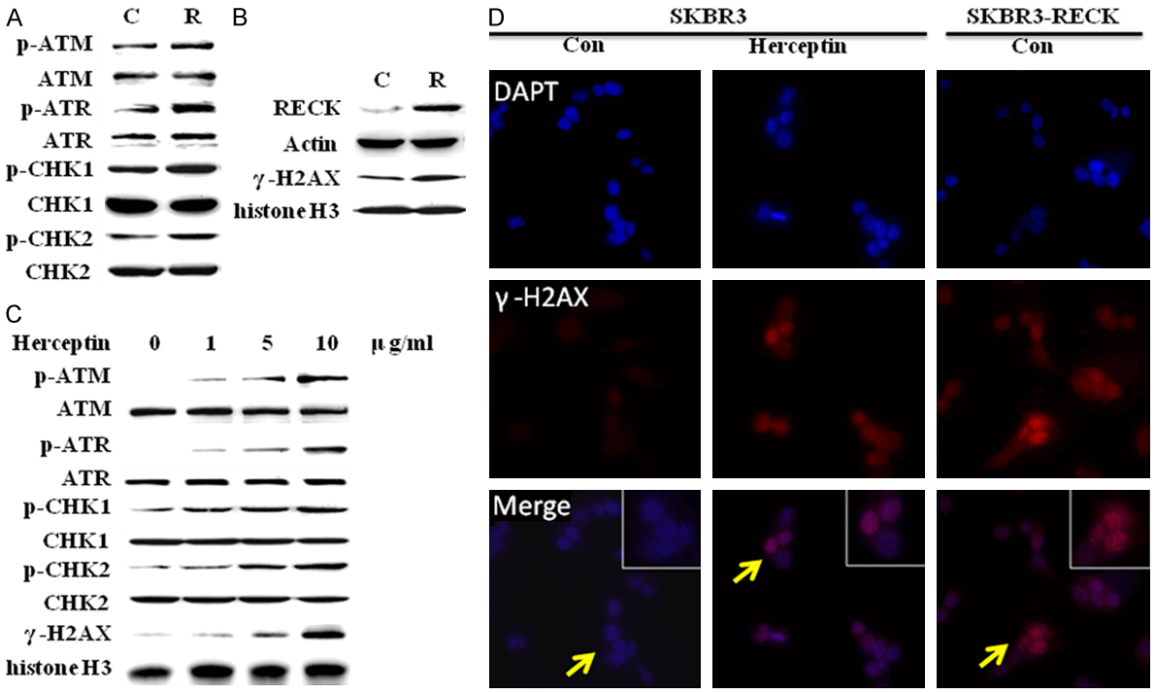

Figure 1. Activation of the ATM/ATR signaling and formation of γ-H2AX foci are enhanced by RECK expression or Herceptin treatment in HER-2-overexpressing breast cancer cells. A. Phosphorylation status and total protein level of ATM, ATR, CHK1 and CHK2 of SKBR3 (C) and SKBR3-RECK (R) cells were investigated by Western blot analysis. B. The protein level of γ-H2AX was also compared in these two cell lines and histone H3 was used as an internal control. C. SKBR3 cells were treated with different doses of Herceptin for 24 h. The cells were harvested and subjected to Western blot analysis to examine the activation of the ATM/ATR signaling and the increase of γ-H2AX. D. γ-H2AX foci formation was detected by immunofluorescent staining in SKBR3 cells treated without or with Herceptin or SKBR3-RECK cells. Nuclei were visualized using DAPI staining. The cells indicated by the arrows were shown in detail in the insets.

ectopic expression of RECK in HER-2-overexpressing breast cancer cells inhibits HER-2 receptor dimerization and autophosphorylation, which leads to down-regulation of HER-2 target genes and attenuation of cellular proliferation [10]. Similarly, Kitajima et al demonstrate that epidermal growth factor receptor (EGFR) activity is transiently upregulated by RECK depletion in mouse embryonic fibroblasts, and continuously downregulated by RECK overexpression in colon cancer cells [11]. EGFR and HER-2 are overexpressed in breast tumor tissues and constitutive activation of EGFR and HER-2 pathways are involved in modulating the repair of DNA damage produced by chemotherapy which contributes to the resistance of chemotherapeutic drugs in breast cancer cells. Because our and others results indicate that RECK is a critical regulator of EGFR and HER-2 signaling, a potential role of RECK in regulating the DNA damage response (DDR) and modulating the chemotherapy sensitivity is suggested. We test our hypothesis in this study and try to elucidate the underlying mechanism by which RECK modulates DDR.

Materials and methods

Cell cultures and reagents

SKBR3 and SKBR3-RECK cells were cultured in McCoys 5A with 10% fetal calf serum (FCS). U2OS cells stably expressed homologous recombination reporter DR-GFP were cultured in the same medium. MDA-MB-231 cells were grown in RPMI 1640 containing 10% FCS. All cells were maintained at 37°C in a 5% CO_2 incubator. Cisplatin, camptothecin and etoposide were obtained from Sigma. Anti-RECK (611513, BD Transduction Laboratories™), Phospho-CHK1 (Ser345), Phospho-CHK2 (Thr68), Phospho-ATM (Ser1981), Phospho-ATR (Ser428), CHK1 (2G1D5), CHK2 (1C12), ATM (D2E2), ATR (#2790), PARP (46D11), Caspase-3 (8G10), Histone H3 (D1H2) were purchased from Cell Signaling Technology. Anti-phospho-Histone

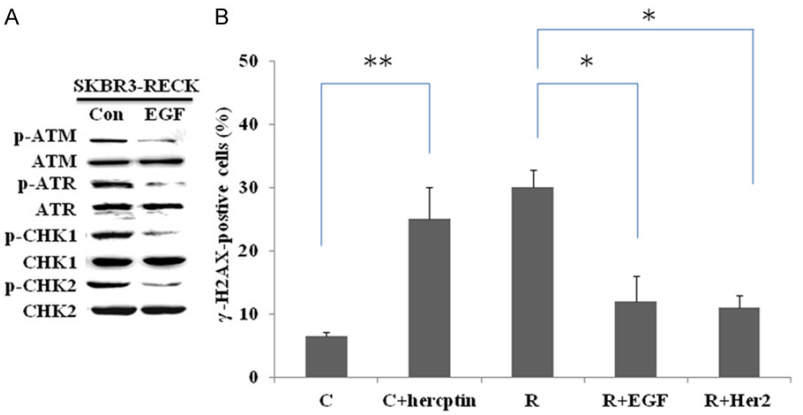

Figure 2. RECK-induced DNA damage response is attenuated by EGF treatment or HER-2 overexpression. A. SKBR3-RECK cells were treated without or with EGF (100 ng/ml) for 24 h. The cells were harvested and subjected to western blot analysis. B. The formation of γ-H2AX foci was analyzed using immunofluorescent staining in SKBR3 (C) cells treated without or with Herceptin. The γ-H2AX-positive SKBR3-RECK (R) cells treated with EGF or ectopically expressed with HER-2 were also examined. The percentage of γ-H2AX-positive cells from three independent assays was expressed as Mean ± SE. $^{*}P<0.05$; $^{**}P<0.01$.

H2A.X (Ser139) and anti-actin antibodies were purchased from Millipore. JAB1 Antibody (N-17) was obtained from Santa Cruz Biotechnology. Rad51 antibody (N1C2) was purchased from GeneTex.

Real-time PCR

RNA was extracted from cells by using Total RNA Mini Kit (Geneaid) and the expression of Jab1 and Rad51 mRNA levels were detected by Applied Biosystems 7500 Fast Real-Time PCR System as described previously [15]. The primers for Jab1: forward 5'-CAAGAAACAGCAGCAAGAAATC-3', reverse 5'-AGCATCAGACC CATCACTTCC-3'; Rad51: forward 5'-CTTTGGCCCACAACCCATTTC-3', reverse 5'-ATGGCCTTTCCTTCACCTCCAC-3'; GAPDH: 5'-AAGGCTGGGGCTCATTTG C-3' and 5'-GCTGATGATCTTGAGGCTGTTG-3'.

Western bolt analysis

Sample preparation and Western blot analysis were performed as described previously [7]. In brief, the treated cells were harvested with a RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM EDTA and 50 mM NaF) and the lysates were incubated on ice for 20 min and centrifugated at 13,000 g for 30 min. Protein concentrations were measured by using protein assay reagents (Pierce). Equal amounts of total protein were mixed with 4X sample loading buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 20% SDS, 2.5% bromophenol blue, and 5% 2-mercaptoethanol), boiled for 10 min and then fractionated by using electrophoresis on 8% or 10% SDS-PAGE. Proteins were electrically transferred to PVDF membranes and treated for 1 h at room temperature with 0.05% TBST and 5% non-fat milk. Blots were subsequently incubated at 4°C overnight with primary antibodies. Immunoreactive proteins were detected after incubation with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The immunoblots were visualized by using enhanced chemiluminescence reagent.

MTT assay

Cells were seeded in a 96-well plate with the density of 10000 cells/well. Cells were incubated with different concentrations of chemotherapeutic drugs for 24 h. After incubation, 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added to each well at a final concentration of 5 mg/ml and the plate was incubated at 37°C for another 4 h. The supernatant was discarded and 100 μl of DMSO was applied to each well. The absorbance was determined at 570 nm with a microplate reader.

Immunofluorescent staining

1×10^3 cells were plated per well in 12-well plates. After drug exposure for 24 h, cells were fixed in 4% paraformaldehyde in PBS overnight, permeabilized in 0.2% Triton X-100 for 5 min, and blocked with 1% bovine serum albumin in PBS for 30 min. Cells were incubated with primary antibodies for 1 h, washed three times in PBS, and then incubated with 546-Alexa-(red) or 488-Alexa-labeled (green) secondary antibodies (Invitrogen) and observed by using a fluorescent microscope (Leica).

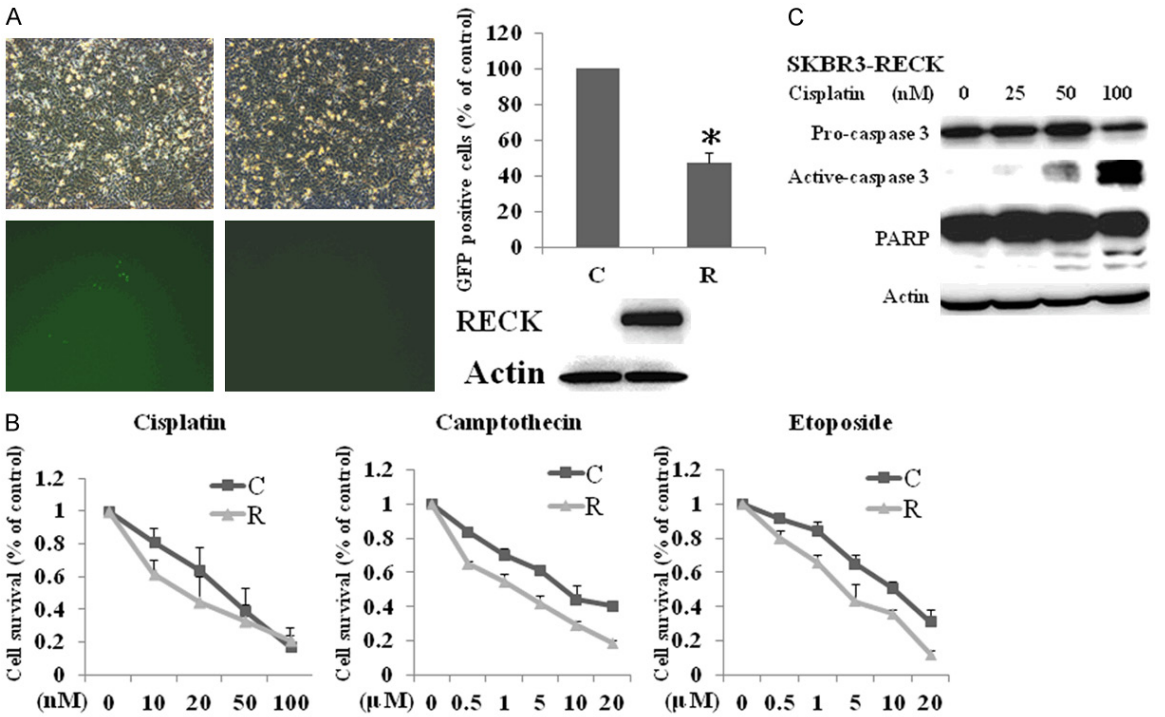

Figure 3. RECK impairs DNA repair and enhances the sensitivity of cells to chemotherapeutic drugs. A. U2OS-DR-GFP cells were transfected with control or RECK expression vector for 48 h and then GFP expression in the cells was analyzed. The relative percentage of GFP-positive (repaired by homologous recombination) cells was expressed as Mean ± S.E. from three independent assays. The expression of RECK protein was determined by western blotting. B. SKBR3 and SKBR3-RECK cells were treated with different concentrations of cisplatin, camptothecin or etoposide at for 24 h and subjected to MTT assay. C. SKBR3-RECK cells were treated with different concentrations of cisplatin for 24 h. The cells were harvested for analysis of caspase-3 activation and PARP degradation.

HR repair assay

U2OS DR-GFP cells, stably transfected with DR-*GFP* reporter gene, were kindly provided by Dr. Liaw HJ (National Cheng Kung University, Taiwan, Republic of China). Cells were transfected with RECK expression vector, I-SceI-expressing plasmid (pCBA-I-SceI) and monomeric red fluorescent protein vector (pCS2-mRFP) by Lipofectamine™ 2000 for 48 h. Cells were subjected for the analysis of GFP expression (recombination induced by double-strand breakage) by flow cytometry [12]. RFP was used as an internal control. Data were expressed as Mean ± SEM from three independent experiments.

Colony formation assay

Cells were seeded onto 6-well plates at a density of 2×10^3 cells per well. After 24 h, vehicle (DMSO, 0.1%) or cisplatin (50 nM) were added into the culture. Cells were maintained with cispltin in the culture for 15 days and medium was changed every 3 days. The colonies were stained with 1% crystal violet for 10 min at room temperature and counted under the microscope. The experiment was repeated four times and the data were presented after statistical processing by Vision Works LS analysis.

Results

RECK expression or Herceptin treatment affects the ATM/ATR signaling and increased γ-H2AX foci in HER-2-overexpressing breast cancer cells

We have previously established a RECK-overexpressing clone (SKBR3-RECK) from the parental SKBR3 breast cancer cells which exhibited HER-2 gene amplification [10]. Interestingly, we found that the activity of ATM, ATR, CHK1 and CHK2 kinases was increased in SKBR3-RECK cells indicating activation of the ATM/ATR signaling pathway (**Figure 1A**). In addition, the level of γ-H2AX was increased (**Figure 1B**). The ATM/ATR signaling could be activated

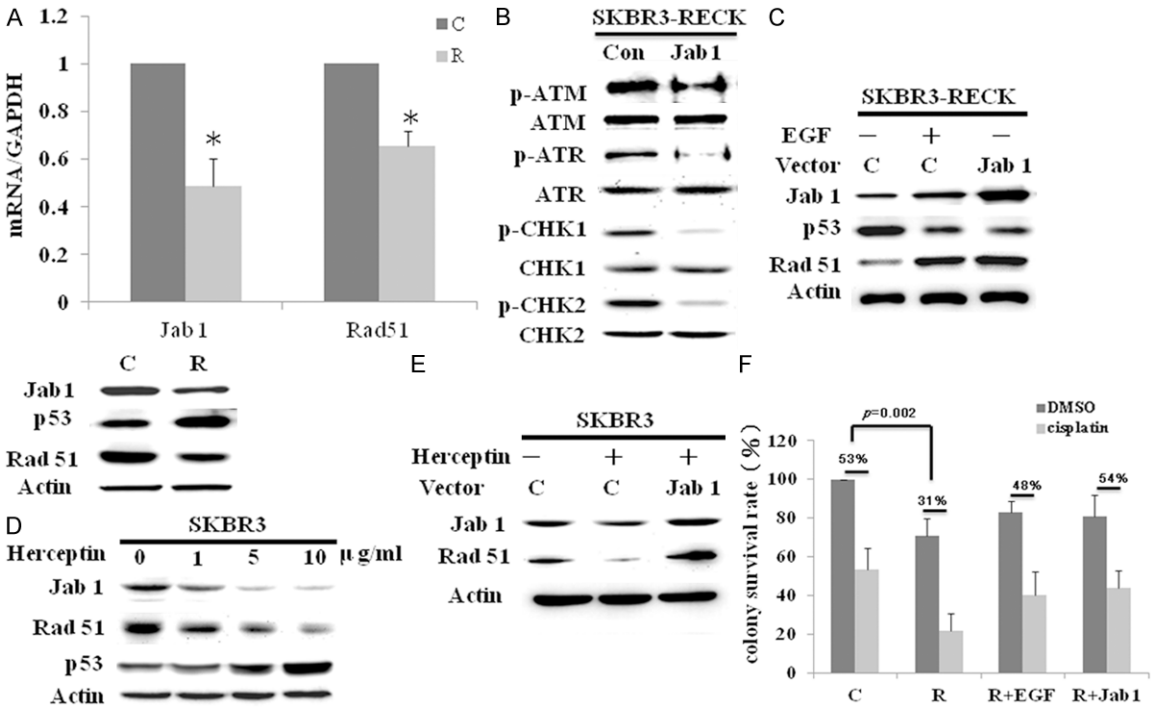

Figure 4. RECK impedes DNA repair by inhibiting the expression of Jab1 and Rad51. A. Upper panel; total RNA was extracted from SKBR3 and SKBR3-RECK cells. The expression of Jab1 and Rad51 was investigated by quantitative RT-PCR and GAPDH was used as an internal control. Lower panel; equal amount of proteins from SKBR3 and SKBR3-RECK cells was subjected to stud the protein level of Jab1, p53, Rad51 and actin. B. SKBR3-RECK cells were transfected with control (Con) or Jab1 expression vector. Twenty four hours later, the cell lysate was prepared and the activation status of the ATM/ATR signaling was studied. C. SKBR3-RECK cells were treated with EGF (100 ng/ml) or transfected with Jab1 expression vector. Twenty four hours later, the expreesion of Jab1, p53 and Rad51 was investigated. D. SKBR3 cells were treated with different doses of Herceptin for 24 h and the protein level of Jab1, p53 and Rad51 was examined. E. SKBR3 cells were treated with Herceptin or transfected with Jab1 expression vector. Twenty four hours later, cells were harvested for analysis of Jab1 and Rad51. F. SKBR3 and SKBR3-RECK cells were treated with vehicle (0.1% DMSO) or cisplatin (50 nM0 for 15 days. The culture medium was changed every 3 days. Colonies were stained with crystal violet and counted. Data from three independent experiments were expressed as Mean ± S.E.

by DNA damage or replication stress, our results implied that SKBR3-RECK cells had either increased DNA breakage or defective DNA repair which caused increase of the ATM/ATR phosphorylation. Previous studies demonstrated that cell lines expressing high levels of HER-2 (like SKBR3), when treated with Herceptin, exhibited marked increases in DNA strand breaks and slowed growth [13, 14]. We found that treatment of SKBR3 breast cancer cells with Herceptin (anti-HER-2 antibody) indeed activated the ATM/ATR signaling and increased γ-H2AX level in a dose-dependent manner (**Figure 1C**). Immunofluorescent staining confirmed the formation of γ-H2AX foci after Herceptin treatment (**Figures 1D** and **2B**). In addition, RECK expression also caused a significant increase in foci formation in SKBR3 cells (**Figures 1D** and **2B**).

Inhibition of HER-2 and EGFR signaling by RECK is required for the induction of DDR

To clarify whether the activation of DDR by RECK was associated with the inhibition of EGFR and HER-2 as reported in our and others studies, we treated SKBR3-RECK cells with EGF and found that the activity of ATM, ATR, CHK1 and CHK2 kinases was dramatically reduced (**Figure 2A**). EGF addition or ectopic expression of HER-2 significantly reversed RECK-induced γ-H2AX foci formation (**Figure 2B**).

RECK inhibits DNA repair and induces hypersensitivity to chemotherapeutic drugs

Because EGFR and HER-2 overexpression has been shown to confer the chemoresistance of

cancer cells, we tested the effect of RECK on DNA repair ability and drug sensitivity. We utilized a classical I-SceI-mediated homologous recombination (HR) reporter system to assess the efficiency of DNA repair [15]. In this assay system, a clone of U2OS cells that stably express a single copy of the HR repair reporter substrate DR-GFP which includes a SceGFP region that contains an I-SceI endonuclease site and an iGFP region that contains homologous sequences for the SceGFP was transfected with control or RECK expression vector. Our data showed that the HR repair activity was reduced by 50% in RECK-expressing cells (**Figure 3A**). Sensitivity of SKBR3 and SKBR3-RECK cells to cisplatin (a platinum-based DNA damage agent), camptothecin (a topoisomerase I inhibitor) and etoposide (a topoisomerase II inhibitor) was compared. Our data demonstrated that RECK expression significantly induced hypersensitivity to chemotherapeutic drugs (**Figure 3B**). The reduced cell survival was mainly caused by induction of apoptosis because activation of caspase-3 and degradation of poly-ADP ribose polymerase were clearly demonstrated in cisplatin-treated SKBR3-RECK cells (**Figure 3C**). The aforementioned effect is not cell line-specific because RECK also induced hypersensitivity to cisplatin in MDA-MB-231 breast cancer cells which express EGFR but not HER-2 (Figure S1). These data point out for the first time that RECK can modulate sensitivity of breast cancer cells to chemotherapeutic drugs via inhibition of the EGFR and HER-2 signaling.

RECK impedes DNA repair by down-regulating Jun activation domain- binding protein 1 (JAB1) and RAD51

We have previously demonstrated that JAB1 is a downstream target transcriptionally activated by HER-2 in breast cancer [16, 17]. Recently, JAB1 has been shown to play an essential role in DNA repair [18]. Tian et al demonstrated that loss of Jab1 expression increased p53 and reduced RAD51 which led to spontaneous DNA damage and HR defects. They also demonstrated that the accumulated p53 directly bound to RAD51 promoter to repress its transcriptional activity. Because RAD51 is a critical DNA repair protein, inhibition of RAD51 by p53 causes impairment of HR repair in these cells. We therefore studied whether RECK-induced inhibition of the erbB signaling is associated with down-regulation of JAB1 and RAD51. Indeed, a 40-50% of reduction of mRNA and protein levels of these two genes was found in RECK-transfected SKBR3 cells (**Figure 4A**). In agreement with the previous findings, p53 was up-regulated. Ectopic expression of JAB1 in SKBR3-RECK cells reduced DDR as demonstrated by the attenuation of the ATR/ATM signaling (**Figure 4B**). In addition, JAB1 expression inhibited p53 and restored RAD51 in SKBR3-RECK cells indicating JAB1 is an upstream regulator of p53 (**Figure 4C**). Similarly, EGF treatment increased JAB1 and RAD51 and reduced p53 in these cells (**Figure 4C**). Conversely, Herceptin reduced JAB1 and RAD51 and increased p53 protein in SKBR3 cells (**Figure 4D**). Moreover Jab1 fully rescued Herceptin-induced down-regulation of RAD51 suggesting JAB1 acts upstream of RAD51 (**Figure 4E**). Finally, we performed clonogenic assay to determine cell reproductive death after treatment of cisplatin. As shown in **Figure 4F**, cisplatin alone suppressed colony formation by 47%. RECK expression caused a 30% of inhibition of colony number. Combination of RECK and cisplatin reduced the colony formation to 20% of that of the control group which could be reversed by EGF treatment or Jab1 over-expression.

Discussion

Although RECK is known to act as a tumor suppressor in vitro and in vivo, this molecule has never been reported to involve in DDR and chemosensitivity. We provide the first evidence that RECK impedes DNA repair activity of cancer cells and induces hypersensitivity to chemotherapeutic agents. Because RECK is significantly down-regulated in most cancers, drugs or chemicals that can re-activate RECK expression are expected to enhance the efficacy of chemotherapeutic agents. We have previously identified two classes of such drugs. The first class is non-steroidal anti-inflammatory drugs [8]. Our data demonstrated that NS398 (a coclyoxygenase-2 inhibitor) and aspirin increased RECK expression in human lung cancer cells. This effect is not cancer type-specific because aspirin also up-regulates RECK in prostate cancer and cholangiocarcinoma cells [19, 20]. The second class is HDAC inhibitors [9]. Because transcription of RECK is frequently

down-regulated by epigenetic repression, we tested the effect of HDAC inhibitors and showed that trichostatin A increased RECK expression to reduce cell invasiveness. HDAC inhibitors have recently been intensively developed and evaluated extensively in clinical trials. A combination of HDAC inhibitors and camptothecin had already been tested to be effective in breast cancer [21, 22]. Our study provides a molecular basis for the combinatory therapy by using HDAC inhibitors and chemotherapeutic drugs. Recently, Murai et al identified 34 compounds as RECK inducers by using a novel screening system [23]. The top-ranking compound is disulfiram. This drug was used to support the treatment of chronic alcoholism and for the therapy of cocaine dependence [24, 25]. Interestingly, disulfiram has been shown to be a potential drug for breast cancer treatment [26, 27]. Further investigations are needed to address the synergistic effect between disulfiram and chemotherapeutic drugs.

Another important finding of this study is the elucidation of the underlying signaling molecules JAB1 and RAD51 involved in RECK-modulated DNA repair. JAB1 is a multifunctional protein that participates in diverse cellular processes including modulation of AP1-mediated transcription activation, promotion of cell cycle progression and regulation of hormone signaling. Our results have demonstrated that Jab1 is overexpressed in breast cancer [16, 17]. Originally identified as a DNA repair protein, RAD51 has also been shown to play multiple roles in the control of DNA recombination, genomic instability and telomere maintenance [28]. Interestingly, overexpression of RAD51 is also found in breast cancer and is associated with poor clinical outcome [29]. Halenaquinone has been suggested to be a RAD51 inhibitor by specifically inhibiting the RAD51-double strand DNA binding [30]. Recently, a small molecule RI-1 which can bind covalently to the surface of RAD51 protein at cysteine 319 to destabilize RAD51 monomers to oligomerize into filaments on DNA has been developed [31]. It will be interesting to study whether these RAD51 inhibitors also potentiate the anti-cancer activity of chemotherapeutic drugs on breast cancer cells. Collectively, we elucidate a novel function of RECK in DNA repair by modulating the erbB/JAB1/RAD51 signaling axis.

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Disclosure of conflict of interest

None.

Abbreviations

DDR, DNA damage response; RECK, Reversion-inducing cysteine-rich protein with Kazal motif; EGFR, epidermal growth factor receptor; MMP, matrix metalloproteinase; JAB1, Jun activation domain-binding protein 1; HDAC, histone deacetylase; MTT, 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

Address correspondence to: Wen-Chun Hung, National Institute of Cancer Research, National Health Research Institutes, No. 367, Shengli Road, Tainan 704, Taiwan, Republic of China. Tel: 886-6-7000123 Ext. 65147; Fax: 886-6-2083427; E-mail: hung1228@nhri.org.tw

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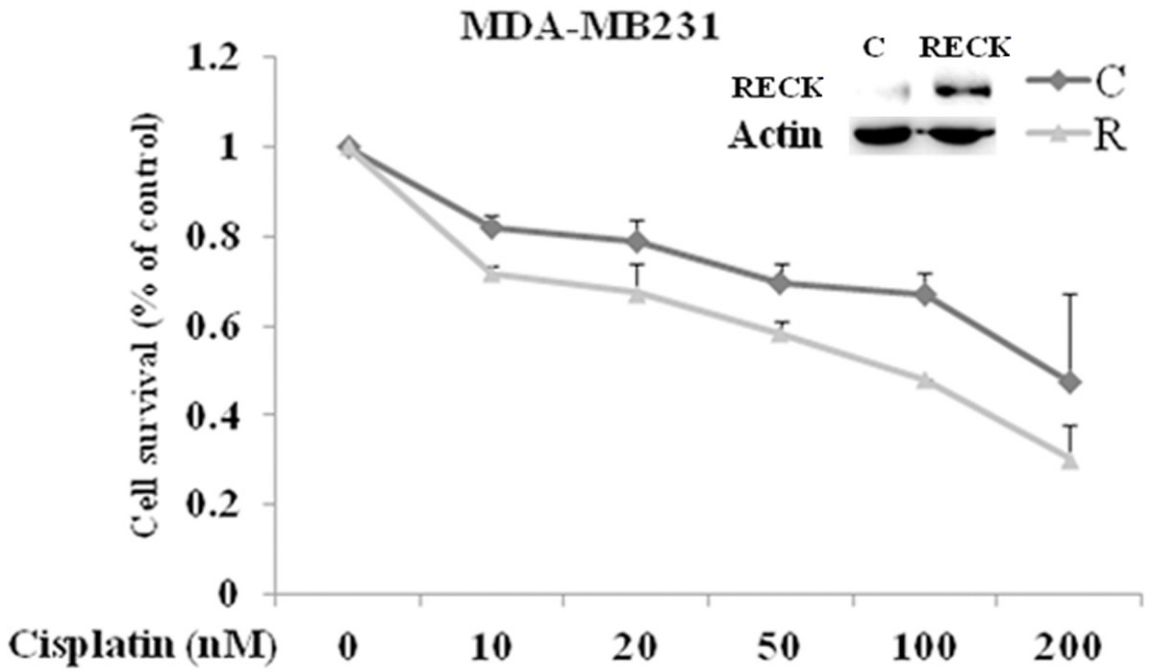

Figure S1. Expression of RECK increased cisplatin-induced cytotoxicity in MDA-MB-231 cells.