

Inhibition of p90RSK activation sensitizes triple-negative breast cancer cells to cisplatin by inhibiting proliferation, migration and EMT

Yujin Jin¹, Diem Thi Ngoc Huynh¹, Keon Wook Kang², Chang-Seon Myung¹, Kyung-Sun Heo^{1*}

¹College of Pharmacy and Institute of Drug Research and Development, Chungnam National University, Daejeon, South Korea

²College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul, 08826, Republic of Korea

Running Title: p90RSK activation in TNBC to cisplatin resistance

Keywords: Cisplatin, Epithelial-mesenchymal transition, p90RSK, Cell proliferation, Breast cancer cells

Corresponding Author's Information:

Kyung-Sun Heo

Tel.: 82-42-821-5927

Fax: 82-42-821-8925

E-mail: kheo@cnu.ac.kr

Materials and methods

Reagents and antibody

Rabbit anti-phospho-p38 Thr180/Thr182, rabbit anti-p38, rabbit anti-phospho-Akt Ser473, rabbit anti-Akt, rabbit anti-phospho-NF- κ B Ser536, rabbit anti-phospho-p90RSK Ser380, rabbit anti-phospho-ERK1/2, and rabbit anti-ERK1/2 antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Mouse anti-NF- κ B, mouse anti-RSK1, and si-RSK1 were purchased from Santa Cruz Biotechnology (Paso Robles, CA, USA). Rabbit anti-MMP2, rabbit anti-MMP9 antibodies, Muse cell cycle kit (#MCH100106), Muse Ki67 proliferation assay (#MCH100114), and immobilon-P (#IPVH00010) were purchased from Merck millipore. (Burlington, Massachusetts, USA). 10X Phosphate buffered saline (PBS, #EBA-1105) and reverse transcription 5X master mix (#EBT-1511) were purchased from ELPIS-BIOTECH (Daejeon, South Korea). Tri-RNA reagent (#FATRR-001) was purchased from Favorgen (Pingtung, China). DDP (#C2210000), doxorubicin (#D1515), ammonium persulfate (#A3678), 2-mercaptoethanol (#M6250), and skim milk (#70166) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Clarity western ECL substrate (#170-5061), iQ SYBR green supermix (#170-8882AP) were purchased for BIO-RAD (Hercules, CA, USA). EzReprobe (#WSE-7240), EzRIPA Lysis kit (#WSE-7240) were purchased for ATTO corporation (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM, #11963-092), RPMI medium 1640 (61870-036), Fetal bovine serum (FBS, #26140-079), Penicillin streptomycin (P/S, #1570-063) and 0.25% Trypsin/EDTA (#25200-072) were purchased from Gibco (Waltham, MA, USA). 3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, #M6494), and Lipofectamine 3000 (#L3000-008) was purchased from Invitrogen (Waltham, MA, USA). EndoFectin™ Max transfection reagent (#EF014) was purchased from GeneCopoeia, Inc (Maryland, USA).

Cell culture and MTT assay

Human mammary carcinoma cell lines MDA-MB-231 (HTB-26™) or MCF-7 (AHTB-22™) and BT549 (HTB-122™) were obtained from the American Type Culture Collection (Manassas, VA, USA). For the cell viability assay, cells were seeded in 96-well at a density of 1×10^4 cells per well. After 24

h of stabilization, the cells were treated with various dose of Cis-DDP for 24 h and then cell viability was determined using the MTT assay (Sigma-Aldrich).

Flow cytometry analysis

The Flow cytometry analysis was used to measure cell proliferation and cell cycle using a Muse cell cycle and Ki-67 proliferation kit (#MCH100106 and #MCH100114) according to the manufacture's protocol. Briefly, MDA-MB-231 cells were seeded in 6-well plate at a density of 1×10^6 cells/well. For cell proliferation experiment, cells were harvested after 36 h treated with Cis-DDP and fixed for 15 min with a provided fix buffer. Then, cells were permeabilized for 15 min using a provided permeabilization buffer and incubated with an anti-IgG1-PE or an anti-Ki67-PE at room temperature (RT) for 30 min. For cell cycle analysis, cells were harvested after 24 h treated with Cis-DDP and fixed in methanol for 4 h at -20°C and then incubated in complete media containing Muse cell cycle reagent. The Cell proliferation and the Cell cycle analyses were performed using a Muse cell analyzer (EMD Millipore) and data were analyzed using a MUSE 1.5 analysis program.

Cell transfection

Plasmids including pcDNA and dominant negative (DN)-p90RSK were transfected with EndoFectin™ Max transfection reagent (#EF014, GeneCopoeia) according to the manufacture's protocol. Rat RSK1 (NM031107) was mutated to K94A/K447A to create a kinase dead protein (DN-p90RSK1) with the QuickChange II site-directed mutagenesis kit (#200521, Agilent) as described previously (1).

Luciferase reporter assay

Cells were transiently co-transfected with pNF- κ B-luc and p-TK-renilla reporter plasmid by the DEAE-dextran methods as described previously (2). After transfection, cells were treated with $10 \mu\text{M}$ FMK for 1 h followed by treatment with $20 \mu\text{g/ml}$ Cis-DDP for 12 h. NF- κ B promoter luciferase activity was assayed using a dual-luciferase reporter assay system.

Real-Time Polymerase Chain Reaction assay

The quantitative RT-PCR (qRT-PCR) assay was used to analyze the mRNA expression of RSK1, RSK2,

MMP2, MMP9, E-cadherin, N-cadherin, vimentin, Snail, Twist, and ZEB1 as described previously (1). The relative gene expression was calculated using the $2^{-\Delta\text{ct}}$ method, and GAPDH was used for normalization. All primer sequences used in qRT-PCR experiments are listed in Supplementary Table 1.

Supplementary Table 1 – Primer sequence of genes

Genes	Forward primer sequence	Reverse primer sequence
RSK1	TGAAGGTGCTGAAGAAGGCA	CAGCTTCACCACGAATGGGT
RSK2	AACCTATGGGAGAGGAGGAGA	AGGATCTGCCTTTTCATGTCC
Snail	CCCCAATCGGAAGCCTACT	GCTGGAAGGTAAACTCTGGATTAGA
Twist	GGAGTCCGCAGTCTTACGAG	TCTGGAGGACCTGGTAGAGG
ZEB1	GCACCTGAAGAGGACCAGAG	TGCATCTGGTGTTCCATTTT
MMP2	ACAAAGAGTTGGCAGTGCAATA	TCTGGTCAAGATCACCTGTCTG
MMP9	CAGTCCACCCTTGTGCTCTT	CCAGAGATTTGACTCTCCAC
E-Cadherin	AATTCCTGCCATTCTGGGGA	TCTTCTCCGCTCCTTCTTC
N-cadherin	TGAGCCTGAAGCCAACCTTA	AGGTCCCCTGGAGTTTTCTG
Vimentin	AGCTAACCAACGACAAAGCC	TCCACTTTGCGTTCAAGGTC
GAPDH	GCACCGTCAAGGGCTGAGAAC	TGGTGAAGACGCCAGTGGA

p90RSK knockdown

To knockdown p90RSK *in vitro*, specific short interference RNA (siRNA) was used (#SC-29475, Santa Cruz Biotechnology). A siRNA that does not match any known human coding cDNA was used as a negative control for silencing (Scramble, sc-37007, Santa Cruz Biotechnology). Transfections were performed using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions.

Western blot analysis

Western blotting analysis were performed as described previously (2). Briefly, total protein samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. Western blotting was performed using an each corresponding specific antibody. Polyclonal mouse anti- α -tubulin (Sigma-Aldrich) was used as an internal control.

***In vitro* wound-healing assay**

MDA-MB-231 cells were seeded in 6-well plate at a density of 1×10^6 cells/well. After 24 h, the

monolayers were scratched with a 200 μ l pipette tip for creating a wound area and washed twice with serum-free media. Cells were treated with FMK or transfected with DN-RSK1 followed by treatment with Cis-DDP for 36 h. The rate of wound closure was assessed and imaged. Each image is derived from the five randomly selected fields.

Immunofluorescent staining

Immunofluorescence assay was performed as described previously (1). Briefly, cells were fixed with 4% paraformaldehyde for 15 min at RT and permeabilized with 0.1% Triton X-100 for 20 min. Cells were incubated with the primary antibodies (p65 1:100) overnight at 4°C followed by incubation with anti-rabbit secondary antibodies (Invitrogen) conjugated with Alexa 488 at a dilution of 1:400 at RT for 1 h. Cell nuclei were counterstained with 40, 6-diamidino-2-phenylindol (DAPI) for 5 min. Slides were mounted with prolong gold antifade mount reagent (#P36930, Invitrogen) and examined with a laser scanning confocal spectral microscope (Nanoscope systems, South Korea). Representative images were automatically taken using a SPOT digital camera.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (version 5.02, GraphPad Software Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by a Bonferroni multiple comparison was performed. A *p* value <0.05 was considered significant. All experiments were expressed as the mean \pm SEM and were performed independently at least 3 times.

Supplementary Figures and Figure legends

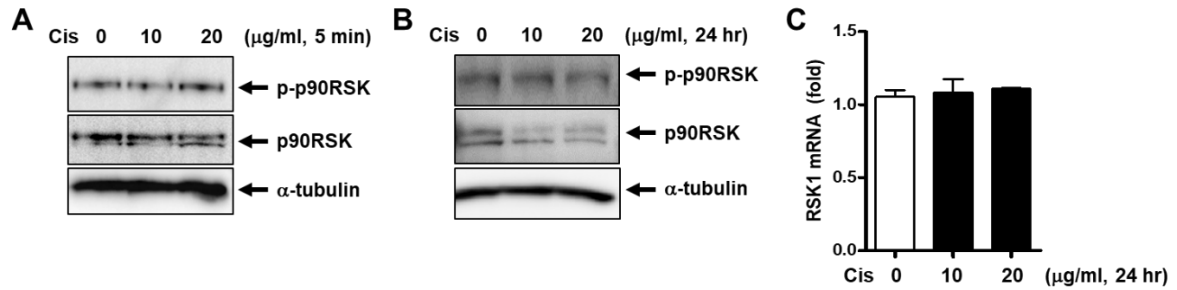


Figure S1

Figure S1. Phosphorylation and protein expression of p90RSK in Cis-DDP-treated MCF-7 cells.

(A-B) MCF-7 cell were stimulated with 0, 10, 20 $\mu\text{g/ml}$ of Cis-DDP for 5 min (A) and 24 h (B) and phosphorylation and protein expression of p90RSK were determined by western blotting against indicated antibodies. (C) Messenger RNA level of p90RSK was determined by qPCR against RSK1 primer.

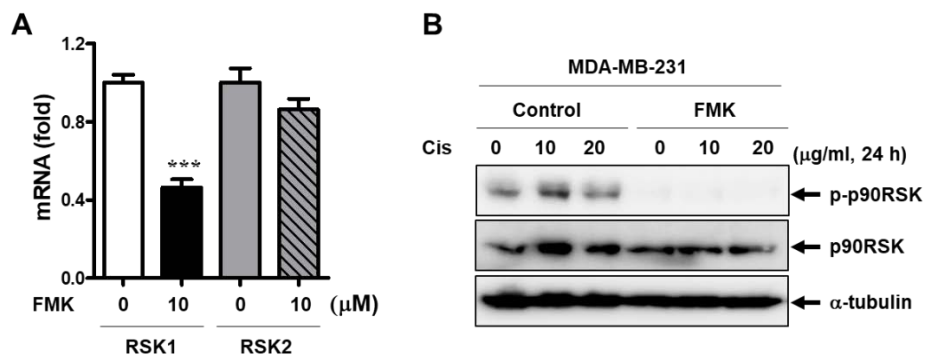


Figure S2

Figure S2. Role of FMK on RSK1 activation and protein expression in MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with 10 μM of FMK for 24 h and total RNAs were subjected to qPCR analysis against RSK1 or RSK2 primers. (B) MDA-MB-231 cells were pretreated with 10 μM FMK for 1 h followed by treatment with 0, 10, 20 μg/ml of Cis-DDP for 24 h. Total protein lysates were subjected by western blotting using anti-phospho or –total p90RSK antibody.

References

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