Attenuation of malignant phenotypes of breast cancer cells through eIF2α-mediated downregulation of Rac1 signaling

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Abstract. Blocking dephosphorylation of eukaryotic transla-tion initiation factor 2α (eIF2α) is reported to alter proliferation and differentiation of various cells. Using salubrinal and guanabenz as an inhibitory agent of dephosphorylation of eIF2α, we addressed a question whether an elevated level of phosphorylated eIF2α attenuates malignant phenotypes of triple negative breast cancer cells (TNBCs) that lack estrogen receptor, progesterone receptor and human epidermal growth factor receptor-2. We determined effects of salubrinal and guanabenz on *in vitro* phenotype of 4T1 mammary tumor cells and MDA-MB-231 human breast cancer cells and evalu-ated their effects on *in vivo* tumor growth using BALB/c mice injected with 4T1 cells. The results revealed that these agents block the proliferation and survival of 4T1 and MDA-MB-231 cells, as well as their invasion and motility. Silencing eIF2α revealed that eIF2α is involved in the reduction in invasion and motility. Furthermore, salubrinal-driven inactivation of Rac1 was suppressed in the cells treated with eIF2α siRNA, and treatment with Rac1 siRNA reduced cell invasion and motility. *In vivo* assay revealed that subcutaneous administration of salubrinal reduced the volume and weight of tumors induced by 4T1 cells. Collectively, the results indicate that these agents can attenuate malignant phenotype and tumor growth of breast cancer cells through the eIF2α-mediated Rac1 pathway. Since salubrinal and guanabenz are known to inhibit bone resorp-tion, this study provides a potential use of eIF2α-mediated Rac1 regulation in suppressing the growth and metastasis of breast cancer.

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Introduction

Various environmental stresses such as oxidation, nutrient deprivation, radiation, and stress to the endoplasmic reticulum induce the integrated stress response in which the elevated phosphorylation level of eukaryotic translation initiation factor 2α (eIF2α) may stimulate cellular apoptosis (1,2). In response to mild stresses, the phosphorylation of eIF2α attenuates translational efficiency and activates the pro-survival signaling (3). However, in response to severe stress, the phosphorylated eIF2α (eIF2α-p) promotes apoptosis (3,4). Salubrinal, a synthetic chemical agent known to elevate the level of eIF2α-p, is considered as a cytoprotective agent as well as an agent to stimulate apoptosis depending on the stress environments and cell types (5). For instance, it is reported that salubrinal could protect against tunicamycin induced cardiomyocyte apoptosis (6). On the contrary, administra-tion of salubrinal to leukemic and chondrosarcoma cells is reported to stimulate apoptosis (7,8). Nonetheless, few studies have been conducted to examine the effect of salubrinal on breast cancer cells.

Breast cancer accounts for ~25% of all cancers in women and its therapeutic strategy heavily depends on the expression levels of three marker genes such as estrogen receptor (ER), progesterone receptor (PgR) and human epidermal growth factor receptor-2 (HER2). When cancer cells exhibit a high expression level of ER and/or PgR, hormonal treatments would be a viable option (9). For cancer cells with an overexpressed level of HER2, treatments with HER2-targeted drugs such as trastuzumab and lapatinib are potentially effective (10). A lack of expression of all three gene products, however, defines a triple negative breast cancer (TNBC) (11-13) that presents a challenge in prognosis and requires a novel treatment option.

In this study, we addressed the question: does administra-tion of salubrinal attenuate the malignant *in vitro* phenotype of TNBCs? If yes, what is the mechanism of salubrinal's action and does its administration to mice injected with TNBCs suppress *in vivo* tumor growth? In response to administration of salubrinal, we examined *in vitro* phenotype of 4T1 mammary tumor cells and MDA-MB-231 human breast cancer cells (14-16). We also employed guanabenz, another synthetic drug

known to elevate eIF2α-p by inhibiting de-phosphorylation of eIF2α-p (17). In order to examine the involvement of eIF2α, silencing by RNA interference was conducted using siRNA specific to eIF2α. Furthermore, the potential linkage between regulation of eIF2α and Rac1 GTPase in cell invasion and motility was evaluated using siRNA specific to Rac1 GTPase. We also employed a fluorescence resonance energy transfer (FRET) technique and evaluated Rac1 activity in response to salubrinal. To test the effects of salubrinal *in vivo*, 4T1 mammary tumor cancer cells were injected to mice and suppression of tumor growth was analyzed.

Materials and methods

Cell culture. 4T1 mouse mammary tumor cells and MDA-MB-231 human breast cancer cells were cultured in DMEM containing 10% fetal bovine serum and anti-biotics (50 U/ml penicillin and 50 μg/ml streptomycin; Life Technologies, Grand Island, NY, USA). Cells were maintained at 37˚C and 5% CO_2 in a humidified incubator. Responses to administration of 10-50 μM salubrinal (18) or 5-50 μM guanabenz acetate (Tocris Bioscience, Ellisville, MO, USA) were evaluated using assays for MTT, adhesion, invasion and motility.

MTT assay. Cells ($5x10^2$/well) were seeded in 96-well plates, and the reduction of MTT to formazan was evaluated by measuring the absorbance at 570 nm with a plate reader (EL800, BioTek, Winooski, VT, USA).

Cell adhesion assay. Ninety-six well plates were coated with poly-L-lysine, fibronectin, laminin (Sigma-Aldrich, St. Louis, MO, USA) or type I collagen (BD Biosciences, Bedford, MA, USA) for 2 h. The plates were then incubated with non-fat dry milk, followed by washing with PBS and serum-free culture medium. Cells ($1x10^4$/well) were added on the plate, and after 30 min and 3 h the attached cells were stained with 0.04% crystal violet (Sigma-Aldrich) for 10 min at room temperature. The wells were washed with PBS, and DMSO was added. Absorbance at 550 nm was measured using the plate reader.

In vitro invasion assay. An invasion assay was performed with a Boyden chamber as described previously (19), with minor modifications. In brief, Matrigel (BD Biosciences) was diluted with ice-cold PBS (100 μg/ml). Matrigel (600 μl) was added to each filter (polyethylene terephthalate membrane, 8-μm pore size, 23.1 mm in diameter, Falcon) and left to polymerize overnight. Prior to assembling the chamber unit, the lower chamber (6-well plate, Falcon) was filled with culture medium consisting of salubrinal or guanabenz. Cells ($1-4x10^5$/well) were added to the culture medium with salu-brinal or guanabenz in the upper chamber and incubated for 24 h. The cells on the filter surface were stained with Giemsa (Sigma-Aldrich) and the number of cells was counted under a microscope.

Two-dimensional motility assay. To evaluate 2-dimensional motility, a wound healing scratch motility assay was carried out as described previously (20). In brief, cells were plated in

12-well plates or 6-cm dishes (Falcon) and on the next day, scratching was performed using a plastic tip. The areas newly occupied with cells in the scratched zone were determined every 3 h up to 24 h using images obtained by a microscope, which were scanned with Adobe Photoshop (CS2, Adobe Systems, San Jose, CA, USA) and quantified with ImageJ.

Western blot analysis. Cells were lysed in a radioimmunopre-cipitation assay (RIPA) buffer containing protease inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and phosphatase inhibitors (Calbiochem, Billerica, MA, USA). Isolated proteins were fractionated using 10-15% SDS gels and electro-transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). The membrane was incubated for 1 h with primary antibodies followed by 45 min incubation with goat anti-rabbit or anti-mouse IgG conjugated with horse-radish peroxidase (Cell Signaling, Danvers, MA, USA). We used antibodies against eIF2α, caspase 3, cleaved caspase (Cell Signaling), Rac1 (Millipore), and β-actin (Sigma). Protein levels were assayed using a SuperSignal west femto maximum sensitivity substrate (Thermo Scientific, Waltham, MA, USA), and signal intensities were quantified with a luminescent image analyzer (LAS-3000, Fuji Film, Tokyo, Japan).

Knockdown of eIF2α and Rac1 by siRNA. Cells were treated with siRNA specific to eIF2α and Rac1 (Life Technologies). Selected target sequences for knockdown of eIF2α and Rac1 were: eIF2α, 5'-CGG UCA AAA UUC GAG CAG A-3', and Rac1, 5'-GCA UUU CCU GGA GAG UAC A -3'; and as a non-specific control, a negative siRNA (Silencer Select no. 1, Life Technologies) was used. Cells were transiently trans-fected with siRNA for eIF2α, Rac1 or control in Opti-MEM I medium with Lipofectamine RNAiMAX (Life Technologies). Six hours later, the medium was replaced by regular culture medium. The efficiency of silencing was assessed with immu-noblotting 48 h after transfection.

Fluorescence resonance energy transfer (FRET). To visualize Rac1 activity in response to salubrinal, FRET imaging was conducted using a cyan fluorescent protein (CFP)-yellow fluo-rescent protein (YFP) Rac1 biosensor. The filter sets (Semrock) were chosen for CFP excitation at 438±24 nm (center wave-length ± bandwidth), CFP emission at 483±32 nm, and YFP emission at 542±27 nm. Time-lapse images were acquired at an interval of 5 min using a fluorescence microscope (Nikon, Tokyo, Japan). The level of Rac1 activity was determined by computing an emission ratio of YFP/CFP for individual cells using NIS-Elements software (Nikon).

In vivo tumor growth. Experimental procedures were approved by the Indiana University Animal Care and Use Committee and were in compliance with the Guiding Principles in the Care and Use of Animals endorsed by the American Physiological Society. Five mice were housed per cage, and fed with mouse chow and water *ad libitum*. Thirty-five BALB/c female mice (6 weeks, Harlan Laboratories) were used. Mice received subcutaneous injection of 4T1 mouse mammary tumor cells (10^6 cells in 100 μl PBS) to the abdomen on day 1. Salubrinal (25 μg) was administered

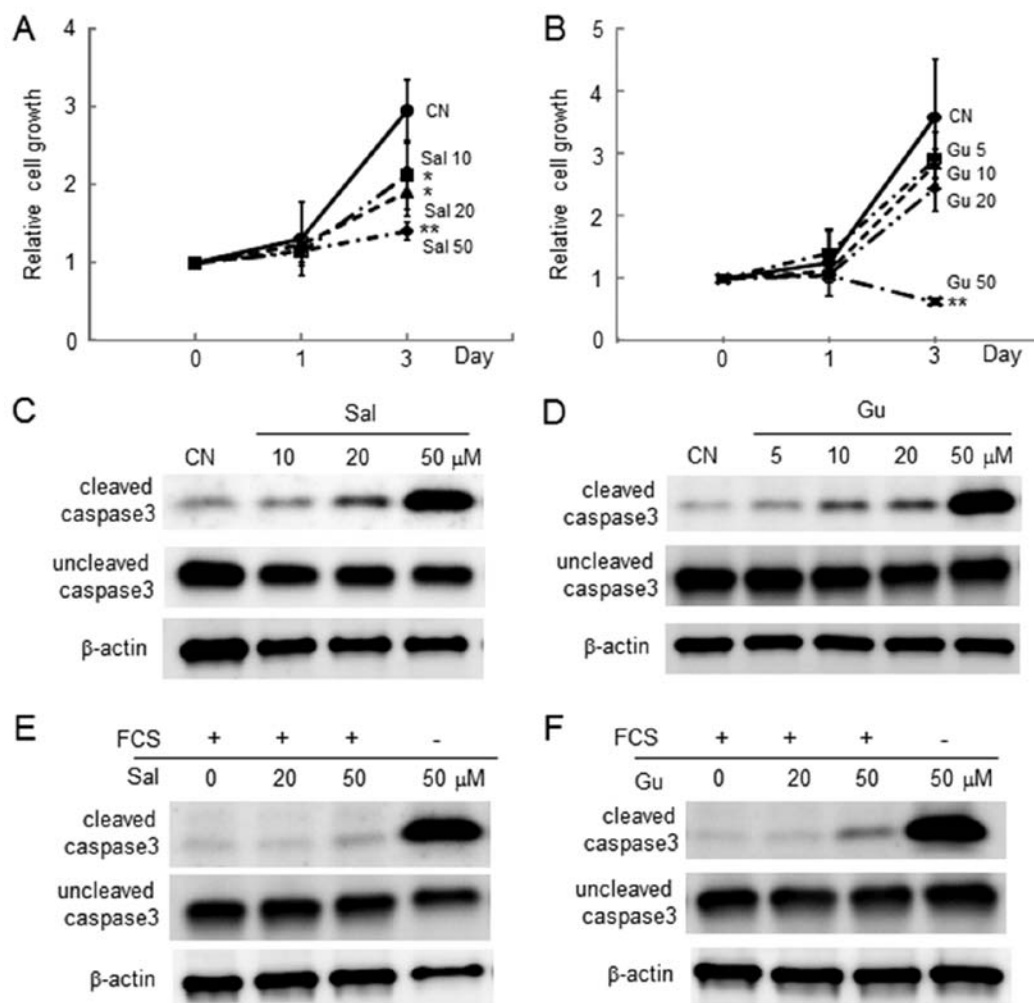Figure 1. Inhibitory effects of salubrinal and guanabenz in proliferation and survival of 4T1 cells. The single and double asterisks indicate p<0.05 and p<0.01, respectively. CN, control; Sal, salubrinal; Gu, guanabenz; and FCS, fetal calf serum. (A) Relative cell growth in response to 10, 20 or 50 μM salubrinal. (B) Relative cell growth in response to 5, 10, 20 or 50 μM guanabenz. (C) Dose-dependent elevation of cleaved caspase 3 to 10-50 μM salubrinal in the absence of serum. (D) Dose-dependent elevation of cleaved caspase 3 to 5-50 μM guanabenz in the absence of serum. (E) Reduced elevation of cleaved caspase 3 to salubrinal in the presence of 10% serum. (F) Reduced elevation of cleaved caspase 3 to guanabenz in the presence of 10% serum.

subcutaneously into the area of cell injection every day, while the control animals received a vehicle. The animals were sacrificed on day 20, and the volume and weight of tumors were determined. The tumor volume was calculated as (long diameter) x (short diameter)^2/2.

Statistical analysis. Three or four-independent experiments were conducted and data were expressed as mean ± SD. For comparison among multiple samples, ANOVA followed by *post hoc* tests was conducted. Statistical significance was set at p<0.05. The single and double asterisks in the figures indicate p<0.05 and p<0.01.

Results

Inhibitory effects of salubrinal and guanabenz in proliferation and survival of 4T1 cells. The MTT assay revealed that in response to 10, 20 and 50 μM salubrinal, the number of live 4T1 cells was reduced in a dose-dependent manner (Fig. 1A). The number of live cells was also decreased by

50 μM guanabenz (Fig. 1B). Consistent with the MTT results, both salubrinal and guanabenz elevated the level of cleaved caspase 3 in the absence and presence of 10% serum in the culture medium, respectively (Fig. 1C-F).

Undetectable changes in cell adhesion by salubrinal and guanabenz in 4T1 cells. The surface was coated with poly-L-lysine, type I collagen, fibronectin, and laminin, and the effects of salubrinal and guanabenz on cell adhesion were examined. The absorbance reading, which indicated the number of adherent cells on the coated surface, did not significantly change in the presence and absence of salubrinal and guanabenz 30 min and 3 h after cell incubation, respectively (data not shown).

Dose-dependent reduction in cell invasion by salubrinal and guanabenz in 4T1 cells. The number of cells invaded through the filter coated with Matrigel was significantly reduced by administration of salubrinal and guanabenz regardless of the presence of serum in the medium in a dose-dependent manner (Fig. 2A-C).
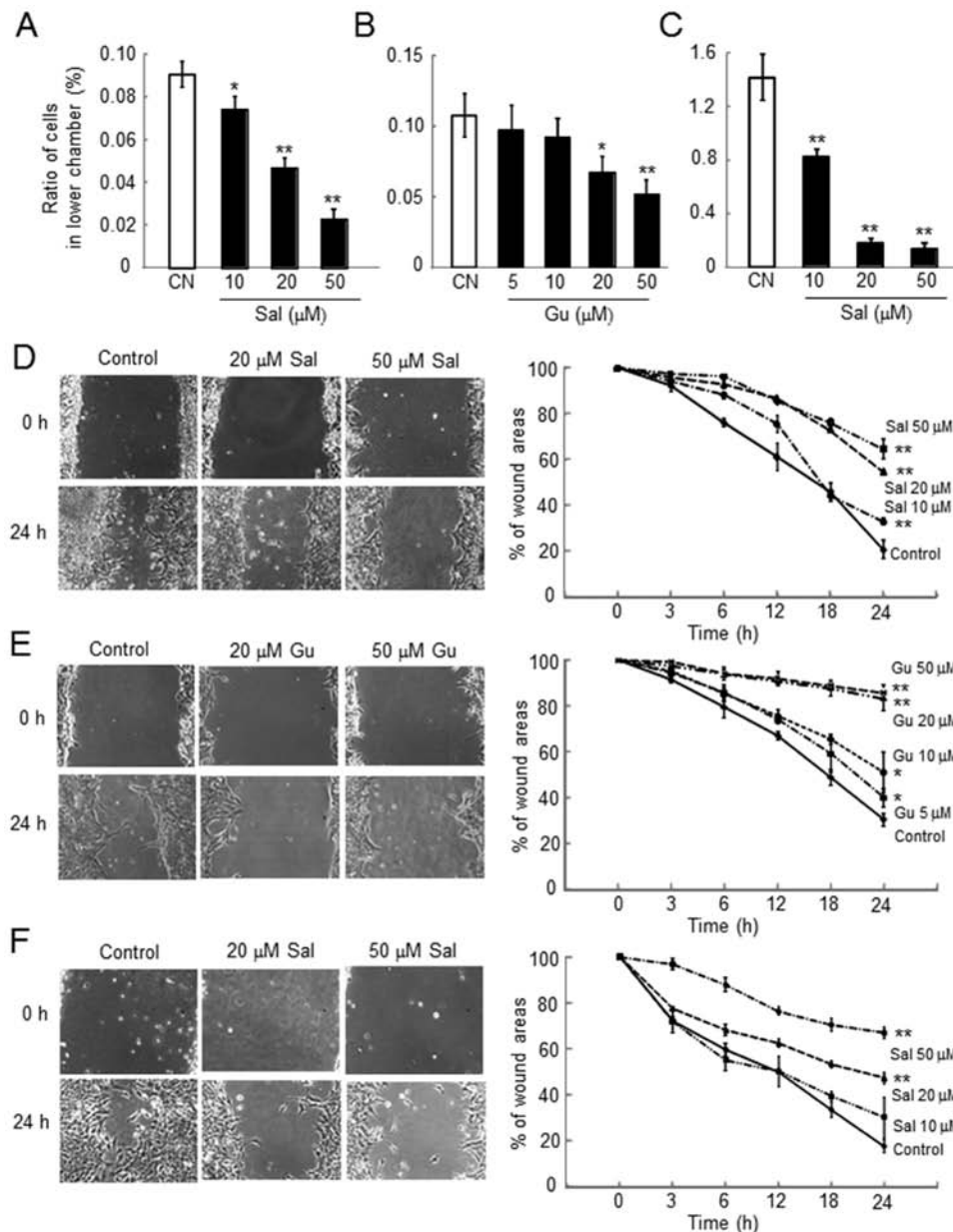
Figure 2. Dose-dependent reduction in invasion and reduction in motility of 4T1 cells. The single and double asterisks indicate p<0.05 and p<0.01, respectively. CN, control; Sal, salubrinal; and Gu, guanabenz. (A) Percent of the invaded cells in response to 10, 20 or 50 μM salubrinal in the absence of serum. (B) Percent of the invaded cells in response to 5, 10, 20 or 50 μM guanabenz in the absence of serum. (C) Percent of the invaded cells in response to salubrinal in the presence of 10% serum. (D) Reduction in cell motility by 20 or 50 μM salubrinal in 24 h in the absence of serum. (E) Reduction in cell motility by 20 or 50 μM guanabenz in 24 h in the absence of serum. (F) Sustained reduction in cell motility by salubrinal in the presence of 10% serum.

Reduction in cell motility by salubrinal and guanabenz in 4T1 cells. Using the scratch-wound assay, the wound area was determined as an indicator of cell motility in which a reduction in motility corresponded with a decrease in wound healing. In response to both salubrinal and guanabenz, cell motility was reduced in a dose-dependent manner (Fig. 2D-F). The results with salubrinal were not affected by the presence of serum in the culture medium.

Reduction in proliferation, invasion, survival, and motility in MDA-MB-231 cells. Consistent with the results in 4T1 mouse mammary tumor cells, we observed reduction in proliferation, invasion, survival and motility in MDA-MB-231 human breast cancer cells (Fig. 3).

Involvement of eIF2α in salubrinal-driven reduction in cell invasion and motility in 4T1 cells. Cell invasion and motility in response to salubrinal were examined using the cells transiently transfected with eIF2α siRNA (Fig. 4A). Compared to the cells transfected with non-specific control (NC) siRNA, salubrinal-driven reduction in cell invasion was significantly suppressed in the cells treated with eIF2α siRNA (Fig. 4B). However, in the control cells treated with NC siRNA, the cell invasion was reduced by salubrinal in a dose-dependent manner. Furthermore, salubrinal-driven reduction in cell motility was also suppressed by eIF2α siRNA (Fig. 4C and D).

Inactivation of Rac1 GTPase by 20 μM salubrinal in 4T1 cells and MDA-MB-231 cells. To evaluate a potential involve-
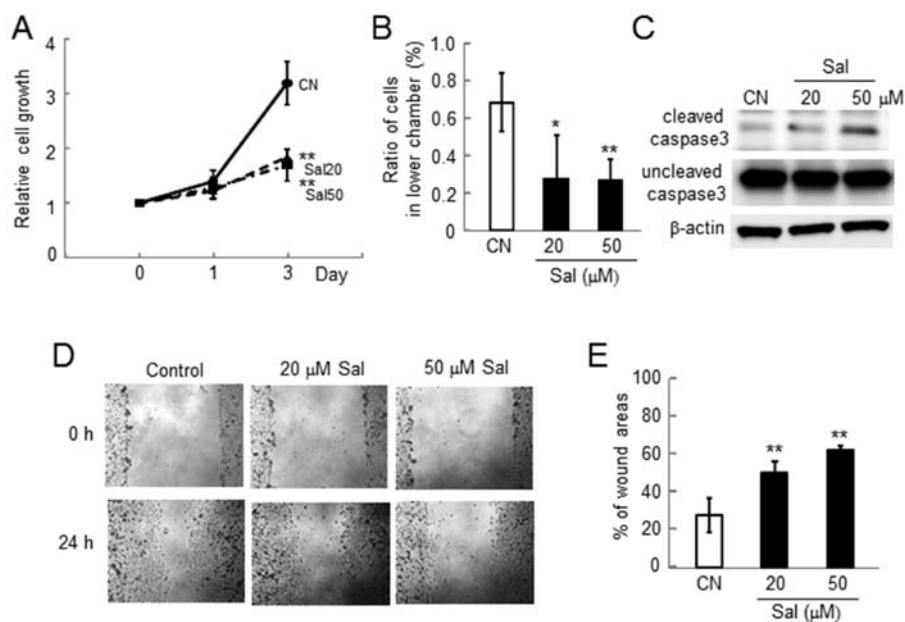
Figure 3. Inhibitory effects of salubrinal in proliferation, invasion, survival, and motility of MDA-MB-231 cells. The single and double asterisks indicate p<0.05 and p<0.01, respectively. CN, control; and Sal, salubrinal. (A) Relative cell growth in response to 20 or 50 μM salubrinal. (B) Reduction in cell invasion by 20 or 50 μM salubrinal in 24 h in the absence of serum. (C) Dose-dependent elevation of cleaved caspase 3 to 20 or 50 μM salubrinal in the absence of serum. (D and E) Reduction in cell motility by 20 or 50 μM salubrinal in 24 h in the presence of 10% serum.
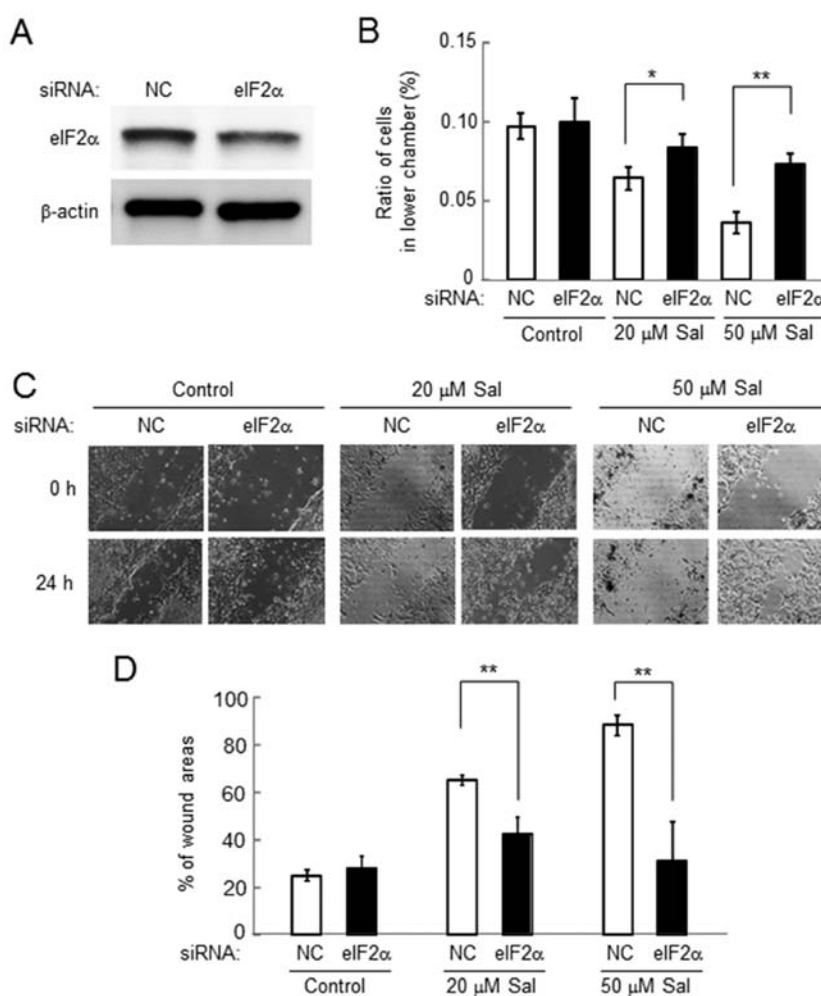

Figure 4. Involvement of eIF2α in salubrinal-driven reduction in cell invasion and motility of 4T1 cells. Salubrinal was given at 20 or 50 μM. The single and double asterisks indicate p<0.05 and p<0.01, respectively. Note that NC designates the samples treated with the non-specific control siRNA. Sal, salubrinal. (A) Reduction in the protein level of eIF2α by RNA interference. (B) Partial suppression of salubrinal-driven reduction in cell invasion by eIF2α siRNA. (C and D) Suppression of salubrinal-driven reduction in cell motility (elevation of the wound areas) by eIF2α siRNA.
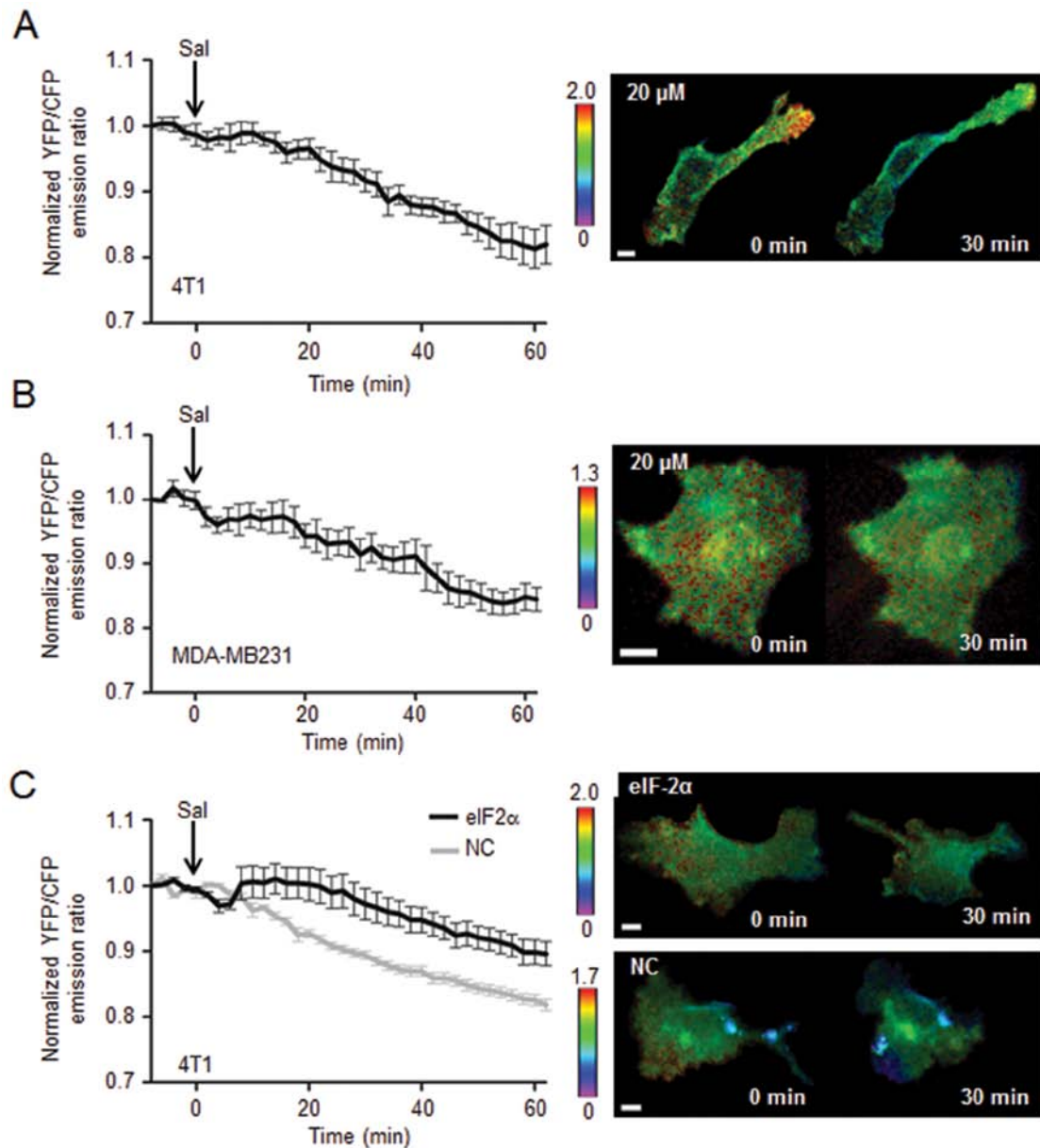
Figure 5. FRET-based detection of inactivation of Rac1 GTPase in response to 20 μM salubrinal. In live cell imaging, the color bar represents the emission ratio of YFP/CFP, an index of Rac1 activation and the arrow indicates the commencement time (t=0) for administration of salubrinal. Scale bar, 10 μm. (A) Normalized Rac1 activity in 4T1 cells. (B) Normalized Rac1 activity in MDA-MB-231 cells. (C) Normalized Rac1 activity in the presence of eIF2α siRNA or non-specific control (NC) siRNA in 4T1 cells.

ment of Rac1 through eIF2α-mediated signaling, the effect of salubrinal on activity of Rac1 GTPase was examined using FRET-based single cell imaging. In response to 20 μM salubrinal, the emission ratio of YFP/CFP was decreased in 4T1 cells as well as MDA-MB-231 cells, indicating that administration of salubrinal reduced the activity level of Rac1 (Fig. 5A and B). The FRET analysis was also conducted using 4T1 cells transfected with eIF2α siRNA, in which salubrinal-driven reduction in Rac1 activity was significantly suppressed (Fig. 5C).

Reduction in cell growth, invasion and motility by Rac1 siRNA in 4T1 cells. In order to examine the role of Rac1 in salubrinal-driven suppression of malignant phenotypes, cell growth, invasion and motility was examined using siRNA specific to Rac1 (Fig. 6A). The result revealed that reduction in the expression level of Rac1 lowered cell growth and elevated the level of cleaved caspase 3 (Fig. 6B and C). Furthermore, silencing Rac1 decreased cell invasion as well as cell motility (Fig. 6D and F).

Inhibitory effects of salubrinal in the volume and weight of tumors. Using the *in vivo* mouse model, the effects of salubrinal on tumor growth were evaluated. A comparison of the tumors isolated from the control mice (N=17) and the salubrinal-treated mice (N=18) revealed that the tumor volume (control, 620.1±271.1 mm^3; and salubrinal, 384.5±248.1 mm^3) and weight (control, 0.51±0.21 g; and salubrinal, 0.32±0.24 g)
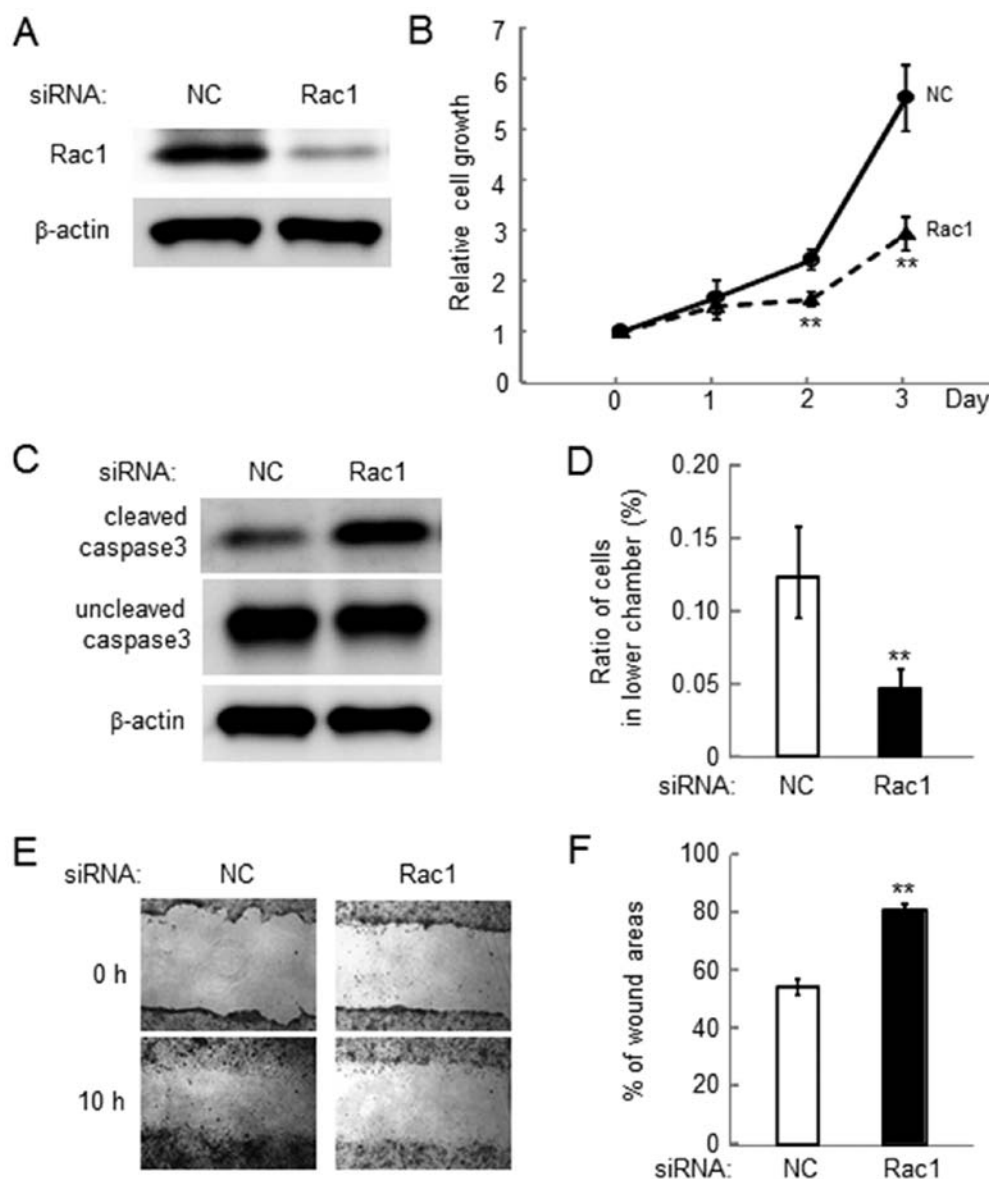
Figure 6. Reduction in cell invasion and motility of 4T1 cells by RNA interference with Rac1 siRNA. Note that NC designates the samples treated with the non-specific control siRNA. The double asterisk indicates p<0.01. (A) Reduction in the protein level of Rac1 by RNA interference. (B) Reduction of cell growth by Rac1 siRNA. (C) Increase in cleaved caspase 3 by Rac1 siRNA. (D) Reduction in cell invasion by Rac1 siRNA. (E and F) Reduction in cell motility by Rac1 siRNA.

were significantly larger in the control group than the salubrinal-treated group (Fig. 7A and B).

Discussion

We demonstrated in this study that salubrinal has inhibitory effects on the malignant phenotypes of 4T1 mammary tumor cells and MDA-MB-231 breast cancer cells that hold a triple negative phenotype. Salubrinal significantly reduced cellular proliferation, invasion, and migration, although it did not alter cellular adhesion to surfaces coated with poly-L-lysine, type I collagen, fibronectin or laminin. The inhibitory effects were commonly observed in response to salubrinal and guanabenz, both of which elevate the level of p-eIF2α. RNA silencing with eIF2α siRNA abolished salubrinal driven reduction in Rac1 and RNA silencing with Rac1 siRNA attenuated malignant phenotypes as seen in the responses to salubrinal. Furthermore,

in vivo tumor size and weight in 4T1 cells injected mice were significantly reduced by daily administration of salubrinal (Fig. 7D).

The elevated level of cleaved caspase 3 indicates that cellular apoptosis is stimulated by salubrinal. An increase in apoptotic death was more significant in the absence of FBS in the medium than that with FBS, suggesting that potency of salubrinal is enhanced in a nutrient poor environment. Previous studies reported that salubrinal could induce either stimulatory or inhibitory effects on cellular death through modulation of the level of p-eIF2α (6,7). Our data are consistent with a notion that in an abnormal growth condition such as in a solid tumor without well-developed vasculature, the elevation of p-eIF2α leads to a pro-apoptotic pathway.

Rac1 GTPase is a regulator of various cellular processes, including cell cycle, motility, invasion and cell-cell adhesion (21). It has been known to play a substantial role in the develop-
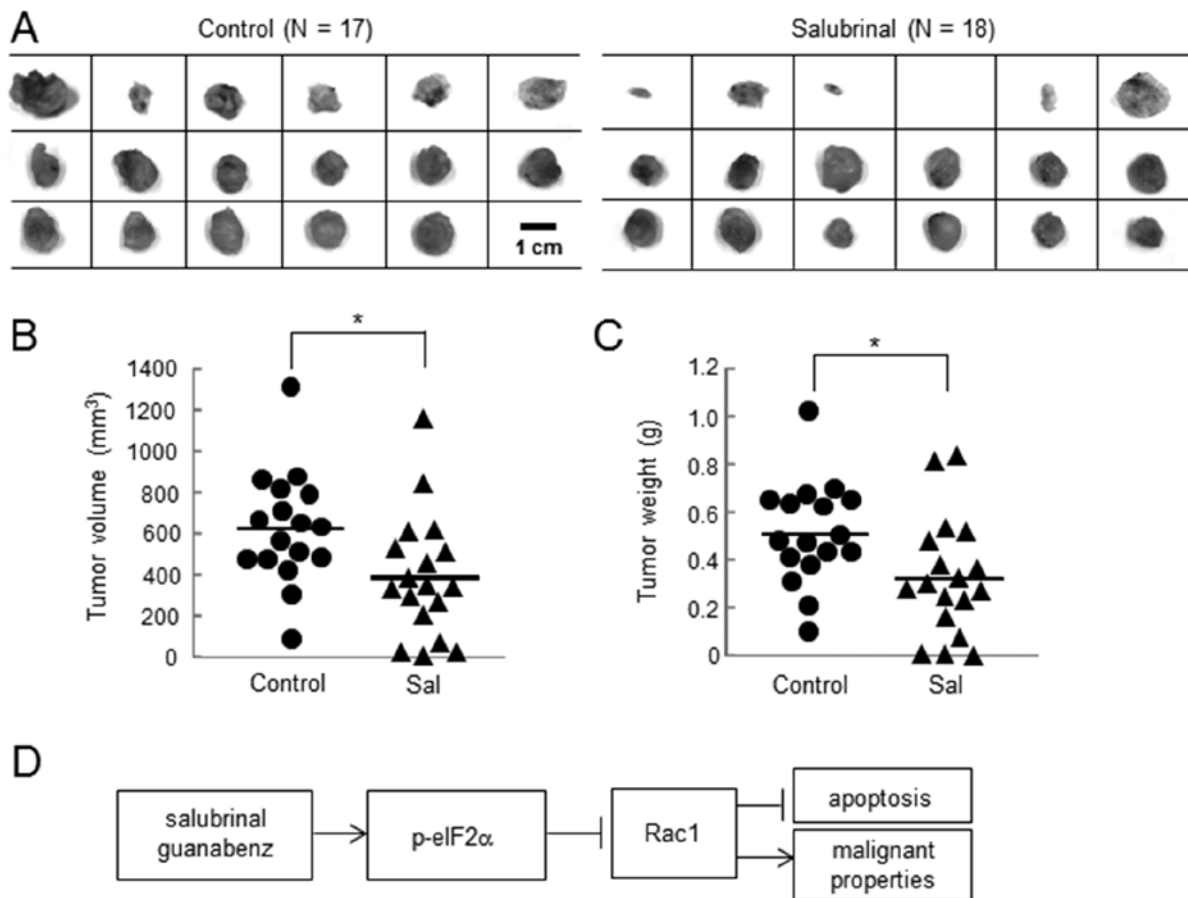
Figure 7. Inhibitory effects of salubrinal on tumor growth with 4T1 cells. (A) Images of harvested tumors in the control and salubrinal-treated groups. (B and C) Comparison of the tumor volume and weight, respectively. The circles and triangles represent the values for the control and salubrinal-treated mice, respectively. The horizontal bars indicate the mean values for each group. N=17 for control and N=18 for the salubrinal-treated mice. Sal, salubrinal. (D) Proposed mechanism of salubrinal's action on breast cancer cells.

ment of various cancers including breast cancer and pancreatic cancer (22,23). It is reported that the Rac-guanine nucleotide exchange factor (GEF) P-Rex1 is an essential stimulator of Rac1 activation, and P-Rex1 is activated by the phosphatidylinositide 3-kinases (PI3K) pathway (24,25). Furthermore, Rac1 can be activated through integrins, tyrosin-kinase receptors, and various stress factors including mechanical stimulation, and the stress to the endoplasmic reticulum (21). Since salubrinal could relieve the stress to the endoplasmic reticulum, there is a possibility that eIF2α-mediated Rac1 suppression is linked to modulation of stress responses in 4T1 mammary tumor cells. In inflammatory cartilage and chondrocytes, it is also reported that the activity of Rac1 is reduced by treatment with salubrinal (26). Further analysis is necessary to identify the mechanistic role of eIF2α in salubrinal-driven regulation of Rac1.

Since 4T1 and MDA-MB-231 cells are considered to be triple negative, the action of salubrinal is indubitably different from that of selective estrogen receptor modulators (SERMs) which target the estrogen receptor. Tamoxifen, for instance, is thought to act as an agonist at the bone and uterus and an antagonist at the breast (9). Unlike salubrinal, however, tamoxifen is not effective for estrogen receptor-negative breast cancer cells. Regarding the involvement of Rac1 in response to salubrinal, it is reported that inhibition of Rac1 using a pharmacological agent (EHT1864) decreases estrogen receptor levels

and proliferation of both tamoxifen-sensitive and resistant cells (27). Although the reported study suggests that inhibition of Rac1 could be a therapeutic strategy for estrogen receptor-positive cells, our study indicates that salubrinal-driven reduction of Rac1 is also effective in attenuating malignant phenotypes of estrogen receptor-negative cells. In this context, MDA-MB-231 cells used in this study have K-Ras mutation and are dependent on Rac1 for growth factor driven invasion and migration (28). Therefore, Rac1 activity may potentially serve as a biomarker of response to salubrinal.

Approximately 20% of breast cancer patients are likely to develop metastatic tumors in distant organs such as the lungs, liver, brain and bone (29). In particular, bone is the most common site for metastasis of breast cancer. The current chemotherapy for the treatment of bone metastasis includes administration of SERMs such as tamoxifen, bisphosphonates (e.g., zoledronate, pamidronate and alendronate), and anti-receptor activator of nuclear factor κ-B ligand (RANKL) antibody (Denosumab) (30,31). Bisphosphonates are the most commonly used medication to reduce bone resorption, bone destruction and tumor growth. However, it does not stimulate bone formation and it often exhibits side effects such as joint inflammation and avascular osteonecrosis of the jaw (32). Denosumab is reported to reduce bone metastasis, but it effects on tumor growth have to be elucidated. Salubrinal

and guanabenz, which inhibit the de-phosphorylation of eIF2α could both stimulate osteoblastgenesis through upregulation of ATF4 and attenuate osteoclastogenesis through down-regulation of nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) (33,34). Since salubrinal was shown to stimulate the growth of new bone and enhance the healing of bone wound, examining its effects on tumor growth would have a significant impact on treatment of breast cancer and bone metastasis.

In conclusion, we demonstrated that an inhibitory agent of dephosphorylation of eIF2α potentially offers a novel therapeutic strategy for attenuating malignant phenotypes of triple negative breast cancer cells. It was shown to downregulate the activity of Rac1 through eIF2α mediated signaling. It may prevent not only tumor growth but also bone resorption associated with metastasis to bone.

Acknowledgements

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