Nitric oxide increases the invasion of pancreatic cancer cells via activation of the PI3K–AKT and RhoA pathways after carbon ion irradiation

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Abstract

Previous studies have shown that serine proteases and Rho-associated kinase contribute to carbon ion radiation-enhanced invasion of the human pancreatic cancer cell line PANC-1. The results presented here show that nitric oxide synthase (NOS) also plays a critical role in this process. Irradiation of PANC-1 cells promoted invasion and production of nitric oxide (NO), which activated the PI3K–AKT signaling pathway, while independently activating RhoA. Inhibition of PI3K, Rho-associated kinase, and serine protease alone or in conjunction with NOS suppressed the radiation-enhanced invasion of PANC-1 cells, suggesting that they could serve as possible targets for the management of tumor metastasis.

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1. Introduction

Tumor metastasis is the major cause of cancer-related mortality, accounting for more than 90% of the deaths [1,2]. A clear understanding of the mechanisms underlying metastasis remains elusive owing to the effects of cumulative mutations in metastatic cells and the heterogeneity of mutated genes among metastatic cancer types.

In the first step of metastasis, cancer cells migrate into the extracellular matrix (ECM) using two modes of migration. Elongated mesenchymal-like cells produce proteases such as matrix metalloproteinases (MMPs), which remodel the ECM by proteolysis to create paths for cell migration (mesenchymal mode of motility). In contrast, rounded ameboid cells squeeze between gaps of the matrix proteins by means of bleb-like protrusions, a movement mediated by actomyosin contractions (amoeboid mode of motility) [3–5]. Some cell lines derived from tumors utilize both modes, which differ fundamentally in terms of mechanism, making it difficult to suppress invasiveness using a single class of reagent.

We previously reported that in two pancreatic cancer-derived cell lines, MIAPaCa-2 and PANC-1, cells underwent mesenchymal–amoeboid (MA) transition upon exposure to an inhibitor specific to either mode of motility [6,7]. Interestingly, these cells showed differential responses to genotoxic stress induced by photon (X-ray) irradiation or particle ion beams. Compared to photons, carbon ion beams deliver a larger mean energy per unit length of their trajectory [8,9]. The relative biological effectiveness of carbon ion beams with respect to reference photon radiation is estimated to be approximately 2–3-fold, as assessed by biological endpoints such as cell death, DNA damage, and chromosomal aberrations, among others. [10,11]. In addition, equivalent doses of carbon ions and photons produce different biological responses in irradiated cells, for instance, differential gene expression [12]; however, the specific mechanisms leading to such differences remain unknown.

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We previously found that MIAPaCa-2 cell invasiveness was enhanced by X-ray irradiation, but was almost completely suppressed by exposure to carbon ion beams; in contrast, invasion was enhanced in PANC-1 cells by carbon ion irradiation, but the enhancement was negligible by X-ray treatment [6,7]. X-ray-induced invasiveness of MIAPaCa-2 cells was almost completely suppressed by treatment with Rho-associated coiled-coil-forming protein kinase (ROCK) or MMP inhibitors, which blocked mesenchymal and amoeboid modes of motility, respectively. Carbon ion-irradiated PANC-1 cell invasion was suppressed by inhibiting ROCK and serine protease but not MMP; however, approximately 50% of cells were still able to migrate through the ECM under these conditions. This limited suppression suggested that an unidentified mechanism contributes to carbon ion radiation-induced cell invasion. The objective of the present study was to identify these additional mechanisms underlying PANC-1 invasiveness in response to genotoxic stress.

2. Materials and methods

2.1. Cell culture and reagents

The human pancreatic cancer cell lines PANC-1 and MIAPaCa-2, and the human glioblastoma cell line SF126 were purchased from the American Type Culture Collection (Manassas, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Co., Ltd.; Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; HyClone; Logan, USA). The nitric oxide synthase (NOS) inhibitors NG monomethyl-\(\cdot\)-arginine (\(\cdot\)-NMA) and 1400W-HCl (Wako; Osaka, Japan), phosphatidylinositol 3-kinase (PI3K) inhibitors LY294002 (Wako) and wortmannin (Merck; Darmstadt, Germany), urokinase-type plasminogen activator (uPA) serine protease inhibitor amiloride hydrochloride hydrate (Sigma-Aldrich, St. Louis, MO, USA), ROCK inhibitor Y27632 (Wako; Osaka, Japan), and nitric oxide (NO) donor S-nitrosoglutathione (GSNO, Santa Cruz Biotechnology Inc.; Santa Cruz, USA) were also used.

2.2. Irradiation

Cells were irradiated with carbon ion at doses of 0.5, 1, 2, or 4 Gy, as previously described [7]. The initial energy of the carbon ion beam was 290 MeV/u; a mono-energetic beam with a narrow Bragg Peak was applied at a depth of 10 cm. Radiation was delivered at a dose rate of approximately 1 Gy/min. An outline of the experimental procedures is shown in Suppl. Fig. 1.

2.3. Inhibitor screening

The inhibitor library consisting of 285 compounds was provided by the Screening Committee of Anticancer Drugs library [13]. Cells were diluted (1 \(\times\) 10\(^5\)) and mixed with each compound for a final concentration of 1 \(\mu\)M [13]. Each sample was transferred to a Transwell membrane on a CultureCoat 96-well BME cell invasion plate (Trevigen; Gaithersburg, USA), and invasion assays were performed according to the manufacturer instructions. The number of invaded cells was calculated for each compound based on a standard curve generated by plotting relative fluorescence units as a function of the number of PANC-1 cells.

2.4. Immunoblotting

Immunoblotting was performed according to standard methods [6,7]. Primary antibodies against human nitric oxide synthase (NOS)1, NOS2 (Santa Cruz Biotechnology Inc.), AKT1 and 2, and phosphorylated (p)-AKT (Cell Signaling Technology; Danvers, USA), along with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Amersham Biosciences; Buckinghamshire, UK) were used.

To evaluate PI3K-mediated AKT activation in NO donor-treated cells, cells were cultured for 2 days before the culture medium was replaced with fresh phenol red-free medium; cells were then incubated with GSNO with or without PI3K inhibitor (LY294002; 1.4 \(\mu\)M) for 24 h. The medium was then collected and used for NO\(_2\) measurements, while cells were washed with PBS and lysed with RIPA buffer (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for immunoblotting.

To investigate the effect of carbon ion radiation on AKT activation in the presence or absence of NOS inhibitor, cells were treated with \(\cdot\)-NMMMA (3 \(\mu\)M) for 7 h on day 1 after irradiation, washed with PBS, then lysed with RIPA buffer.

2.5. Immunofluorescence labeling and image acquisition

Cells were cultured on coverslips in DMEM supplemented with 10% FBS for 48 h. The cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, then permeated with 3\% fetal calf serum, followed by incubation with primary antibody for 30 min at room temperature. For the primary antibodies, AKT1, AKT2, and p-AKT (Cell Signaling Technology; Danvers, USA) were used. The slides were washed with PBS and mounted with ProLong Gold Antifade Reagent containing the nuclear counterstain 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). Immunostaining of invaded cells that reached the undersurface of the Transwell membrane was performed by placing the Transwell directly onto the antibody solution on Parafilm for 30 min at room temperature. The membranes were cut with a needle and mounted with ProLong Gold Antifade Reagent.

Cells were visualized and images were acquired using a BZ-9000 fluorescence microscope and associated software (Keyence; Osaka, Japan). DAF-2DA-treated NO-producing live cells stained with DAPI, or fixed cells labeled with Alexa Fluor 555- or 488-labeled anti-mouse IgG or anti-rabbit IgG secondary antibodies (Invitrogen; Carlsbad, USA). The slides were washed with PBS and mounted with ProLong Gold Antifade Reagent.

Cells were visualized and images were acquired using a BZ-9000 fluorescence microscope and associated software (Keyence; Osaka, Japan). DAF-2DA-treated NO-producing live cells stained with DAPI, or fixed cells labeled with Alexa Fluor 555 or 488 were photographed at room temperature using a 20 \(\times\) Plan objective fluorescent lens (N.A. 0.45) with BZ filters for green fluorescent protein (GFP) and DAPI (live cells), or tetramethylrhodamine-5- and-6-isothiocyanate, GFP, and DAPI (fixed cells). Representative images were uniformly processed in Adobe Photoshop using the brightness and contrast tools.

2.6. Cell invasion and viability assays

The invasive potential of PANC-1 cells was examined as previously described [6,7]. The four randomly selected fields of invaded cells reached the undersurface of the Transwell membrane were photographed and the number of invaded cells in each field was counted under a light microscope. Percentages of viable and dead cells were determined by fluorescence-activated cell sorting and by using a cell viability double-staining kit (Dojindo Molecular Technologies Inc.; Tokyo, Japan). The proportion of viable cells was calculated by dividing the number of viable cells by the total number of seeded cells.

2.7. Detection of NO-producing cells

Diaminofluorescein-2 diacetate (DAF-2DA) (Santa Cruz Biotechnology Inc.) was used for the detection of NO-producing cells.
according to the manufacturer’s protocol [14]. Briefly, cells were washed with Krebs–Ringer phosphate (KRP) buffer, followed by staining with DAF-2DA with or without L-NMMA in PBS for 1 h. The cells were then washed with KRP buffer and treated with the nuclear stain NucBlue (Invitrogen). For detection of NO-producing invaded cells, a Transwell invasion assay was performed with cells grown in a 24-well plate; invaded cells that reached the undersurface of the Transwell membrane were stained with DAF-2DA. Three randomly selected fields were imaged with a fluorescence microscope, and the total number of PANC-1 cells in each field was assessed by counting the number of DAPI-stained nuclei, while the percentage of NO-producing cells was calculated by dividing the number of DAF-2DA-positive cells by the total number of cells.

To measure the NO content of the medium, the cells were first washed with PBS, then incubated in fresh medium with or without L-NMMA for 6 h. Cells were washed with KRP buffer and treated with diaminofluorescein-2 (DAF-2 DA) (Sekisui Medical; Tokyo, Japan) at concentrations of 495 nm and 515 nm for excitation and emission, respectively. Nitrite (NO2⁻) levels in the medium were measured after growing cells for 17 h using the NO2/NO3 Assay Kit-FX (Fluorometric). The invasive potential of carbon ion-irradiated PANC-1 cells was assessed in the presence of 1 μM of each compound according to recommendations of The Screening Committee of Anticancer Drugs.

The standard curve for the cell invasion assay is shown in Suppl. Fig. 2. Of the screened compounds, 19 were found to inhibit PANC-1 cell invasion by more than 40% relative to controls (Table 1). Among these, several targeted the same molecule, including the NOS inhibitors 1400W-HCl, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine, and L-NMMA; the PI3K inhibitors LY294002 and wortmannin; and the glycosylation inhibitors tunicamycin and deoxynojirimycin. To clarify the effects of these seven compounds, their ability to inhibit invasion at previously reported concentrations was tested (Table 2). Under these conditions, the NOS and PI3K inhibitors still inhibited invasion to a similar degree; thus, these concentrations were used in subsequent experiments (Fig. 1). These data suggest that PANC-1 cell invasion is regulated by NOS and PI3K; however, the contribution of other enzymes could not be ruled out, since the conditions of the initial screen could not be used due to the presence of NO [16]. As predicted, increased NO2⁻ levels were also measured to corroborate the presence of NO [16].

### 2.8. RhoA activity assay

To measure endogenous small GTPase activity, a RhoA activation assay kit was used according to the manufacturer’s instructions (Cell Biolabs Inc.; San Diego, USA). Briefly, cells were lysed on ice and centrifuged at 4 °C for 10 min at 14,000 × g, and the supernatant was incubated with Rhotekin RBD-agarose beads at 4 °C for 1 h on a rotator. Protein samples were eluted and processed for immunoblotting with anti-RhoA antibodies provided by the kit.

### 2.9. Statistical analysis

Statistical analyses were performed using the unpaired Student’s t-test. A P-value < 0.05 was considered significant.

### 3. Results

#### 3.1. Carbon ion radiation-enhanced invasiveness is regulated by NOS and PI3K

The invasive potential of carbon ion-irradiated PANC-1 cells was assessed in the presence of 1 μM of each compound according to recommendations of The Screening Committee of Anticancer Drugs.

The maximum suppression of radiation-enhanced PANC-1 cell invasion (77%) was observed by treatment with the general NOS inhibitor L-NMMA (Fig. 1). NO production by PANC-1 cells increased slightly upon irradiation, and this was also suppressed by treatment with the inhibitor (Fig. 2A). Under these conditions, the NOS and PI3K inhibitors still inhibited invasion to a similar degree; thus, these concentrations were used in subsequent experiments (Fig. 1). These data suggest that PANC-1 cell invasion is regulated by NOS and PI3K; however, the contribution of other enzymes could not be ruled out, since the conditions of the initial screen (1 μM concentration) were not optimized for all compounds in the library.

### 3.2. Carbon ion radiation-enhanced invasiveness increases the proportion of NO-producing cells

The maximum suppression of radiation-enhanced PANC-1 cell invasion (77%) was observed by treatment with the general NOS inhibitor L-NMMA (Fig. 1). NO production by PANC-1 cells increased slightly upon irradiation, and this was also suppressed by treatment with the inhibitor (Fig. 2A). Under these conditions, the NOS and PI3K inhibitors still inhibited invasion to a similar degree; thus, these concentrations were used in subsequent experiments (Fig. 1). These data suggest that PANC-1 cell invasion is regulated by NOS and PI3K; however, the contribution of other enzymes could not be ruled out, since the conditions of the initial screen (1 μM concentration) were not optimized for all compounds in the library.

### Table 1

| Compound     | Description                        | Concentration used for the assay | % Reduction in invasion |
|--------------|------------------------------------|----------------------------------|-------------------------|
| 1400W, HCl   | NOS2 inhibitor                      | 40 μM [37]                       | 59                      |
| AMT, HCl     | NOS2 inhibitor                      | 4.2 nM [38]                      | 42                      |
| L-NMMA       | Inhibits all NOS1, NOS2, NOS3       | 3 μM [14]                        | 87                      |
| LY294002     | PI3K inhibitor                      | 1.4 μM [39]                      | 41                      |
| Wortmannin   | PI3K inhibitor                      | 5 nM [40]                        | 45                      |
| Tunicamycin  | GlcNAc phosphotransferase inhibitor | 6 μM [41]                        | 6                       |
| Deoxynojirimycin | Alpha-glucosidase I and II inhibitor | 0.4 mM [42]                    | 0                       |

All compounds were used for assay at the same concentration used in other in vitro experiments (n = 3).

### Table 2

| Compound     | Description | Concentration used for the assay | % Reduction in invasion |
|--------------|-------------|----------------------------------|-------------------------|
| 1400W, HCl   | NOS2 inhibitor | 40 μM [37]                     | 59                      |
| AMT, HCl     | NOS2 inhibitor | 4.2 nM [38]                     | 42                      |
| L-NMMA       | Inhibits all NOS1, NOS2, NOS3 | 3 μM [14]                     | 87                      |
| LY294002     | PI3K inhibitor | 1.4 μM [39]                     | 41                      |
| Wortmannin   | PI3K inhibitor | 5 nM [40]                      | 45                      |
| Tunicamycin  | GlcNAc phosphotransferase inhibitor | 6 μM [41]                | 6                       |
| Deoxynojirimycin | Alpha-glucosidase I and II inhibitor | 0.4 mM [42]     | 0                       |

All compounds were used for assay at the same concentration used in other in vitro experiments (n = 3).
NO production in PANC-1 cells was also determined in real-time using DAF-2DA. Interestingly, only 4% of cells produced NO (Fig. 2C and D). This proportion increased to 20% upon irradiation, an effect that was suppressed following treatment with L-NMMA (Fig. 2D and E).

To observe whether NO production was correlated with invasiveness, the fraction of NO-producing cells among those that reached the undersurface of the Transwell membrane was determined. Most (90%) of the invaded cells produced NO, and this proportion was unaffected by irradiation (Fig. 2F and G), suggesting that NO production is associated with both spontaneous and radiation-induced invasion. In accordance with the increase in radiation-induced NO production, the total number of invaded cells was also higher (Fig. 1A).

Since PANC-1 was the only pancreatic cell line in which invasiveness increased as a result of carbon ion irradiation so far, invasiveness after the carbon ion irradiation (2 Gy) was assessed in 30 other cell lines derived from other types of tumors. Of these, the human glioblastoma cell line, SF126, showed an increase in invasiveness upon carbon ion irradiation (Suppl. Fig. 4A). Consistent with what was observed in PANC-1 cells, elevated levels of NO2 were detected in SF126 culture medium (Suppl. Fig. 4B). Thus, the enhanced NO2 content of SF126 upon carbon ion irradiation may be related to the increased invasiveness of irradiated SF126. We previously reported that radiation suppressed MIAPaCa-2, BxPC-3, and AsPC-1 cell invasion [7]. In the present study, decreased percentage of NO-producing cells was observed in MIAPaCa-2 after carbon ion irradiation (Suppl. Fig. 4C and D). Furthermore, NO2 levels of MIAPaCa-2 culture medium were decreased upon irradiation (Suppl. Fig. 4E), suggested that decreased NO2 may be related to the reduced invasiveness observed in irradiated MIAPaCa-2.

3.3. Upregulation of NOS expression and increased invasiveness by carbon ion irradiation

Increased levels of NOS1 and NOS2 protein were detected in nearly all invaded PANC-1 cells (Fig. 3A and B), irrespective of exposure to radiation. NOS expression was also upregulated in invaded SF126 glioblastoma cells (Suppl. Fig. 5).

3.4. NO-induced, PI3K-dependent AKT activation in PANC-1 cell invasion

In addition to NOS inhibitors, PI3K inhibitors were also effective in suppressing radiation-induced PANC-1 cell invasion. To determine whether NO plays a role in the activation of the serine/threonine-specific kinase AKT via PI3K, the phosphorylation status of endogenous AKT was examined in the presence of the NO donor GSNO. NO2 levels increased in a dose-dependent manner in GSNO-treated cells, concomitant with the phosphorylation-dependent activation of AKT (Fig. 4A), in accordance with previous reports [17]. GSNO-dependent AKT activation was abolished in the presence of PI3K inhibitors, indicating that NO activated AKT via stimulation of the kinase activity of PI3K (Fig. 4B). Indeed, levels of AKT1 and AKT2 protein were unaltered by GSNO treatment (Fig. 4C). In addition, GSNO-induced PANC-1 cell invasion was observed (Fig. 4D). Treatment with GSNO at concentrations of >6 mM failed to enhance invasiveness, possibly because of an increase in cell death (Fig. 4E, Suppl. Fig. 6).

AKT was activated at radiation doses of 2 and 4 Gy (Fig. 4F and G), an effect that was abolished by l-NMMA treatment, indicating that the effect was dependent on NO production. Interestingly, the AKT2 protein level was also upregulated at these doses, but this was unaffected by application of l-NMMA, indicating that AKT2 expression is not induced by NO (Fig. 4F and G). Similar to what was observed in PANC-1 cells, AKT was activated in SF126 cells at a radiation dose of 2 Gy and was associated with enhanced invasiveness (Suppl. Fig. 7A).

Consistent with NO-dependent activation of AKT, expression of p-AKT was detected only in invaded PANC-1 or SF126 cell lines (Fig. 5A, Suppl. Fig. 7B), which also produced high levels of NO. AKT2, but not AKT1, primarily localized to the cell membrane (Fig. 5B, Suppl. Fig. 7C). Interestingly, distribution of p-AKT in invaded cells was also observed at the cell membrane. Furthermore, we observed that this activation of p-AKT was reduced by...
The enhanced invasiveness upon irradiation and reduced invasion by treatment with NO inhibitors (Fig. 5C). The enhanced invasiveness was confirmed and presented in Fig. 6B. Recently, the actin-binding AKT substrate GIRDIN was reported to play a significant role in AKT-dependent cell motility [18]. The phosphorylation of GIRDIN by activated AKT is essential for actin organization and lamellipodia formation, and is thus required for invasion. 

Fig. 2. NO production in non-irradiated and carbon ion-irradiated PANC-1 cells. (A) NO level in the culture medium, without treatment or after treatment with L-NMMA (3 μM), as measured by DAF-2. RFU, relative fluorescence units. (B) Relative ratios of NO2− content in non-irradiated vs. carbon ion-irradiated cells. Data are presented as mean ± SD of triplicate samples. *P < 0.05. (C) Representative images of NO-producing cells. Cells treated with DAF-2DA were visualized by green fluorescence. Left, bright field; middle, epifluorescence; right, merged. Scale bars: 200 μm (top), 50 μm (bottom). (D) NO-producing non-irradiated and carbon ion-irradiated cells, untreated or treated with L-NMMA. NO production was visualized by green fluorescence, with DAPI-stained nuclei emitting a blue signal. Scale bar: 200 μm. (E) Percentages of NO-producing non-irradiated or carbon ion-irradiated cells, untreated or treated with L-NMMA. (F) Invaded PANC-1 cells at the undersurface of the Transwell membrane treated with DAF-2DA (green; NO-producing cells) and DAPI (blue). Scale bar: 200 μm. (G) Number of NO-producing invaded cells counted from three randomly selected fields, divided by the total number of invaded cells in the same field. The percentages of invading non-irradiated and carbon ion-irradiated cells are shown. Data represent the mean ± SD of triplicate samples. *P < 0.05 vs. control.
directional migration and invasion. It is worth noting that here, p-GIRDIN was observed at the leading edge of directionally invading cells: p-GIRDIN-positive cell processes were seen emerging from a pore in the Transwell membrane and were immunoreactive for p-AKT (Fig. 5D).

3.5. NOS–PI3K-independent activation of RhoA by NO

We previously showed that carbon ion-irradiated PANC-1 cells have the ability to undergo MA transition [7]. Among invaded cells, a higher proportion of cells with a round morphology was observed in cells exposed to radiation than in non-irradiated cells, a difference that was abolished by inhibiting NOS (Fig. 1B). In accordance with these morphological changes, RhoA activation was enhanced in irradiated cells, which was suppressed by NOS inhibitor treatment (Suppl. Fig. 8A). To determine whether the activation of RhoA is regulated by the NOS–PI3K signaling pathway, irradiated PANC-1 cells were treated with PI3K inhibitors. No effect on RhoA activation was observed, suggesting that NO activates RhoA through a distinct pathway (Suppl. Fig. 8B).

3.6. Suppression of invasive potential by serine protease, PI3K, ROCK, and NOS inhibition

Treatment of irradiated PANC-1 cells with a PI3K inhibitor caused a decrease in invasiveness to baseline levels (Fig. 6A). The combined application of ROCK and PI3K inhibitors, which blocked NOS-dependent RhoA and PI3K pathways, respectively, suppressed invasion to a greater degree than either compound alone, indicating an additive effect. We previously showed that radiation-induced invasion decreased by approximately 50% upon combined treatment with inhibitors of uPA and ROCK [7]. Here, combined application of ROCK, uPA inhibitors, or NOS and uPA inhibitors, led to a greater inhibition of invasive potential than individual application of any one of these agents (Fig. 6A and B). These results suggest that NO produced as a result of genotoxic stress increases invasiveness of PANC-1 cells via activation of the PI3K–AKT and RhoA pathways (Fig. 6C).

4. Discussion

Radiation therapy is a standard method of treatment for most types of cancer, and recent studies have evaluated its efficacy in pancreatic cancer treatment [19]. However, the successful application of any treatment strategy requires a thorough understanding of resultant physiological changes, whether intended or inadvertent. Therefore, better understanding of the mechanism underlying changes will facilitate the development of treatments to prevent these effects and further increase the benefit to patients receiving radiation therapy.

It was previously observed that genotoxic stress resulting from the high linear energy transfer carbon ion radiation enhanced invasiveness of PANC-1 cells through the activation of serine proteases such as uPA. Treatment with serine protease inhibitor alone induced MA transition in cells, with cells continuing to invade the ECM by a protease-independent mechanism. Although inhibition
Fig. 4. PI3K-mediated AKT activation in NO donor-treated PANC-1 cells. (A) Expression of p-AKT, AKT1, and AKT2 was determined by Western blotting after treating cells with different concentrations of GSNO. NO$_2^-$ levels were measured as an index of NO production. (B) Effect of treatment with PI3K inhibitor on GSNO (3 mM)-induced AKT activation, as determined by Western blotting. LY294002 concentration was 1.4 μM. (C) Quantitative densitometric results for p-AKT, AKT1, AKT2 are shown. Data represent the mean ± SD of triplicate samples. *P < 0.05 vs. control. (D) PANC-1 cells were trypsinized and washed with PBS, then resuspended in serum-free DMEM with the indicated dose of GSNO and used for the invasion assay, which was performed over 24 h before invaded cells were fixed and stained. Data represent the mean ± SD of triplicate samples. *P < 0.05 vs. control. (E) PANC-1 cells treated with the indicated dose of GSNO were cultured in DMEM supplemented with 10% FBS or serum-free DMEM for 24 h. Cells were then fixed and stained with Diff-Quick solution (Sysmex, Kobe, Japan). (F) Effect of carbon ion irradiation on AKT activation in the presence or absence of L-NMMA (3 μM), as determined by Western blotting. (G) Expression levels of p-AKT, AKT1, and AKT2 were measured by quantitative densitometry. Data represent the mean ± SD of triplicate samples. *P < 0.05 vs. control.
of both mesenchymal and amoeboid cell motility via the combined administration of serine protease and ROCK inhibitors decreased radiation-induced cell invasion, approximately half of cells still exhibited invasive potential. In this study, NOS was observed to play a significant role in radiation-enhanced invasiveness through activation of the PI3K–AKT and RhoA signaling pathways, both of which have been implicated in actin cytoskeleton remodeling [20]. NOS and uPA are activated in response to genotoxic stress; plasmin produced by uPA degrades ECM proteins [21], while NO produced by NOS activates both PI3K and Rho kinase. Accordingly, simultaneous inhibition of uPA, PI3K, and ROCK, or uPA and NOS, resulted in almost complete suppression of radiation-induced cell invasion (Fig. 6).

NO is known to activate PI3K–AKT signaling and prevents tumor cell apoptosis [17,22]. Furthermore, NO-dependent mechanisms that promote cell survival are induced by ionizing radiation [23]. While these reports used cell viability as the end-point for assessing PI3K–AKT pathway activation, the present study demonstrated that NOS–PI3K–AKT signaling also mediates cell invasion. Two closely related members of the AKT/PKB family, AKT1 and AKT2, differ in their physiological roles, with AKT1 regulating proliferation and survival and AKT2 promoting migration and invasion [20,24]. Due to the presence of highly conserved serine residues, immunodetection of a specific, activated AKT family member is difficult [25]. However, activated AKT1 and AKT2 localize, respectively, to the nucleus and plasma membrane [24,25]; therefore, examining the subcellular localization of AKT could serve as a means of distinguishing between activated AKT1 and AKT2. In this study, localization of p-AKT was predominantly associated with the plasma membrane, with little nuclear staining, implying that AKT2 is activated in invading PANC-1 cells, consistent with the known function of AKT2 [20,24].

Distinct populations of invaded PANC-1 cells were observed, with carbon ion irradiation specifically enhancing a population of NO-producing cells. In the SF126 glioblastoma cell line, NOS is constitutively expressed [26], and likely produces higher levels of NO than pancreatic cell lines. These experiments confirmed that the expression of NOS proteins was upregulated in invading, compared to whole culture, SF126 cells, an observation that was also made in PANC-1 cells. Accordingly, NO2/C0 content increased concomitantly with SF126 cell invasion upon carbon ion irradiation (Suppl. Fig. 4). Conversely, irradiation suppressed NO2/C0 formation and invasiveness in MIAPaCa-2 cells. Therefore, invasive potential is modulated by NO in a variety of cell types, but the specific effects differ significantly.

NO has a role in tumorigenesis, including in pancreatic cancer [27,28], and NOS expression is correlated with tumor stage or metastatic progression [29–31] as well as tumor growth and metastasis in a murine mammary tumor model [32]. Tumor cells with high levels of NOS expression derived from highly metastatic mice showed strong invasive potential in the Transwell invasion assay, and lung metastasis in these mice was reduced upon treatment
with the NOS inhibitor L-NMMA, implicating NOS in the metastasis of tumor cells to the lungs [33]. In cancer patients receiving \( \gamma \)-ray radiotherapy, NOS levels are correlated with post-radiotherapy metastatic progression; moreover, the upregulation of NOS expression is associated with decreased survival and a greater likelihood for metastasis in cervical cancer patients receiving radiotherapy.

**Fig. 5.** Upregulation of p-AKT in non-irradiated and carbon ion-irradiated invading PANC-1 cells. (A) Relative levels of protein were compared for whole culture and invaded cells stained with antibodies against AKT1, AKT2, or p-AKT. Scale bar: 50 μm. (B) Representative images of AKT1, AKT2, and p-AKT localization in invaded cells. Arrows indicate plasma membrane localization of AKT2 and p-AKT. Scale bar: 20 μm. (C) Non-irradiated or carbon ion-irradiated invading cells, untreated or treated with L-NMMA and labeled with an antibody against p-AKT. Scale bar: 50 μm. (D) Invaded PANC-1 cells were stained with antibodies against p-GIRDIN and p-AKT. Left panel, p-GIRDIN (green); the second left panel, p-AKT (red); middle two panels, merged images; right panel, phase contrast images. Scale bar: 10 μm.
Fig. 6. Effect of the NOS activity on invasive potential of carbon ion-irradiated PANC-1 cells. (A) Invasion assay performed with the addition of PI3K inhibitor (LY294002; 1.4 µM), PI3K and ROCK (Y27632; 10 µM) inhibitors, or PI3K, ROCK, and uPA (amiloride hydrochloride hydrate; 50 µM) inhibitors. (B) Invasion assay performed with the addition of NOS (L-NMMA; 3 µM) or uPA inhibitor, or both. Data represent the mean ± SD of triplicate samples. *P < 0.05 vs. control. (C) Model of events leading from carbon ion radiation-enhanced genotoxic stress to PANC-1 invasion.

molecular basis for these differences is unclear, although heterogeneity in the genetic background of cell lines could be an underlying cause. Mutations in the oncogenes P53 and KRAS have been identified in both PANC-1 and MIAPaCa-2 pancreatic cells [36]; however, cell-line specific mutations in other oncogenes from commercially available cancer panels have not been found (data not shown). Whole exon/genome analysis of various cell lines using next-generation sequencing could be useful for characterizing differential mutation patterns. In addition, further studies are required to identify the characteristics of cells that increase NOS activity, which could provide an explanation for why only a fraction of cells increases NO production in response to genotoxic stress. Moreover, several groups have reported that a high concentration of NO can induce apoptosis in cancer cells. In this study, we showed that relatively high doses of carbon ion irradiation and high concentrations of NO caused cell death, but it was not possible to distinguish whether this was the result of DNA damage or NO-induced apoptosis. Resolving these issues will provide insight into the mechanisms of cancer metastasis, and enable the development of less toxic therapies to inhibit cancer progression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.07.006.

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