Suppressive effects of a proton beam on tumor growth and lung metastasis through the inhibition of metastatic gene expression in 4T1 orthotopic breast cancer model

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Abstract. A proton beam is a next generation tool to treat intractable cancer. Although the therapeutic effects of a proton beam are well known, the effect on tumor metastasis is not fully described. Here, we investigated the effects of a proton beam on metastasis in highly invasive 4T1 murine breast cancer cells and their orthotopic breast cancer model. Cells were irradiated with 2, 4, 8 or 16 Gy proton beam, and changes in cell proliferation, survival, and migration were observed by MTT, colony forming and wound healing assays. 4T1 breast cancer cell-implanted BALB/c mice were established and the animals were randomly divided into 4 groups when tumor size reached 200 mm^3. Breast tumors were selectively irradiated with 10, 20 or 30 Gy proton beam. Breast tumor sizes were measured twice a week, and breast tumor and lung tissues were pathologically observed. Metastasis-regulating gene expression was assessed with quantitative RT-PCR. A proton beam dose-dependently decreased cell proliferation, survival and migration in 4T1 murine breast cancer cells. Also, growth of breast tumors in the 4T1 orthotopic breast cancer model was significantly suppressed by proton beam irradiation without significant change of body weight. Furthermore, fewer tumor nodules metastasized from breast tumor into lung in mice irradiated with 30 Gy proton beam, but not with 10 and 20 Gy, than in control. We observed correspondingly lower expression levels of urokinase plasminogen activator (uPA), uPA receptor, cyclooxygenase (COX)-2, and vascular endothelial growth factor (VEGF), which are important factors in cancer metastasis, in breast tumor irradiated with 30 Gy proton beam.

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Proton beam irradiation did not affect expressions of matrix metalloproteinase (MMP)-9 and MMP-2. Taken together, the data suggest that, although proton beam therapy is an effective tool for breast cancer treatment, a suitable dose is necessary to prevent metastasis-linked relapse and poor prognosis.

Introduction

Although the 5-year survival rate of breast cancer patients is increasing due to advanced therapeutic techniques, lower survival rate and poor prognosis are still observed in patients diagnosed with metastatic breast cancer (1). Actually, ~98% of breast cancer patients with a localized tumor can survive 5 years, but just 27% of patients diagnosed with metastatic breast cancer can live >5 years (2). This indicates the importance of cancer metastasis prevention to improve therapeutic efficacy in breast cancer patients.

To metastasize, cancer cells must be released from the primary tumor and travel to a distant site by attachment and invasion through degradation of biological barriers, such as basement membrane (BM) and extracellular matrix (ECM). The degradation is predominantly regulated by membrane-degrading enzymes, such as matrix metalloproteinases (MMPs), urokinase plasminogen activator (uPA), and uPA receptor (uPAR) (3). Among MMPs, the gelatinases MMP-2 and -9 play important roles in breast cancer metastasis and angiogenesis (4). In breast cancer cell systems, several studies have revealed that metastatic potential was suppressed by inhibition of MMP-2 and/or -9 (4-6). Also, a clinical study showed that expression levels of MMP-2 and -9 in metastatic breast cancer patients were higher than those in non-metastatic breast cancer patients (7). uPA is also closely related to metastatic potential. uPA accelerates ECM degradation and MMP-9 activation through the catalysis of plasmin production from plasminogen and it is also a well-known prognostic factor in breast cancer (8,9). In clinical evaluation, poor prognosis and high rate of relapse were shown in node-negative breast cancer patients with high levels of uPA in breast tumor tissue or high concentrations of serum uPA, whereas patients with low levels of tissue uPA had a low risk of recurrence (8,10,11). Consequently, the inhibition of these proteases is a key strategy to prevent breast cancer metastasis.

Proton beam therapy is a promising radiotherapeutic tool due to the characteristic delivery of specifically designed doses to tumor tissue with minimized damage to surrounding normal tissues because the ionizing proton beam is deposited in an exact region with a dramatic end point increase of the specific energy per unit in a body (Bragg Peak) (12). Proton beam therapy is widely applied to treat various types of cancer including breast cancer (13). In general, the molecular biological study of therapeutic effects in a proton beam has focused on tumor cell apoptosis caused by direct or indirect DNA damage (14,15). However, the effects on metastasis, a major cause of recurrence and poor prognosis, have not been fully investigated.

We previously showed that a proton beam diminished the metastatic potential of MCF-7 and MDA-MB-231 human breast cancer cells via the inhibition of MMP-9 activity and expression (12,16). Furthermore, proton beam irradiation suppressed angiogenic activity in MCF-7 human breast cancer cells through the inhibition of vascular endothelial growth factor (VEGF) expression, an important angiogenic factor (17). Here, we investigated the efficacy of proton beam irradiation on breast cancer metastasis using aggressive 4T1 murine breast cancer cells isolated from naturally developed mammary tumor in BALB/c mice and its orthotopic breast cancer model (18,19). We analyzed the effects of proton beam irradiation on cell proliferation, viability and metastatic potential in 4T1 murine breast cancer cells and also measured tumor growth, lung metastasis and metastatic gene mRNA expression in 4T1 orthotopic animal models.

Materials and methods

Cell culture. The 4T1 murine breast cancer cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) and routinely maintained in Dulbecco's modified Eagle's medium (DMEM, Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) and 1% antibiotic-antimycotic solution (Welgene) in 5% (v/v) CO_2 atmosphere at 37°C.

Cell proliferation assay. To evaluate the effect of proton beam irradiation on cell proliferation, we conducted 3-(4,5-dimethyl-thiazol-2-yl)-2,5 diphenyl-tetrazolium bromide (MTT) assays. 4T1 murine breast cancer cells were plated at 2,000 cells per well into 96-well plates and incubated for 24 h. Cells were then irradiated with a dose of 2, 4, 8, or 16 Gy by spread-out Bragg peak (SOBP) with the 100 MeV proton accelerator (Korea Multi-Purpose Accelerator Complex, Gyeongju, Korea), grown for 24 or 48 h, and then incubated with MTT for 4 h in the dark. Produced insoluble formazan was dissolved in dimethyl sulfoxide (DMSO) and the absorbance of formazan was measured at 570 nm.

Clonogenic cell survival assay. Clonogenic cell viability was assessed by colony forming assay. Three hundred cells were seeded in each well of a 6-well plate and attached for 24 h. Then, the cells were irradiated with a proton beam in a dose-dependent manner and additionally grown for 6 days. The colonies were fixed with 10% formalin for 20 min, stained with 1% crystal violet for 40 min and then captured.

Wound healing assay. Cell migration was evaluated with a wound healing assay. Cells were plated on 6-well plates coated with collagen IV (Corning, Bedford, MA, USA) and cultured to confluence. Cell monolayers were scratched with a blue tip and then detached cells were removed by excluding media and washing with phosphate-buffered saline. Media were replaced to DMEM supplemented with 2% FBS and cells were irradiated with a proton beam in a dose of 2, 4, 8 or 16 Gy. The wound areas were observed and captured at 0 and 24 h after proton beam irradiation, respectively.

Animal model and tumorigenesis. Animal experiments were performed with the approval of the Institutional Animal Care and Use Committee at Dongguk University (IACUC-2014-008). Female BALB/c mice were purchased from Orient Bio Inc. (Gapyung, Korea) and housed under a 12 h light/dark cycle at 25±2°C with a relative humidity of 50±5%. After adaptation of one week, $1x10^5$ 4T1 murine breast cancer cells were implanted into the mammary fat pad of 5-week-old female BALB/c mice to establish the orthotopic animal breast cancer model. When the tumor volumes reached ~200 mm^3, mice were randomly divided into 4 groups: 0 (control), 10, 20 and 30 Gy. Mice were protected by an acryl plate (50 mm thickness) and mammary tumors were selectively irradiated with a proton beam at a dose of 10, 20 or 30 Gy using the 100 MeV proton accelerator. Tumor sizes and body weights were measured twice a week. Tumor volume (mm^3) was calculated with the following formula: tumor volume = width^2 x length x 0.5. Mice were sacrificed after 14 days and breast tumors and lungs were dissected.

Quantitative RT-PCR. The proton beam irradiation-mediated changes in metastasis-regulating genes, such as MMP-9, MMP-2, uPA, uPAR, cyclooxygenase (COX)-2, and VEGF, were analyzed. Total RNA was extracted from breast tumor tissues using the easy-BLUE™ Total RNA Extraction kit (iNtRON Biotechnology Inc., Sungnam, Korea) according to the manufacturer's protocol. cDNA was synthesized with Goscript™ Reverse Transcriptase (Promega, Madison, WI, USA). Real-time PCR reactions were performed with QGreen 2X SybrGreen Master Mix (Cellsafe, Suwon, Korea) using Eco™ Real-Time PCR (Illumina, San Diego, CA, USA). The reaction was preheated at 95°C for 10 min, followed by 40 cycles of 90°C for 10 sec, 60°C for 15 sec, and 72°C for 20 sec; products were verified by melting curve analysis. Relative expression levels compared with control were automatically evaluated by Eco Software v3.1.7. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Sequences of the primers used for the reaction were as follows: MMP-9: forward, 5'-GCGAGTG TCTCCCTCAAACG-3'; reverse, 5'-TCCTCACGGTGATGCT GTTC-3'. MMP-2: forward, 5'-GCTGATACTGACACTGGT ACTG-3'; reverse, 5'-CAATCTTTT CTGGGAGCTC-3'. uPA: forward, 5'-ATGCATGGTGCATGACTGCTCT-3'; reverse, 5'-ATTCGTGCAAGATGAGCTGCTC-3'. uPAR: forward, 5'-CAGAGCACAGAAAGGAGCTTGA-3'; reverse, 5'-CTG AAAGGTCTGGTTGCTATGGA-3'. COX-2: forward, 5'-CCT GCTGCCCGACACCTTCA-3'; reverse, 5'-AGCAACCCGGC CAGCAATCT-3'. VEGF: forward, 5'-GTACCTCCACCAT GCCAAGT-3'; reverse, 5'-GCATTCACATCTGCTGTGCT-3'.
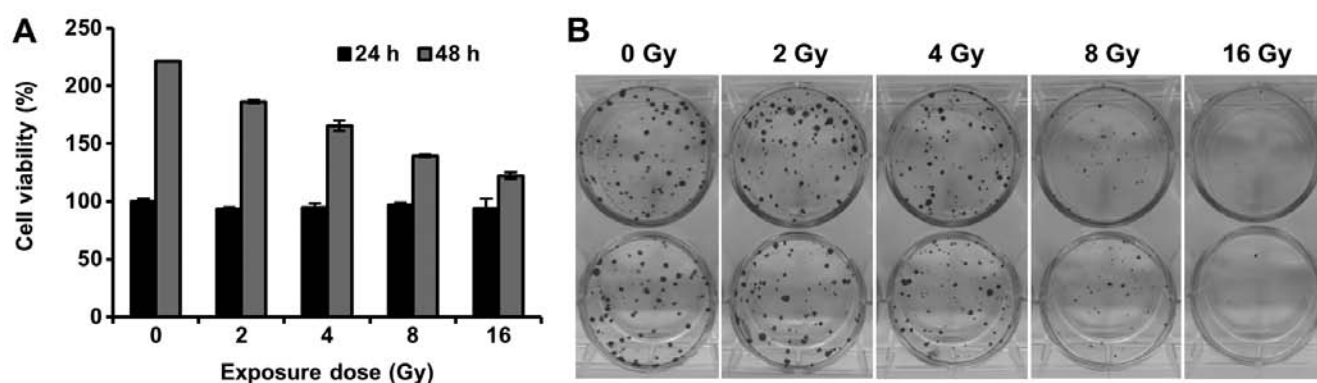
Figure 1. A proton beam decreased proliferation and clonogenic survival of 4T1 murine breast cancer cells. (A) Cell viability of 4T1 cells after irradiation with a proton beam. Cells were seeded in 96-well plates and irradiated with 2, 4, 8 or 16 Gy proton beam and then cell viability was measured 24 and 48 h later with MTT reagent, respectively. (B) Representative images of colonies. Cells were seeded and grown for 24 h prior to irradiation by 2, 4, 8 or 16 Gy proton beam, respectively. Cells were additionally grown for 6 days, and then the formed colonies were fixed with 10% formalin and stained with 1% crystal blue.
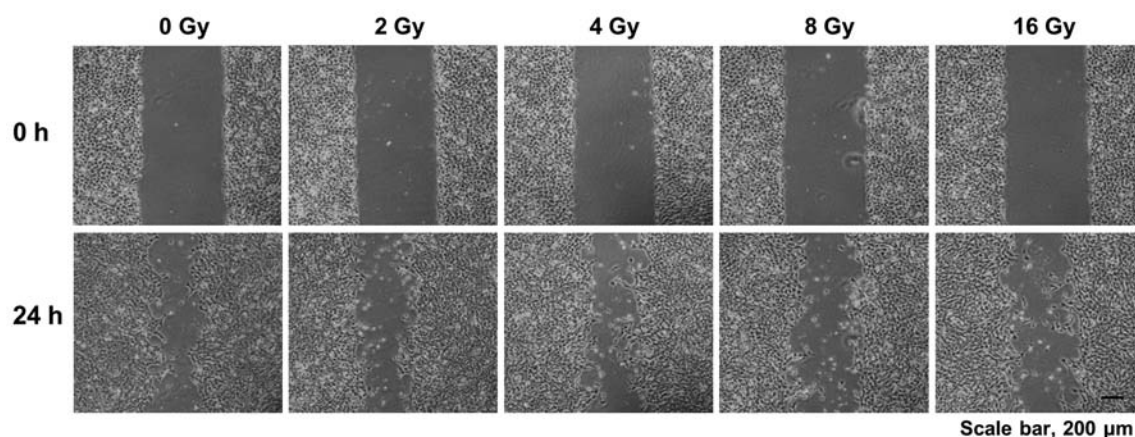

Figure 2. A proton beam inhibited the cell migration of 4T1 murine breast cancer cells. Cells were plated in 6-well plates and irradiated with 2, 4, 8 or 16 Gy proton beam. Wounded areas were captured at 0 and 24 h after proton beam irradiation using an optical microscope.

GAPDH: forward, 5'-GTATGACTCCACTCACGGCAAA-3'; reverse, 5'-GGTCTCGCTCCTGGAAGATG-3'.

Pathological analysis. To observe metastasized tumor cells in the lung, lung tissues were collected from mice, fixed with 10% formalin, and embedded in paraffin. Then the paraffin-embedded lung tissues were cut into 4-mm-thick slices and stained with haematoxylin and eosin (H&E). Tissue sections were randomly selected and tumor nodules were counted using optical microscopes; the sizes of the tumor nodules were also measured. The sizes of the tumor nodules were calculated with the following formula: size of tumor nodule = (the longest diameter + the shortest diameter)/2.

Statistical analysis. Statistical significance was determined using the Student's t-test or one-way ANOVA. All cell experiments were conducted in triplicate, and results are presented as mean ± SD. P-values of <0.05 were considered significant.

Results

Effects on proliferation and survival of 4T1 murine breast cancer cells. We assessed the effect of a proton beam on cell prolifera-

tion and clonogenic survival in 4T1 murine breast cancer cells. Although cell proliferation was not affected by proton beam irradiation within 24 h, the proton beam inhibited the proliferation at 48 h in MTT assay (Fig. 1A). In addition, the colony forming assay showed that clonogenic viability was significantly decreased by proton beam irradiation (Fig. 1B). These results are consistent with several studies that revealed inhibition of cell growth by proton beam irradiation in human solid tumors, such as pancreas, prostate, and breast cancer (14,15,20).

Effects on cell migration in 4T1 murine breast cancer cells. Cell migration is crucial for cancer cells to metastasize from a primary tumor tissue to distant sites in the body. Therefore, we also evaluated the effects of proton beam irradiation on cell migration of 4T1 murine breast cancer cells in the wound healing assay. We found that cell migration of 4T1 murine breast cancer cells was effectively prevented by proton beam irradiation ranging from 2 to 16 Gy (Fig. 2). This result demonstrates that a proton beam may suppress the metastatic potential of 4T1 murine breast cancer cells.

Effects on breast tumor growth and lung metastasis in 4T1 orthotopic animal model. In vitro experiments showed the
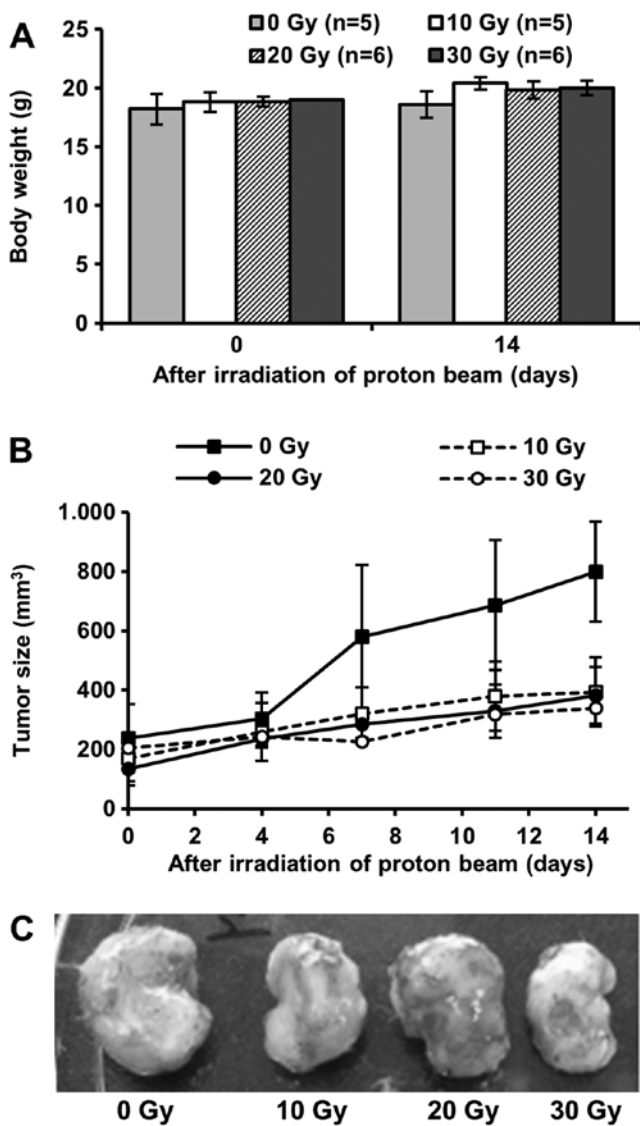
Figure 3. Proton beam delayed tumor growth in 4T1 orthotopic breast cancer model. (A) Changes of mouse body weight. Values indicate average body weight in each group. (B) Changes of breast tumor growth by proton beam irradiation. Tumor sizes were measured every 3 or 4 days. (C) Image of the breast tumor in each group. Breast tumor in each group was irradiated with indicated proton beam dose and then additionally grown for 14 days.

suppressive effects of proton beam irradiation against cell proliferation, clonogenic survival and cell migration in 4T1 murine breast cancer cells (Figs. 1 and 2). Based on the results, we assessed the effects of proton beam irradiation on breast tumor growth and lung metastasis in BALB/c mice implanted with 4T1 murine breast cancer cells. The growth of breast tumors irradiated by a proton beam was significantly repressed without body weight changes (Fig. 3A and B). Furthermore, fewer tumor nodules were found in lungs of mice irradiated with 30 Gy proton beam (Fig. 4A). Also, although not significant, the size of tumor nodules in mice irradiated with 30 Gy proton beam was smaller than others (Fig. 4B and C). However, 10 and 20 Gy proton beam could not prevent lung metastasis of breast cancer. The results correspond with those of *in vitro* cell proliferation and cell migration assays (Figs. 1 and 2).

Effects on metastatic gene expression in 4T1-implanted breast tumors. We determined the proton beam irradiation-mediated change in transcription of metastatic genes, such as COX-2, uPA, uPAR, MMP-9 and MMP-2, in breast tumors. As shown in Fig. 5A and B, COX-2, uPA and uPAR expressions were inhibited by 30 Gy proton beam irradiation. However, proton beams did not suppress MMP-2 and -9 transcription (Fig. 5C). Surprisingly, mRNA expression of COX-2, uPA, MMP-2 and MMP-9 genes were enhanced in breast tumor irradiated with 20 Gy proton beam (Fig. 5A-C), suggesting that optimized dose irradiation of a proton beam is important for improving cancer treatment and preventing metastasis. We also clarified the effect of proton beam irradiation on VEGF gene expression. Lower levels of VEGF mRNA were also observed in breast tumors irradiated with 30 Gy proton beam (Fig. 5D). The suppression of VEGF mRNA expression corresponded with the change of metastatic gene expression by proton beam irradiation.

Discussion

Radiation therapy is a treatment technique to kill cancer cells and prevent tumor growth using high energy beams, such as X-ray, gamma ray or proton beam. X-ray is the most common energy source for radiation therapy. However, this technique can induce various side effects, such as radionecrosis, blood dyscrasia, inappropriate hormone secretion and so on, as a result of normal tissues damage caused by the physical characteristics of X-ray. Proton beam therapy is a new high technology radiation therapy. A proton beam enters the body, releasing the highest energy when it reaches the target and then the energy quickly fades away. Because of these physical properties, proton beam irradiation is used to treat various cancers, including breast, bladder, head and neck, to minimize normal tissue damage. Global proton beam facilities to treat cancer are increasing (21).

We found in previous studies that metastatic potential in MCF-7 and MDA-MB-231 human breast cancer cells was diminished by proton beam irradiation and cell viabilities in both were also decreased (12,16,17,22). The results demonstrated that a proton beam should increase the efficacy of breast cancer treatment while inhibiting metastasis. However, previous studies did not elucidate the *in vivo* anti-metastatic potential of a proton beam. Here, we assessed the effects of proton beam irradiation on *in vivo* breast cancer metastasis by using the 4T1 orthotopic breast cancer model. 4T1 cells are known as an adequate cancer cell model to research human breast cancer because the cells can easily develop into a primary tumor in the mammary gland and spontaneously metastasize with a similar pattern as human breast cancer metastasis (23). Therefore, we used 4T1 cells to clarify the anti-metastatic effects of a proton beam. Prior to *in vivo* analysis, we found that proton beam irradiation prevented cell proliferation and clonogenic survival in an *in vitro* system with 4T1 murine breast cancer cells (Fig. 1). Also, cell migration of 4T1 murine breast cancer cells was suppressed by proton beam irradiation in the wound healing assay (Fig. 2). The results show that metastatic activity in 4T1 murine breast cancer cells should be prevented by proton beam irradiation. Therefore, the data support that 4T1 murine breast cancer cells are suitable
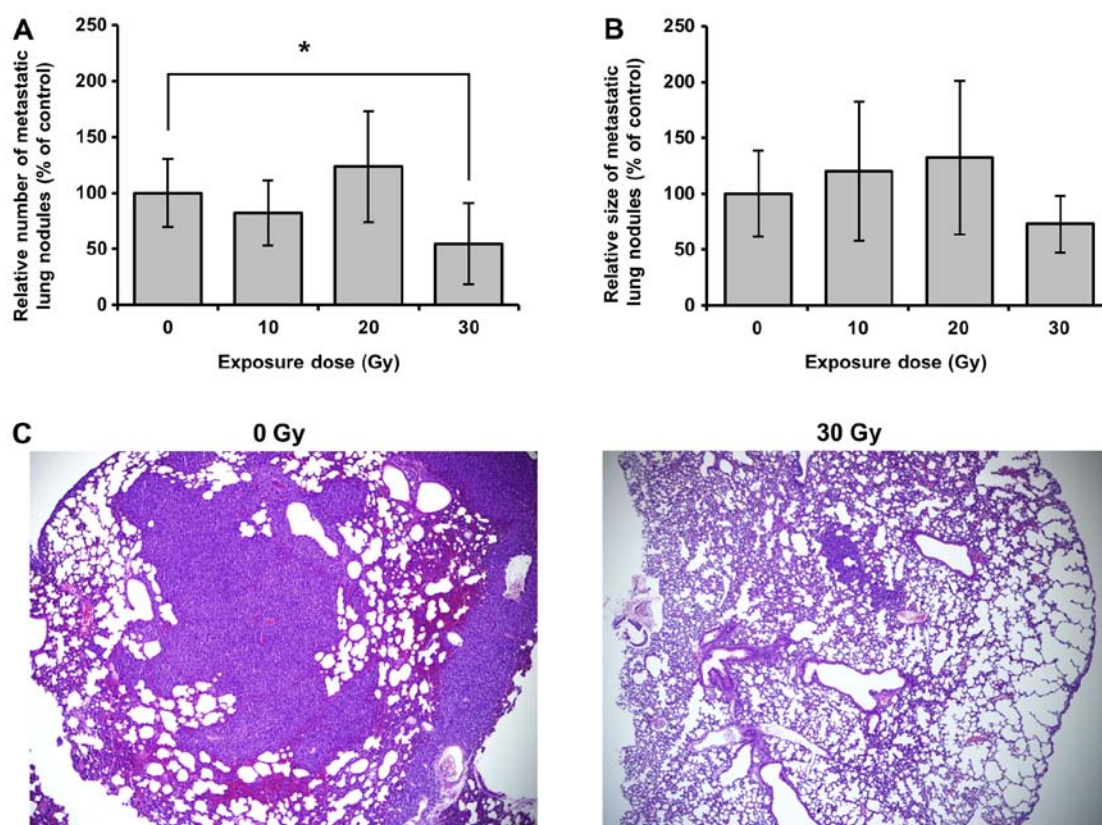
Figure 4. Lung metastasis was significantly suppressed by 30 Gy proton beam. The tumor nodules were microscopically observed. (A) Relative number of metastasized tumor nodules. The values are presented as relative percent compared to control (0 Gy). (B) Relative size of metastasized tumor nodules. The values are presented as relative percent against control (0 Gy). Significant difference was considered as P-values <0.05. *P<0.05. (C) Lung tissues in 4T1 orthotopic breast cancer model were observed by optical microscope. Tissues were stained with haematoxylin and eosin and pathologically analyzed in a blinded manner. 0 Gy indicates lung tissue in control mouse and 30 Gy indicates lung tissue in mouse irradiated with 30 Gy proton beam. Magnification, x40.
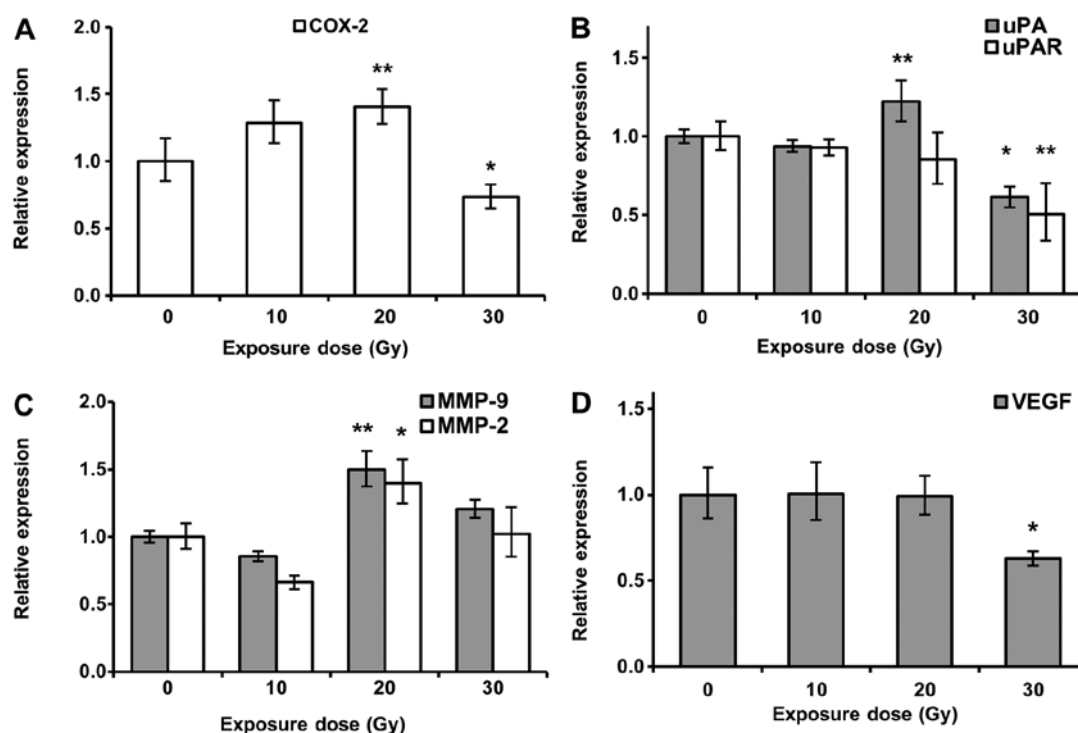

Figure 5. Metastasis-enhancing gene transcription was significantly repressed by 30 Gy proton beam in 4T1 orthotopic breast tumor. Relative expression levels of metastasis-enhancing genes and VEGF gene were determined by quantitative real-time PCR. Relative expression level of (A) COX-2, (B) uPA and uPAR, (C) MMP-9 and MMP-2 and (D) VEGF in 4T1 orthotopic breast tumors in BALB/c mice irradiated with 10, 20, or 30 Gy proton beam, respectively. Values are presented as means ± SD. * and ** indicate P-value <0.05 and P-value <0.01, respectively.

to investigate the *in vivo* anti-metastatic effects of a proton beam. Based on these results, we established the BLAB/c 4T1 orthotopic breast cancer model and then irradiated the breast cancers with 10, 20 and 30 Gy proton beams.

The 5-year survival rate in breast cancer patients is predominantly determined by whether or not the cancer metastasizes (24). Therefore, inhibition of metastatic potential in breast cancer is important to improve prognosis of patients. Wang *et al* in 2012 showed that endothelial-mesenchymal transition (EMT) in human epithelial cells was enhanced by proton beam irradiation of <2 Gy (25). Although not all cancer cells undergo EMT prior to metastasis, many cancers go through EMT for successful metastasis (26-28). Therefore, the data demonstrate that <2 Gy proton beam should enhance metastatic potential in cancer. Here, we found that breast tumor growth was significantly suppressed by proton beam irradiation without changes of body weight (Fig. 3), and 30 Gy proton beam significantly inhibited lung metastasis in the animal model (Fig. 4A and C). However, although 10 and 20 Gy proton beams critically delayed breast tumor growth, neither dose of proton beams prevented lung metastasis of breast cancer in the animal model (Figs. 3 and 4A and C). Consequently, both studies implicate the importance of appropriate exposure dose to improve therapeutic efficacy in proton beam therapy.

We further investigated the effects of proton beam irradiation on the expression of metastasis-regulating genes, such as COX-2, uPA and uPAR, in 4T1 orthotopic breast cancer model. The results showed lower levels of COX-2, uPA and uPAR transcription in breast cancer in mice irradiated with 30 Gy proton beam (Fig. 5A and B). Furthermore, 30 Gy proton beam downregulated VEGF mRNA expression (Fig. 5D). However, the expression of MMP-2 and -9 genes was not changed (Fig. 5C). The importance of these factors in metastasis has been well explained in previous studies. Enhanced COX-2 expression triggers metastasis with the induction of VEGF expression, and anti-metastatic agents inhibit COX-2 and MMPs expression (29-31). Other reports demonstrated that the uPA system facilitates cell invasion, adhesion and metastasis in breast cancer (32,33). Furthermore, Andres *et al* suggested that the uPA system should be a useful biomarker for predicting the prognosis of breast cancer patients (34). Based on these reports, metastatic potential is closely correlated with the expression level of these factors and should be suppressed by their downregulation. Therefore, the present results demonstrate that 30 Gy proton beam prevents lung metastasis of breast cancer through the suppression of COX-2, uPA, uPAR and VEGF in the 4T1 orthotopic breast cancer animal model.

In conclusion, we found that cell proliferation, viability and migration of 4T1 breast cancer cells were significantly decreased by proton beam irradiation. Also, *in vivo* research showed that tumor growths in 4T1 orthotopic breast cancer model were drastically delayed by proton beam irradiation. Furthermore, 30 Gy proton beam inhibited lung metastasis via suppression of COX-2, uPA, uPAR and VEGF gene expression. However, prevention of lung metastasis was not observed in mice irradiated with 10 and 20 Gy proton beams. Taken together, our data suggest that a proton beam is a useful tool in metastatic breast cancer treatment and demonstrate that selection of a suitable exposure dose is important to improve

therapeutic efficacy through the inhibition of metastasis to distant organs in breast cancer.

Acknowledgements

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