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Exercise affects high-fat diet-stimulated breast cancer metastasis through irisin secretion by altering cancer stem cell properties

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ABSTRACT

Background: Regular physical activities reduce the growth of breast cancer, but research on the effects of steady exercise on metastasis and its mechanisms is limited. In this study, the effects of steady exercise on breast cancer metastasis and its possible mechanism were demonstrated.
Methods: Experimental metastasis was induced after 8 weeks of steady exercise using a mouse model. Furthermore, one of the myokines, irisin, was studied to elucidate the effects of metastasis-regulating protein expression, and colony and sphere formation, which are cancer stem cell properties.
Results: Low- and moderate-intensity exercise significantly reduced the number and volume of metastasized tumors. Among myokines, only irisin was significantly increased by steady exercise but decreased by a high-fat diet. In vitro studies, irisin significantly decreased the number of colonies and sphere formation. Irisin also inhibited cell migration and invasion and suppressed the malignancy of breast cancer cells by reducing the expression of vimentin, MMP-2, MMP-9, and HIF-1 and by increasing the expression of TIMP-1 and TIMP-2.
Conclusion: Steady exercise modulates myokine secretions and among them, irisin suppresses breast cancer metastasis by decreasing self-renewal properties and invasion regulating protein expressions. Thus, regular exercise may be beneficial in the prevention of breast tumor metastasis.

1. Introduction

Breast cancer is the main cause of death in women and by far the most frequent cancer affecting women worldwide [1,2]. According to the latest data from the GLOBOCAN 2020 database, over two million new cases of breast cancer were diagnosed in 2020; globally, it is the second-most common cause of cancer deaths following lung cancer, and its standardized mortality rate is 6.9 (per 100,000) [3,4].

Studies on the prevention and treatment of breast cancer have focused on cancer stem cells (CSCs) as treatment targets because they affect the tumorigenesis and metastasis of breast cancer [5–7]. CSC exhibit self-renewal, tumorigenesis, invasion, and metastasis capabilities, and they additionally facilitate invasion and metastasis by regulating the process of epithelial-to-mesenchymal transition (EMT) through the activation of matrix metalloproteinases (MMPs) [8,9]. MMP proteins play crucial roles in tissue remodeling and organ development, but when their function or expression fails, they can contribute to all steps of tumor progression [9].

Contributing to various cancer outbreaks, a high-fat diet is one of the important factors that increase cancer mortality [10–12]. Consuming excessive fat increases the plasma level of inflammatory proteins, which promote the incidence and growth of tumors in the liver, pancreas, colon, prostate, and breast [12–14]. A high body mass index caused by excessive fat intake is associated with breast cancer incidence and poor prognosis by altering insulin resistance, estrogen levels, and levels of inflammatory cytokines and adipokines [15–22].

Along with dietary changes, the amount of physical activity should be increased to reduce body fat. Regular physical activities can reduce

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the incidence of various cancers [23–25]. They also enhance the effects of anti-cancer treatment on patients with cancer and may improve treatment outcomes [25,26]. An increase in blood flow rate through physical activity also reduces the survival rate of cancer cells and decreases metastasis [27,28]. Furthermore, proteins called myokines are implicated in cancer. They are produced by skeletal muscle cells during exercise, and they interact in the muscles, liver, adipose tissues, brain, and other organs [29]. An example of myokine is irisin, which induces heat generation by activating fat metabolism; it also reduces the survival rate of breast cancer cells, indicating that irisin and breast cancer are closely related [30–33]. Therefore, in this study, we investigated whether steady exercise modulates high-fat-stimulated breast cancer metastasis by altering myokine secretion through changing the characteristics of CSCs.

2. Materials and methods

2.1. Experimental animals and diets

The design of the experimental study design is shown in Fig. 1. Female BALB/c mice (Koatech Inc., Gyeonggi-do, Korea), aged five weeks, were separated by weight and randomly assigned to four groups (n = 9 per group): (i) AIN93G-fed normal control (Ctrl), (ii) a high-fat diet (HFC) control group, (iii) high-fat diet combined with low-intensity exercise (HFLE), and (iv) high-fat diet combined with moderate-intensity exercise (HFME). Exercise training began after a one-week adaptation period to the high-fat diet (Research Diets in New Brunswick, NJ, USA) and was followed by 11 weeks of training before and after injection of breast tumor cells through the tail vein to initiate breast cancer metastasis. The exercise groups underwent training sessions of either low-intensity (10 m/min) or moderate-intensity (15 m/min on a 2.5° incline) exercise for 1 h, five days per week. The mice were housed in well-maintained cages with controlled temperature and humidity of 22 ± 3 °C and 55% ± 5%, respectively, and followed a 12-h light/dark cycle. The metastasis experiment was terminated on week 11, and the Chung-Ang University Animal Ethics Committee approved the animal experiment (2016–00099).

2.2. Cell culture and reagents

The murine mammary cancer cell line 4T1 was provided by Prof. Seungmin Lee, while the human breast cancer cells (MDA-MB-231) were obtained from ATCC (Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640; WelGENE, Gyeongsangsi, Korea) medium supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 1% penicillin-streptomycin (100 mg/ml; Invitrogen, Carlsbad, CA, USA). They were maintained at 5% CO_2 at 37 °C.

2.3. Experimental metastasis

For experimental metastasis, breast cancer cells (4T1) were injected into the tail vein. An infrared lamp was used to dilate the caudal venous vessels. BALB/c mice were injected with 5×10^3 4T1 cells in RPMI media (WelGENE, Gyeongsan-si, Korea) to 100 μl final volume per mouse.

2.4. Measurement of lung metastasis

After 3 weeks of metastasis, exercise was conducted before sacrificing the experimental animals to measure myokines production. Isoflurane (Piramal Critical Care, Bethlehem, PA, USA) inhalation anesthesia was used to euthanize the mice. India black ink (15% India ink, 85% water, and three drops of NH_4OH/100 ml) was intratracheally injected to examine pulmonary metastasis. The lungs were removed, washed, and fixed in Fekete's solution for 16 h. The diameter of metastatic tumors was measured by counting white tumor nodules against a black lung background.

2.5. Blood sampling and plasma preparation

After the experimental animals were anesthetized, blood was collected, placed in a vacuum blood collection tube, mixed with heparin for 20 min, and centrifuged (1500 rpm × *g*, 4 °C) to obtain the plasma. Plasma samples were rapidly frozen in liquid nitrogen and stored at −80 °C until the time of analysis.

2.6. Myokine measurement

Plasma levels of the myokines oncostatin-M, secreted protein acidic and rich in cysteine (SPARC), irisin and interleukin-15 (IL-15) were measured by enzyme-linked immunosorbent assay (ELISA; LSBio, Shirley, MA, USA) according to the manufacturing protocol. Myokine concentrations were measured using SpectraMax M Series multimode microplate readers (Molecular Devices, San Jose, CA, USA) at 595 nm absorbance.

2.7. Cell viability assay

MDA-MB-231 cells were incubated with or without different

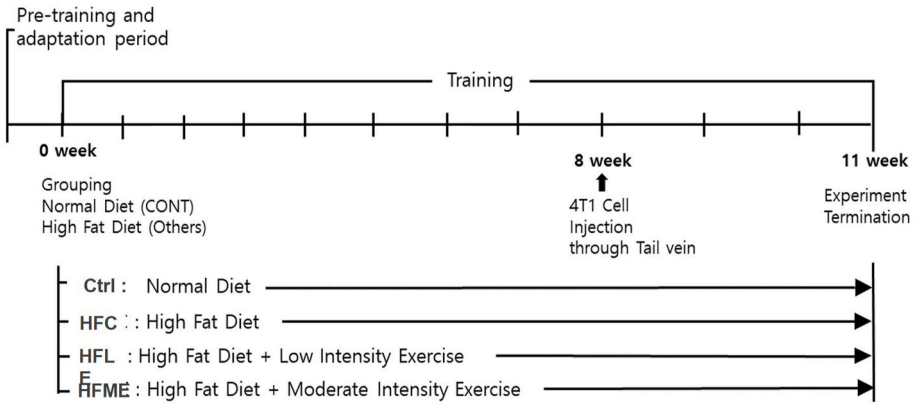

Fig. 1. Experimental study design
After one week of adaptation, eight-week-old Balb/c mice received either a control or a high-fat diet for 8 weeks, followed by tail vein injection of 4T1 mouse mammary tumor cells (5×10^3). The low-intensity and moderate-intensity exercise groups had 5 days of pre-training. After metastasis, HFLE and HFME groups trained 5 days per week until termination.

concentrations of irisin (Cayman Chemical, Ann Arbor, MI, USA) in a 96-well plate for 24 h. Afterward, cell viability was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MI, USA). Absorbance was read at 595 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Cell viability was set to 100% of the control group, and the absorbance of each treatment group was expressed as a relative percentage.

2.8. Migration assay

MDA-MB-231 cells were grown to confluency on 60 mm cell culture dishes, and the cell layer was scratched using a yellow tip. Cells were washed and incubated with or without different irisin concentrations for 24 h. The scratched areas were photographed under a microscope (Olympus, Tokyo, Japan) at 0 and 24 h. The distance was measured with Image J (National Institute of Health, Bethesda, MD, USA). Cell movement was calculated as the relative percentage of the change rate over time for each concentration by setting the value at 0 h for each concentration to 100%.

2.9. Invasion assay

Cell invasion was measured using Matrigel™-coated Transwell® chambers (Corning Inc., Corning, NY, USA). A medium containing 5% FBS or various irisin concentrations as separately added to the lower chamber. After 24 h of incubation, the medium in the chambers was removed. Then, the cells were fixed, washed, and stained (Sigma, St. Louis, MO, USA). The cells adhering to the upper surface of the membrane were removed using a cotton applicator, and the cells on the lower side of the membrane were counted.

2.10. Colony formation assay

MDA-MB-231 cells (1.5×10^2) were treated with 20 nM irisin for 10 days. During the culture, the medium was changed every 3 days. After 10 days, the colonies formed in the wells were visualized by staining with a staining solution (0.1% crystal violet). The number of colonies grown to a certain size was counted.

2.11. Sphere formation assay

MDA-MB-231 cells (1.5×10^2) were seeded in a poly-HEMA coated six-well plate and treated with the indicated irisin concentration. Dulbecco's modified Eagle's medium–high glucose (Sigma-Aldrich Korea, Yongin, Korea) with 30% BSA solution, human epidermal growth factor (PeproTech Korea, Seoul, Korea), fibroblast growth factor (PeproTech Korea), B-27 supplement (Gibco, Billings, MT, USA), Ham's F-12 nutrient mixture (Sigma-Aldrich Korea), and 2% penicillin was added. Irisin-treated plates were incubated with medium changed every 3 days. The MDA-MB-231 spheres that formed 10 days later were photographed under an inverted microscope at 50 × magnification and counted.

2.12. Western blot assay

MDA-MB-231 cells were treated with various irisin doses for 24 h. Proteins were extracted with a RIPA lysis buffer and quantified with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). The quantified protein was then loaded onto SDS-PAGE gel and subjected to electrophoresis. The protein loaded on the SDS-PAGE gel was transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA) in a cold room, blocked with 5% BSA or 5% skim milk, and reacted with primary antibodies detecting MMP-2, MMP-9, TIMP-1, TIMP-2 (Santa Cruz Biotechnology, Dallas, TX, USA), vimentin (Abcam, Cambridge, MA, USA), and HIF-1 (Cell Signaling Technology, Danver, MA, USA) at 4 °C overnight. The membrane was washed and reacted with secondary antibodies. After 30 min of washing, an enhanced chemiluminescence reagent (Animal Genetics Inc., Suwon, Korea) was used to detect bound antibodies.

2.13. Statistical analyses

Results were represented as mean ± standard error of the mean. Significance was determined via one-way ANOVA with the Newman–Keuls method as a post hoc test. Data with $p < 0.05$ were considered significant, and those labeled with alphabet letters on top of the bar of the obtained graph were significantly different ($p < 0.05$). All experiments were performed at least three times.

3. Results

3.1. Effect of steady exercise with different intensities on breast tumor metastasis

We first determined whether high-fat diet and exercise affected body weight before and after breast tumor metastasis (Table 1). There was no significant difference between the groups at the beginning of the experiment, before tumor injection, and at the end of the experiment. Next, we tested whether exercise intensity affected the number of lung metastases (Fig. 2B, Supplementary Fig. 1). The results showed that the number of metastases was significantly higher in the HFC group than in the Ctrl, HFLE, and HFME groups ($p < .05$; Fig. 2B, Supplementary Fig. 1). Furthermore, the volume of metastasized tumors in the HFC group was significantly greater than in the Ctrl, HFLE and HFME groups ($p < 0.01$; Fig. 2C). These results suggested that low- and moderate-intensity steady exercise suppressed the number and volume of breast cancer metastasis.

3.2. Effect of steady exercise with different intensities on plasma myokine levels

To determine the effect of exercise on myokines release, mice performed either low- or moderate-exercise for an hour and then sacrificed immediately. Plasma OSM had the highest secretion from HFME ($p < 0.05$), and was about 4.5 times more secreted than Ctrl, HFC, and HFLE (Fig. 2D). Plasma SPARC decreased in secretion from HFC compared to other groups, but there was no statistical difference among groups (Fig. 2E). Plasma IL-15 indicated a significant increase in the high-fat diet groups ($p < 0.05$), and secretion tended to increase as exercise intensity increased (Fig. 2F). Finally, plasma irisin was significantly decreased in the secretion of HFC compared to HFME (($p < .05$), and the secretion tended to increase depending on the intensity (Fig. 2G).

3.3. Effect of irisin on the viability and self-renewal ability of MDA-MB-231 cells

Since plasma irisin levels but not others were significantly decreased by the high-fat diet but restored by steady exercise, we investigated whether irisin modulated breast cancer metastasis. First, we determined whether irisin modulates the viability of breast tumor cells. The results showed that irisin treatment for 24 h slightly increased cell viability; however, the difference was less than 10% of the Ctrl group in all treated

Table 1
Effects of low- and moderate-intensity of exercise on body weight.

Body Weight (g)	Ctrl	HFC	HFLE	HFME
Initiation	18.75 ± 0.41	18.78 ± 0.31	18.71 ± 0.37	18.84 ± 0.16
Before tumor injection	20.13 ± 0.31	21.42 ± 0.76	19.62 ± 0.39	22.24 ± 0.49
Termination	20.46 ± 0.36	21.85 ± 0.68	20.21 ± 0.36	21.37 ± 0.93

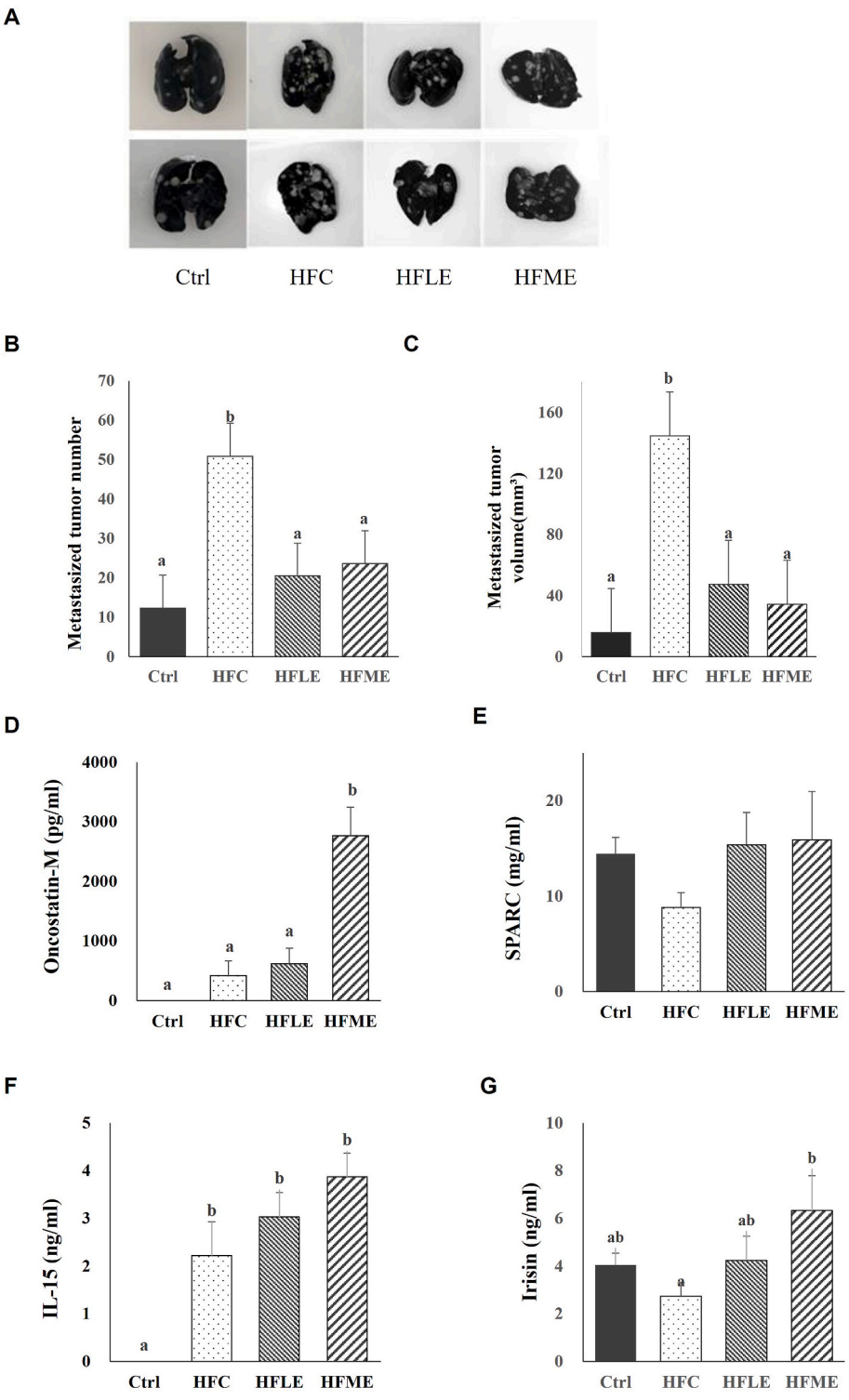

Fig. 2. Effects of low- and moderate-intensity exercise on cancer metastasis and myokine secretion
After 8 weeks of diet and exercise, tumor cells were metastasized. The lung tissues were stained with India black ink after 3 weeks of metastasis (A). The number (B) and volume (C) of metastasized tumors were measured from lung tissues. Before the termination of the experiment, the mice were subjected to low- or moderate-intensity exercises, and plasma was collected. Plasma levels of OSM (D), SPARC (E), IL-15 (F), and irisin (G) were measured using an ELISA kit. At least three replicates were performed for each experiment. Each bar represents the means ± SEM, and different letters on the bar indicate significant differences among groups ($p < 0.05$).

groups ($p < 0.05$; Fig. 3A).

We also determined whether irisin altered self-renewal ability. Fig. 3B shows the effects of 20 nM irisin treatment for 10 days on the colony formation of cells. The 20 nM treated group showed significantly decreased colonies compared to the Ctrl group ($p < 0.05$). Furthermore, 20 nM irisin significantly reduced the number and size of the spheres formed ($p < 0.05$; Fig. 3C).

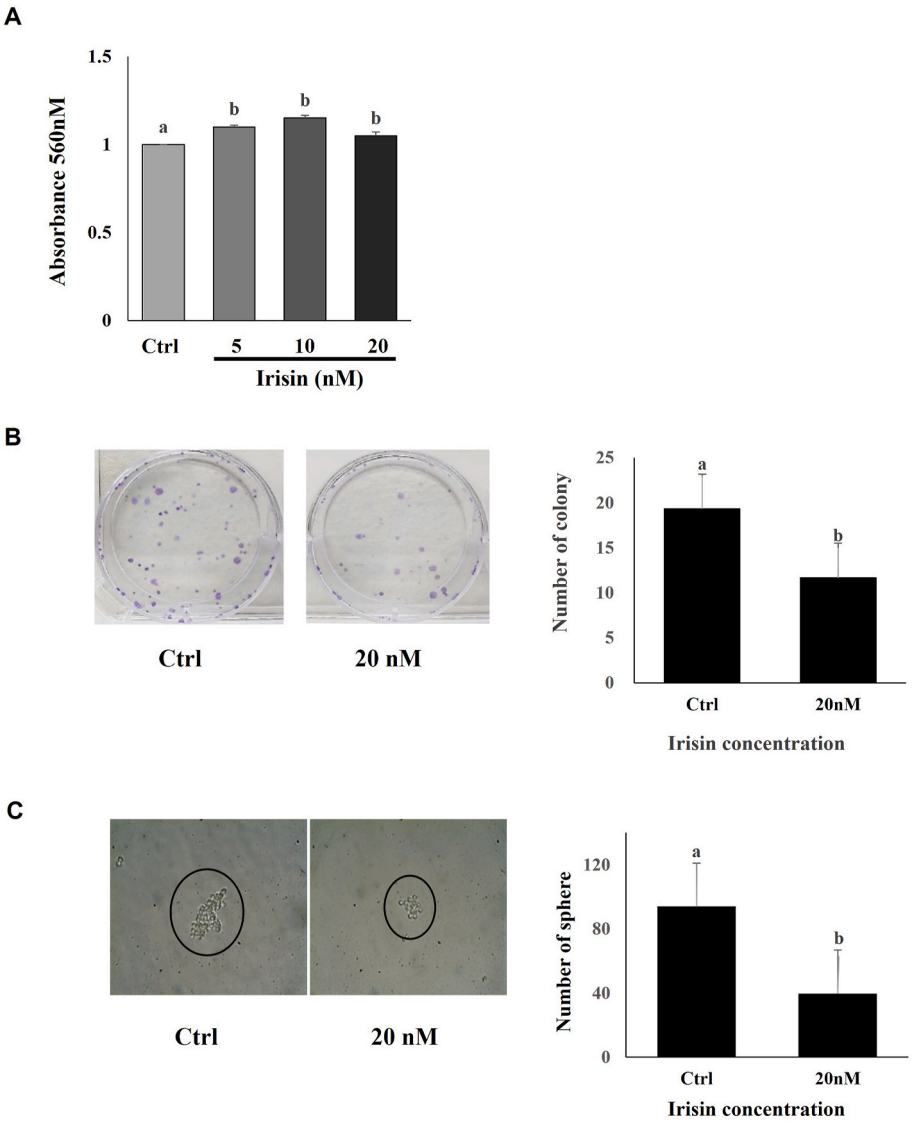

Fig. 3. Effects of irisin on MDA-MB-231 cell viability and self-renewal ability
MDA-MB-231 cells were incubated in media with or without different concentrations of irisin (5, 10, and/or 20 nM) for 24 h. Afterward, cell viability was measured by an MTT assay (A). MDA-MB-231 cells were treated with 20 nM of irisin, and colony formation (B), and sphere formation (C) were assessed. The photographs illustrate the size of colonies and spheres (magnification, 100×) in the irisin-treated and control (Ctrl) groups. The bar graph presents the number of colonies and spheres with or without irisin, and the values shown are the means ± SEM derived from three repeated tests.
*$p < 0.05$.

3.4. Effect of irisin on migration, and invasive potential of MDA-MB-231 cells

To determine the effect of irisin on breast cancer cell metastasis in vitro, cell migration and invasion assays were performed (Fig. 4A and B). As shown in Fig. 4A, the cells in the Ctrl group migrated farther than the cells treated with 20 nM irisin did ($p < 0.01$). Although 10 nM irisin had a trend toward slower cell movement than the Ctrl group, the difference was not significant. Furthermore, in MDA-MB-453 cells, compared to the Ctrl group, 10 nM and 20 nM irisin significantly reduced cell movement (Supplementary Fig. 2). Fig. 4B presents the results of the invasion assays on the effect of irisin on the direct invasive ability of breast cancer cells. Invasive cells in the irisin-treated groups were significantly fewer than those in the Ctrl group in a dose-dependent manner. ($p < 0.01$). In particular, the number of invasive cells in the 20 nM irisin group decreased by about 80% compared with that in the Ctrl group ($p < 0.001$).

3.5. Effect of irisin on EMT expressions

Since irisin modulated tumor migration and invasion, we determined whether irisin altered tumor invasion by changing EMT (Fig. 5). The expression of MMP-2 protein was significantly reduced in the groups treated with 10 nM and 20 nM of irisin as compared to the expression in the Ctrl group ($p < 0.001$ for 10 nM and $p < 0.01$ for 20 nM; Fig. 5A). The MMP-9 protein expression in the 20 nM irisin-treated group showed a significant decrease compared to the Ctrl group ($p < 0.01$). The vimentin expression was significantly downregulated in the irisin-treated groups compared with that in the Ctrl group ($p < 0.01$ for 5 and 10 nM irisin; $p < 0.001$ for 20 nM irisin; Fig. 5B). The TIMP-1 expression was significantly upregulated in the cells treated with 20 nM irisin as compared to the expression in the Ctrl cells ($p < 0.01$). In addition, the TIMP-2 expression significantly increased in the cells treated with 20 nM irisin, as detected in TIMP-1 ($p < 0.05$; Fig. 5C). Finally, the expression of the EMT-regulating protein, hypoxia-inducible protein-1 (HIF-1), was significantly downregulated in the irisin-treated

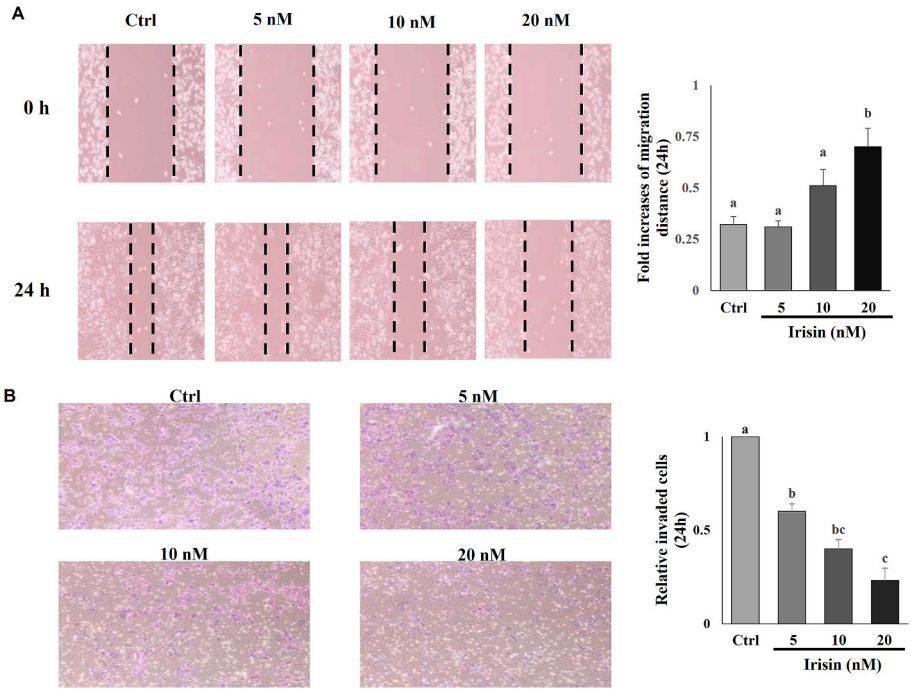

Fig. 4. Effects of irisin on MDA-MB-231 cell migration, and invasive ability
(A) MDA-MB-231 cells were grown to confluence on 60 mm cell culture dishes. The cell layer was scratched with a yellow tip, washed with PBS, and incubated in media with or without different concentrations of irisin (5, 10, and 20 nM). Images were taken at 24 h and compared with those captured at 0 h to measure wound healing. The black dotted lines indicate the boundaries of the wound at the beginning of the assay and were recorded 24 h after the scratch. Relative gap distance was calculated as the ratio of the remaining scratched gap at a given time compared to the original gap at 0 h. (B) The effects of irisin on MDA-MB-231 cell motility were assessed by Boyden chamber Transwell invasion assays after 24 h of treatment with irisin (5, 10, and 20 nM). The photographs are representative of cell invasion for each condition. The bar graph was set based on the control (Ctrl) group's invasive level to indicate each group's relative invasive level. Each experiment was performed at least three times. Each bar represents the means ± SEM, and different letters on the bar indicate significant differences among groups ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cells in a dose-dependent manner ($p < 0.05$; Fig. 5D). In addition, one of the angiogenic factors, vascular endothelial growth factor (VEGF), was significantly decreased in cells treated with 10 and 20 nM irisin ($p < 0.05$; Supplementary Fig. 3).

4. Discussion

With the increasing incidence of breast cancer due to the rising prevalence of obesity, the importance of physical activity in breast cancer prevention is being highlighted. Intensive physical activities can reduce cancer incidence and mortality; in addition, by improving a patient's ability to physically tolerate high doses of cancer treatment, regular physical activity may reduce cancer-related recurrence [26–28,33]. Regular physical activities reduce breast cancer tumorigenesis, and growth by decreasing weight, reducing inflammation, and normalizing the tumor microenvironment, potentially increasing systemic treatment delivery to cancer cells [27,28]. However, few studies have been conducted to determine whether regular exercise can modulate tumor metastasis and its mechanism. Therefore, in this study, we tested whether low- or moderate-intensity exercise could modulate breast tumor metastasis in the animal model. The results showed that the size and number of metastatic tumors in low- and moderate-intensity exercised mice were significantly reduced compared to those in HFC mice.

Several studies have investigated how exercise affects tumor inflammatory responses [26,28,32]. Myokines secreted by the skeletal muscle affect muscle and bone mass, glucose and lipid metabolism, and inflammation, contributing to the pathogenesis of obesity, diabetes, cancer, and other chronic diseases [30,34,35]. Recent studies have also revealed that myokines reduce the plasma level of inflammatory cytokines and growth factors affecting tumor formation after exercise [28,32]. The link between myokines and various cancer types has been investigated, but specific mechanisms have not been identified because the effects of exercise vary depending on cancer type and stage; furthermore, studies have yet to clarify whether such effects are caused by myokines acting on tumors [33,35,36].

Irisin is released from skeletal muscle after exercise and is a proven modulator of thermogenesis and body weight [31,32]. Recent studies have shown reduced plasma irisin levels in patients with type II diabetes, chronic kidney disease, and breast cancer [32,33]. In addition, irisin suppresses EMT of osteosarcoma, lung cancer, and gastric cancer cells, thereby inhibiting proliferation and invasion; high irisin concentrations may reduce tumor viability due to hyperthermia by lipid metabolism [37–39]. However, another study reported that irisin did not affect endometrial, colon, thyroid, and esophageal cancer cells, so irisin differentially affected cancer cell proliferation and invasion. In this study, we showed that plasma irisin secretion was decreased by high-fat diet, but restored by continuous exercise. Therefore, we tested whether irisin could modulate breast tumor metastasis.

CSCs, a small fraction of cells in solid tumors, are responsible for drug resistance, tumor recurrence, and metastasis [5–7]. Therefore, studies have been conducted to target CSCs in tumors to prevent tumorigenesis, tumor recurrence, and metastasis. In this study, we showed that irisin significantly suppressed self-renewal characteristics by reducing colony and sphere formation. Irisin also reduced the expression of vimentin in a dose-dependent manner. Furthermore, we showed that irisin decreased the migration and invasion of breast cancer cells without affecting cell viability, downregulated the expression levels of MMP-2 and MMP-9, and upregulated the expression levels of TIMP-1 and TIMP-2 in a dose-dependent manner. Irisin also decreased the expression levels of HIF-1, which regulated angiogenesis, cell proliferation, CSC specification, invasion, and metastasis [40,41]. Thus, irisin might show an anti-metastatic potential by regulating these

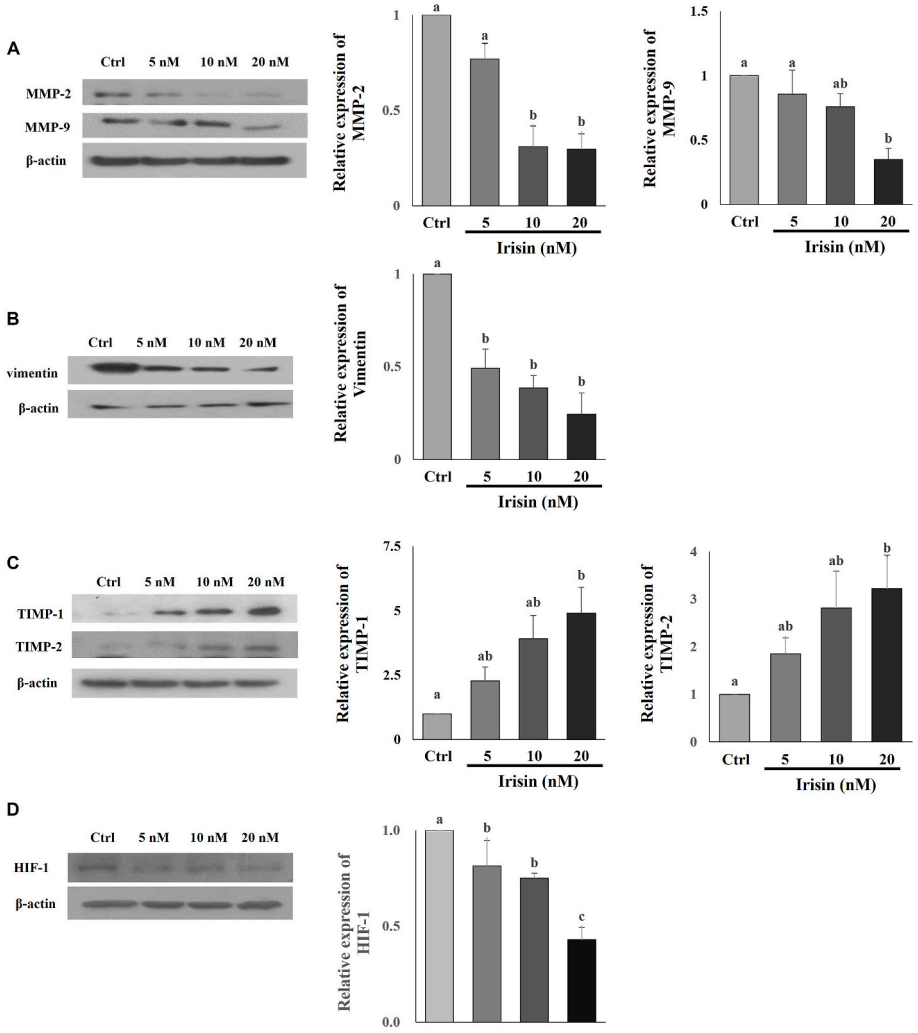

Fig. 5. Effects of irisin on the expression of MMP-2, MMP-9, vimentin, TIMP-1, TIMP-2, and HIF-1
MDA-MB-231 cells were incubated in media with or without various concentrations of irisin (5, 10, and 20 nM) for 24 h. Afterward, cell lysates were analyzed via western blotting with the indicated antibodies such as MMP-2 and MMP-9 (A), vimentin (B), TIMP-1 and TIMP-2 (C), and HIF-1 (D). Photographed chemiluminescence blots are shown. The relative abundance of each band with respect to its own β-actin was quantified, and the level of the control (Ctrl) was set to 1. Different letters on the bar indicate significant differences among groups ($p < 0.05$), and the values shown are the means ± SEM derived from three repeated tests. Each bar represents the means ± SEM, and different letters on the bar indicate significant differences among groups ($p < 0.05$).

metastasis-related proteins and exhibiting self-renewal properties.

In conclusion, the present study demonstrated that continuous exercise reduced high-fat diet-induced breast tumor metastasis and restored irisin secretion compared with the high-fat diet. Irisin suppressed CSC characteristics such as self-renewal, migration, invasion, and EMT expression. The results provide the potential mechanism of steady exercise on breast tumor metastasis.

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Ethics and dissemination

This study does not involve individual patients and does not raise any ethical issues.

CRediT authorship contribution statement

YuJin Lee: Data curation, Formal analysis, Investigation. **SoDam Park:** Data curation, Formal analysis, Investigation. **SeungHwa Park:** Data curation, Investigation. **Hye Ji Kwon:** Data curation. **Sang-Ho Lee:** Supervision, Writing – review & editing. **Yuri Kim:** Conceptualization, Supervision, Writing – review & editing. **Jung-Hyun Kim:** Investigation, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101684.

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