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A combination of myokines and genistein suppresses cancer stemness in MCF-7 human breast cancer cells

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ABSTRACT

BACKGROUND/OBJECTIVES: Breast cancer is considered a serious health issue worldwide and is influenced by risk factors, including physical inactivity and unhealthy diet. Myokines secreted by muscles during physical activity play a crucial role in cancer development and the immune system. Genistein (Gen), an isoflavone primarily in legumes, induces anti-cancer activity by regulating cancer stem cells (CSCs). Therefore, this study investigated the potential anti-cancer effect of a combination of myokine and Gen on the human breast cancer MCF-7 cells.
MATERIALS/METHODS: MCF-7, a human breast cancer cell line, was used for *in vitro* study. The cell viability of MCF-7 cells was evaluated in response to treatment with myokines, irisin (Iri), oncostatin M (OSM), and Gen using the MTT assay. Clonogenic and sphere formation assays were used to evaluate the self-renewal capacity of breast CSCs. The mRNA expression levels of stem cell markers were analyzed in MCF-7 breast cancer cells.
RESULTS: Administering Iri or OSM with Gen significantly inhibited the self-renewal capacity of MCF-7 cells. In addition, mRNA expression of breast CSC markers *SOX2* and *OCT4*, which are characteristic of CSCs, was suppressed by both myokine and Gen. However, combining Iri or OSM with Gen was the most effective treatment.
CONCLUSION: These results suggested that combining Iri or OSM with Gen has an additive effect on breast CSCs by regulating self-renewal capacity and expression of CSCs markers. Therefore, the combination of myokines and Gen may have the therapeutic potential for treating breast cancer and improving the quality of life of cancer patients.

Keywords: Breast neoplasms; cancer stem cells; genistein; myokines

INTRODUCTION

Breast cancer in females is a major global health problem globally. In 2023, breast cancer was the most commonly diagnosed cancer and exerted the highest mortality rate among women worldwide [1]. Breast cancer incidence varies according to socioeconomic development with higher rates in developed countries [2]. These trends imply that changes in lifestyle during economic development can increase breast cancer risks. A high-fat diet and an inactive lifestyle contribute to obesity by increasing body fat [3], which can promote the initiation and growth of breast cancer [4].

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Conflict of interest

The authors declare no potential conflict of interests.

Author Contributions

Conceptualization: Kim JH; Funding acquisition: Kim JH; Investigation: Kwon H, Kim Y, Kim JH; Methodology: Kwon H, Kim Y, Kim JH; Supervision: Kim JH, Kim Y; Visualization: Kwon H; Writing - original draft: Kwon H; Writing - review & editing: Kim JH, Kim Y.

Cancer stem cells (CSCs) regulate cancer aggressiveness and metastasis [5]. CSCs are a small portion of tumor cell populations and possess characteristics similar to normal stem cells, with self-renewal potential and differentiation [6]. However, unlike normal stem cells, CSCs initiate and promote tumor development and metastasis [5]. They are resistant to radiation therapy and chemotherapy [7]. Thus, developing alternative anti-cancer therapies targeting CSCs is crucial to enhancing patient outcomes and quality of life.

Regular physical exercise has been demonstrated to be effective in preventing cancer [8]. It has been shown that physical activity can prevent the onset or metastasis of cancer by regulating numerous biological pathways, including systemic inflammation, DNA repair capacity, and metabolic hormones [9]. During regular physical activity, muscles secrete various cytokines as they contract and relax [10]. Myokines, which are cytokines from muscles, play a role in cancer development and the regulation of the immune system [11,12]. However, the specific molecular mechanisms through which myokines and physical activity prevent cancer are not fully understood. Irisin (Iri) or oncostatin M (OSM) has been shown to inhibit the development of breast cancer. Iri, a type of myokine produced by fibronectin type III domain-containing protein 5 in various malignant tissues, is released from muscles in response to physical activity [13]. OSM, a type of interleukin-6 cytokines, acts in autoimmunity, inflammation, and cancers [14]. A recent study has confirmed that OSM suppresses proliferation and augments cell detachment in breast cancer cells [15,16]. And OSM has been shown to suppress the self-renewal capacity of breast cancer cells by downregulating the stem cell markers, such as BMI1 and POUSF1 [17].

The intake of dietary and functional foods has been associated with suppressing breast cancer. Genistein (Gen; 4', 5, 7-trihydroxyisoflavone) is one of the most examined isoflavones, which is rich in soy-derived foods [18]. It has been reported that 17β-estradiol (E2) and Gen are structurally and functionally similar [19]. Therefore, it is expected that Gen supplementation might provide anti-estrogenic effects by blocking the binding of estrogen receptors, which in turn exerts anti-cancer benefits [19]. Several studies have demonstrated that Gen inhibited tumor growth, promoted cell differentiation, and reduced the invasiveness of cancer cells through controlling the activity of tyrosine kinase, inflammation, and angiogenesis [20-22]. In addition, Gen induced apoptosis by regulating Bcl-2 and Bax [18].

Previous *in vivo* studies have reported that the combination of exercise and daidzein, the phytoestrogen, has a synergistic effect on suppressing breast cancer when compared to single treatments by modulating signaling pathways such as tumor growth, natural killer cells, and apoptosis [23]. As such, combining the two therapies has been reported to be more effective in preventing and treating cancer, as well as improving the quality of life of cancer patients. However, the effects of the combination of physical exercise and Gen on CSCs and the underlying mechanisms have not been extensively studied. Therefore, the present study investigated the anti-CSC effects of the combination of myokine and Gen in human breast cancer *in vitro* using MCF-7 cells.

MATERIALS AND METHODS

Cell culture and reagents

The MCF-7 human breast cancer cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in Minimum Essential Medium (Welgene, Daegu,

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Korea). The medium was supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin–streptomycin (100 U/mL and 100 μg/mL) (Invitrogen, Carlsbad, CA, USA). The breast cancer cells were cultured at 37°C in humidified 5% CO_2 conditions. Gen was purchased from BOC Sciences (Upton, NY, USA) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). Iri was purchased from Cayman Chemical (Ann Arbor, MI, USA), and OSM was purchased from Sino Biological (Beijing, China) and dissolved in distilled water.

Cell viability assay

MTT assay was performed to assess the proliferative capacity of the cells. To examine the impact of Gen, Iri, and OSM on viability, cells were seeded at a density of 1.4×10^4 cells per well in 96-well plates. After 24 h, cells were treated with various doses of Gen (0, 100, 125, 150, 175, and 200 μM), Iri (0, 10, 20, 40, and 80 nM), OSM (0, 5, 10, 20, and 40 ng/mL). After 72 h, the medium was extracted, and then 200 μL MTT solution (0.5 mg/mL, Sigma-Aldrich) was added to each well. Next, the mixture was then incubated at 37°C for 3 h. Then, after the supernatant was removed, the formazan crystals were dissolved in each well by adding 100 μL of DMSO solution (Sigma-Aldrich). An analysis was conducted at 570 nm using a microplate reader (Molecular Device, Sunnyvale, CA, USA) to determine the absorbance values of each well.

Clonogenic assay

MCF-7 cells (4×10^3 cells/well) were seeded into each well of a 6-well plate. After 24 h, cells were treated with DMSO alone, Gen (117.5 μM), or myokines (Iri [20 nM], OSM [10 ng/mL]). After 10–14 days, the colonies were fixed with a 0.9% sodium chloride solution and stained with crystal violet (Sigma-Aldrich). The number of stained colonies was counted using the following formula: (colony number/seeded cell number) × 100% [24].

Sphere formation assay

MCF-7 cells (1×10^4 cells/well) were seeded in 6-well plates and covered with a 10% stock solution of poly-(2-hydroxyethyl methacrylate) (Sigma-Aldrich). The composition of the sphere medium was DMEM–F12 (1:1, Welgene) supplemented with 2% B27 (Invitrogen), 20 ng/mL epidermal growth factor (Pepro Tech, London, UK), and 40 ng/mL basic fibroblast growth factor 2 (bFGF; Pepro Tech). After 24 h, cells were treated with DMSO alone, Gen (117.5 μM), or myokines (Iri [20 nM], OSM [10 ng/mL]). Phase contrast microscopy (Nikon, Tokyo, Japan) was used to count and take pictures of the number of spheres after treating with Gen and myokines for 10–14 days.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (PCR)

Following the manufacturer's instructions, TRIzol reagent (Invitrogen) was used to extract total RNA from cells. Complementary DNA (cDNA) was synthesized using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). Rotor-Gene SYBR Green PCR (Qiagen, Hilden, Germany) was used for quantitative real-time PCR. The amplification conditions were as follows: 95°C for 5 min, 95°C for 5 s, and 60°C for 10 s. The mRNA expression levels of the target gene were normalized to glyceraldehyde 3-phosphate dehydrogenase. The primer sequences of the primers are listed in **Table 1**.

Statistical analysis

The presented data are described as mean ± SE of the mean. Data were analyzed by one-way analysis of variance and the Newman-Keuls test using GraphPad Prism software (GraphPad

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Table 1. List of primer sequences

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
SOX2	CAAGATGCACAACTCGGAGA	GCTTAGCCTCGTCGATGAAC
OCT4	GTGAGAGGCAACCTGGAGAA	GAACCACACTCGGACCACAT
GAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC

SOX2, sex-determining region Y-box 2; *OCT4*, octamer-binding transcription factor 4; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

Software, Inc., San Diego, CA, USA). A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Effects of myokines, Gen, or their combination on MCF-7 cell viability

MTT assays were conducted to assess the possible anti-cancer effects of myokines, Gen, or their combination on cell viability. MCF-7 cells were treated with different concentrations of Iri (0, 10, 20, 40, and 80 nM), OSM (0, 5, 10, 20, and 40 ng/mL), and Gen (0, 100, 125, 150, 175, and 200 μM) for 72 h. Cell viability was reduced in a dose-dependent manner by both Iri and Gen (**Fig. 1A and C**). However, OSM had no significant effect on the viability of MCF-7 cells (**Fig. 1B**). Based on these data, the treatment dose for the subsequent *in vitro* experiments was determined using the median value for the Iri and OSM and the IC_{50} value for Gen (**Fig. 1D**). The IC_{50} value for Gen was 117.5 μM.

Effects of myokines, Gen, or their combination on colony formation ability of MCF-7 cells

A colony formation assay was performed to determine the self-renewal capacity of CSCs *in vitro*. MCF-7 cells were treated with myokines (Iri and OSM) and Gen alone or in combination to investigate the effect of myokines and Gen on suppressing colony formation. The number of colonies was decreased by each myokine and Gen treatment (**Fig. 2A and B**). Furthermore, colony formation was suppressed by the combination of Iri and Gen (61.1%, $P < 0.001$) and the combination of OSM and Gen (66.5%, $P < 0.001$), compared with the control (Ctrl) group ($P < 0.001$). In both cases, the combination treatment exhibited a larger reduction in colony formation than each individual treatment.

Effects of myokines, Gen, or their combination on sphere formation by MCF-7 cells

The Sphere formation assay is another experiment that analyzes the self-renewal capacity of CSCs *in vitro*. MCF-7 cells were treated with myokines (Iri and OSM) and Gen alone or in combination to examine the effect of myokines and Gen on the suppression of cancer spheroid formation. Each myokine and Gen treatment decreased the size and the number of spheres (**Fig. 3A and B**). When compared with the Ctrl group, the combination of Iri and Gen suppressed sphere formation by 55.0% ($P < 0.001$), and the OSM and Gen combined treatment group suppressed by 56.3% compared with the Ctrl group ($P < 0.001$). In both cases, the combination treatment exhibited the highest reduction in sphere formation among the groups.

Effects of myokines, Gen, or their combination on the mRNA expression of CSC markers in MCF-7 cells

The expression levels of the CSCs markers (*SOX2* and *OCT4*) were evaluated using quantitative real-time PCR. The mRNA expression of *SOX2* and *OCT4* were downregulated by treatment

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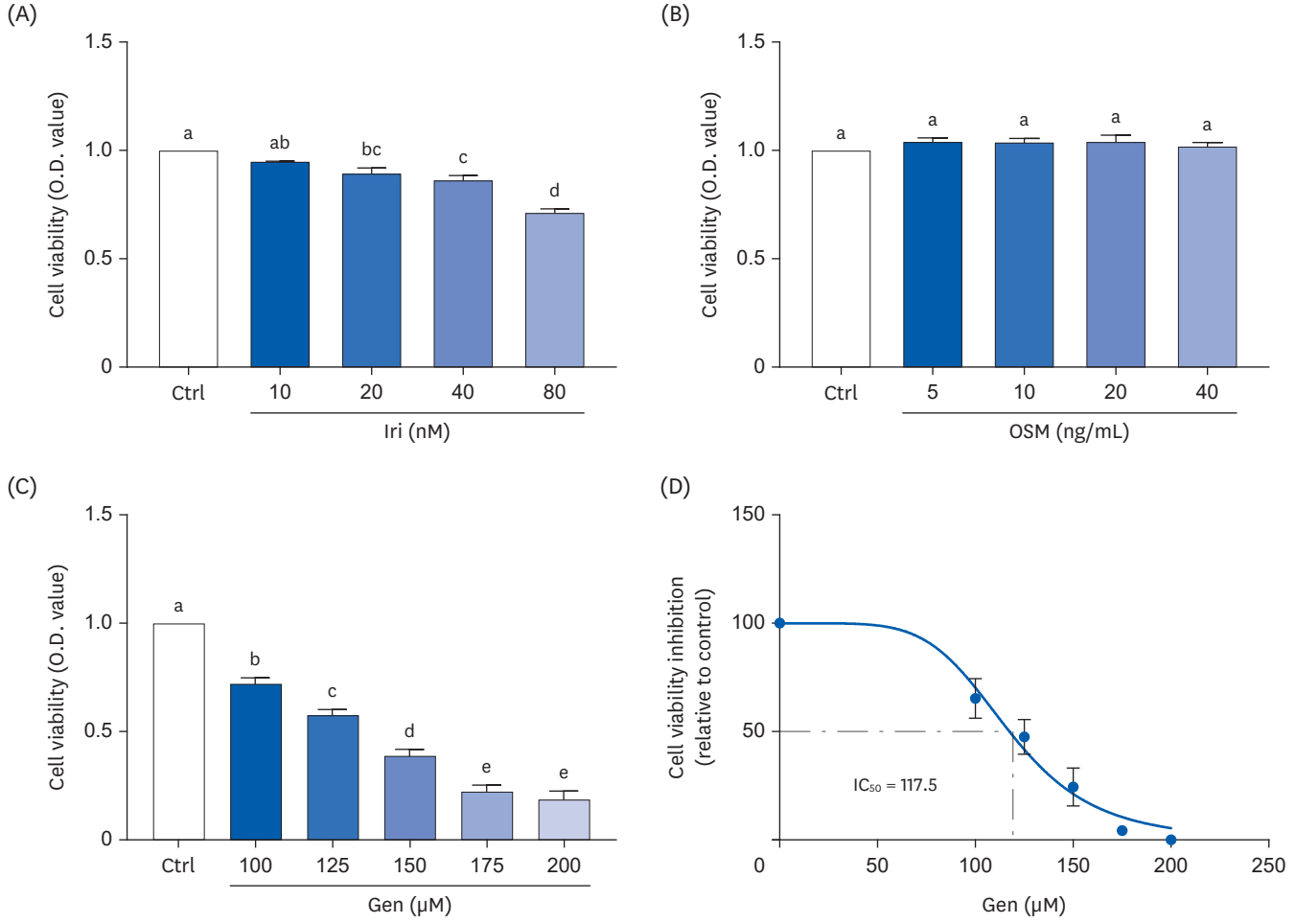

Fig. 1. Effects of myokines, Gen, or their combination on MCF-7 cell viability. MCF-7 cells were applied with various concentrations of Iri (0, 10, 20, 40, and 80 nM), OSM (0, 5, 10, 20, and 40 ng/mL), and Gen (0, 100, 125, 150, 175, and 200 μM) for 72 h. (A) Iri, (B) OSM, (C) Gen, and (D) IC_{50} values of Gen in MCF-7 cells are shown. All data are represented as mean ± SE of the mean from at least 3 independent experiments (n = 3) and analyzed by one-way analysis of variance with Newman-Keuls *post hoc* test. Different alphabet letters indicate significant differences ($P < 0.05$).
O.D., optical density; Ctrl, control; Iri, irisin; OSM, oncostatin M; Gen, genistein; IC_{50}, half maximal inhibitory concentration.

with myokines (Iri and OSM), Gen, or their combination in the MCF-7 cell line (**Fig. 4A and B**). Compared with the Ctrl group, the combination treatment group showed the largest reduction among the groups. The co-administration of Iri and Gen led to a decrease in *SOX2* (66.2%; $P < 0.001$) and *OCT4* (57.4%; $P < 0.001$) the mRNA expression, respectively. The mRNA expression of *SOX2* and *OCT4* was reduced by 56.46% ($P < 0.01$) and 46.7% ($P < 0.001$), respectively, when OSM and Gen were administered together. These results showed that the myokines (Iri and OSM), Gen, or their combination, have anti-cancer stemness effects on MCF-7 breast cancer cells. In addition, combining myokines and Gen was more effective than each treatment alone.

DISCUSSION

The present study aimed to investigate whether myokines and Gen or their combination have anti-cancer effects by regulating cancer stemness in MCF-7 breast cancer cells. Our results presented that a combination treatment of myokines with Gen suppressed the self-renewal properties of CSCs and downregulated the expression of SOX2 and OCT4 in MCF-7 breast

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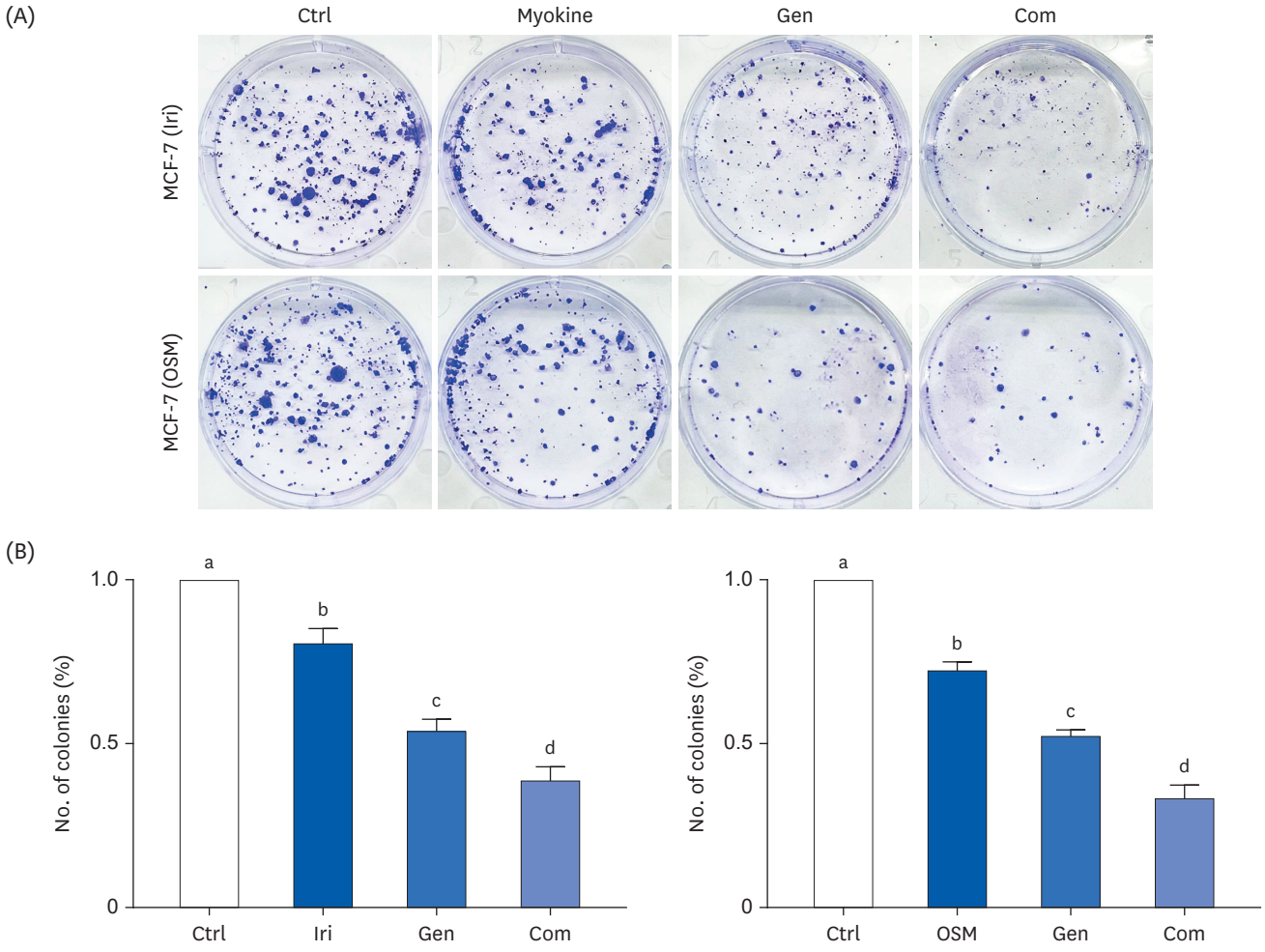

Fig. 2. Effects of myokines, Gen, or their combination on colony formation ability of MCF-7 cells. (A) MCF-7 cells were seeded into 6-well plates and treated with Iri, OSM, and Gen. After 10–14 days, colonies were stained and photographed. (B) The number of colonies was counted. All data are represented as mean ± SE of the mean from at least 3 independent experiments (n = 3) and analyzed by one-way analysis of variance with Newman-Keuls *post hoc* test. Different alphabet letters indicate significant differences ($P < 0.05$). Data are presented as below: MCF-7 (Iri): Ctrl, control; Iri, 20 nM Iri; OSM, 10 ng/mL OSM; Gen, 117.5 μM Gen; Com, 20 nM Iri + 117.5 μM Gen. MCF-7 (Osm): Ctrl, control; OSM, 10 ng/mL OSM; Gen, 117.5 μM Gen; Com, 10 ng/mL OSM + 117.5 μM Gen.
Iri, irisin; Ctrl, control; Gen, genistein; Com, combination; OSM, oncostatin M.

cancer cells. Therefore, myokines and Gen could be a novel therapeutic strategy for targeting specific CSCs in breast cancer.

CSCs play a crucial role in different stages of cancer development, such as tumor initiation, metastasis, and resistance to chemotherapy and radiotherapy, ultimately leading to cancer recurrence [6,7], which is closely related to the ability of CSCs to self-renew. The ability of CSCs to generate colonies and spheroids from single cells is known to offer a suitable assay for evaluating their self-renewal ability [25]. The present study observed that MCF-7 cells formed colonies and spheroids without myokines and Gen. However, treatment with Gen Iri, or OSM significantly inhibited the formation of the colonies and spheroids. The mRNA expression levels of stem cell markers, SOX2 and OCT4 were also significantly reduced in the groups treated with myokines and Gen, both individually and in combination.

A previous study demonstrated that Gen treatment suppressed CD44+CD24- MCF-7 CSCs and inhibited colony and sphere formations compared to the control group by downregulating

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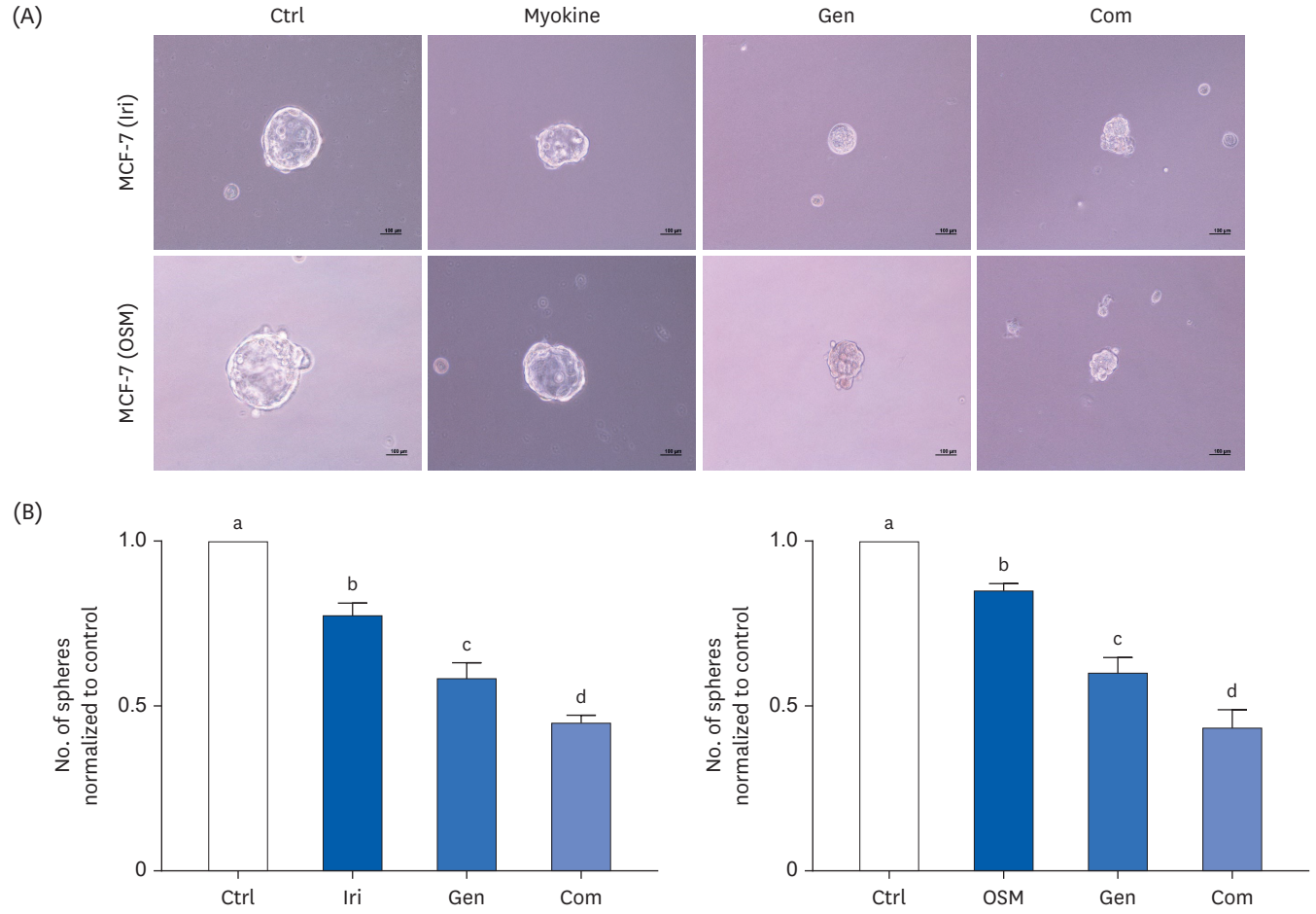

Fig. 3. Effects of myokines, Gen, or their combination on sphere formation by MCF-7 cells. MCF-7 cells were cultured in poly-HEMA coated 6-well plates and treated with Iri, OSM, Gen, or their combination. Spheres were formed and maintained in sphere media for 10–14 days. (A) Spheres were photographed using phase contrast microscopy (magnification, 100 ×). (B) Several spheres were counted. All data are represented as mean ± SE of the mean from at least 3 independent experiments (n = 3) and analyzed by one-way analysis of variance with Newman-Keuls *post hoc* test. Different alphabet letters indicate significant difference ($P < 0.05$).
Data are presented as below: MCF-7 (Iri): Ctrl, control; Iri, 20 nM Iri; OSM, 10 ng/mL OSM; Gen, 117.5 μM Gen; Com, 20 nM Iri + 117.5 μM Gen. MCF-7 (Osm): Ctrl, control; OSM, 10 ng/mL OSM; Gen, 117.5 μM Gen; Com, 10 ng/mL OSM + 117.5 μM Gen.
Iri, irisin; Ctrl, control; Gen, genistein; Com, combination; OSM, oncostatin M.

the Hedgehog–Gli1 signaling pathway [26]. In addition, Iri inhibited the invasion of CSCs by mediating the PI3K/AKT/Snail pathway and suppressed epithelial to mesenchymal transition in lung cancer cells [27]. The present study provided evidence of the inhibitory effects of Gen and myokines on breast CSCs by regulating colony and sphere formation. In addition, these results showed that the combination treatment was more effective than each individual treatment.

The study found that OSM did not affect cell viability, as measured by the MTT assay. However, it significantly inhibited colony and sphere formation and the expression of *SOX2* and *OCT4* mRNA, which are recognized as CSC markers. Previous studies have indicated that OSM decreased stem cell markers, such as BMI1 and POU5F1, which are known to be required for self-renewal, in EpCAM HuH1 and HuH7 liver cancer cells [28]. Furthermore, OSM was confirmed to inhibit the growth of breast cancer cells by regulating the activation of the transcription factors STAT1 and STAT3 and the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway [29]. This suggests that OSM does not have a toxic effect on the cells themselves but instead inhibits self-renewal and growth, which are characteristics of the CSCs.

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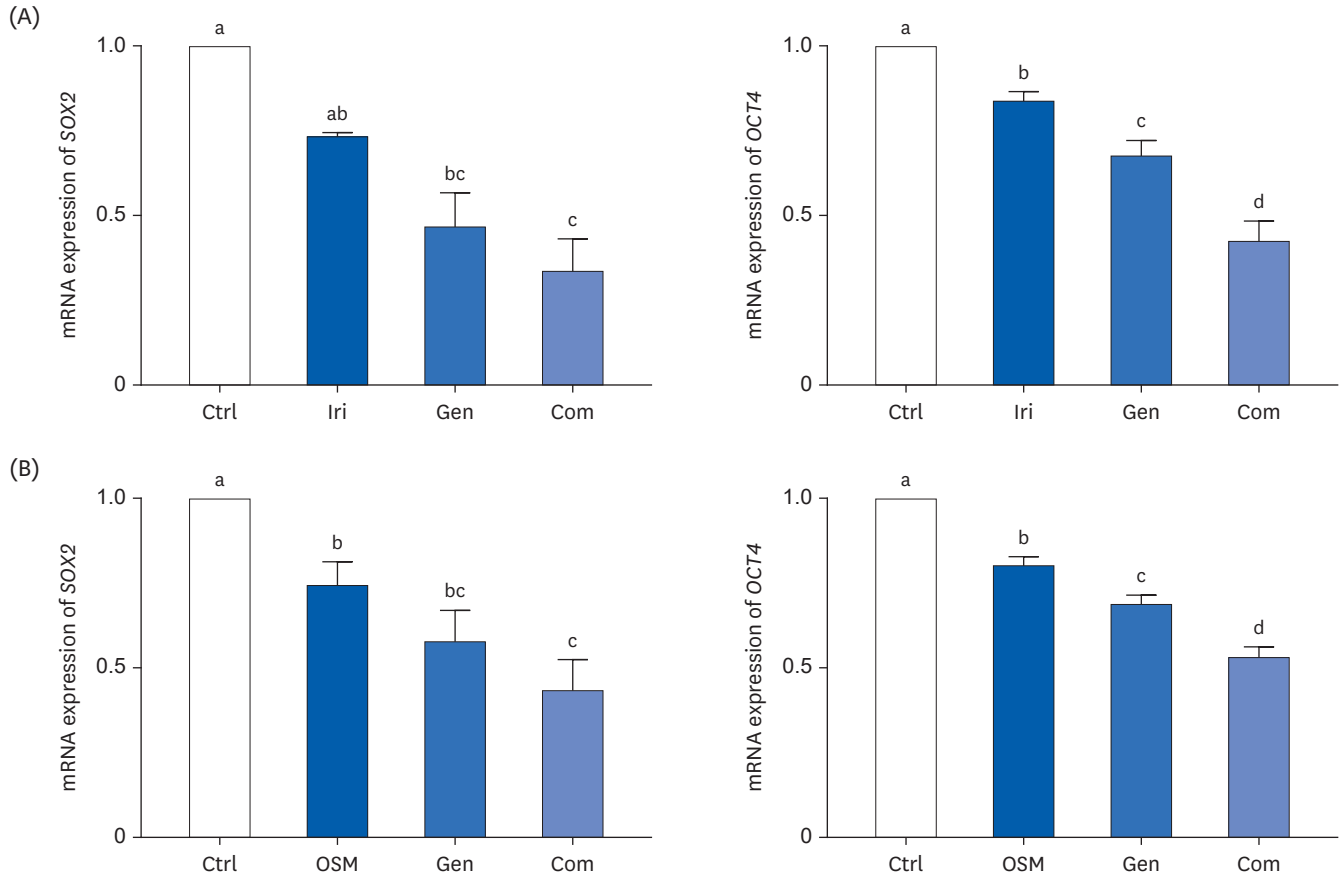

Fig. 4. Effects of myokines, Gen, or their combination on the mRNA expression of CSC markers in MCF-7 cells. MCF-7 cells were treated with myokines (Iri and OSM), Gen, or their combination for 72 h. (A) After treatment of MCF-7 cell lines with (A) Iri and Gen and (B) OSM and Gen, the mRNA expression of *SOX2* and *OCT4* are presented by quantitative real-time polymerase chain reaction. *GAPDH* was used as a loading control. All data are represented as mean ± SE of the mean from at least 3 independent experiments (n = 3) and analyzed by 1-way analysis of variance with Newman-Keuls *post hoc* test. Different alphabet letters indicate significant difference ($P < 0.05$).
Data are presented as below: MCF-7 (Iri): Ctrl, control; Iri, 20 nM Iri; OSM, 10 ng/mL OSM; Gen, 117.5 μM Gen; Com, 20 nM Iri + 117.5 μM Gen. MCF-7 (Osm): Ctrl, control; OSM, 10 ng/mL OSM; Gen, 117.5 μM Gen; Com, 10 ng/mL OSM + 117.5 μM Gen.
Ctrl, control; Iri, irisin; Gen, genistein; Com, combination; OSM, oncostatin M.

Most tumors initially respond to cytotoxic therapies but gradually acquire a multidrug resistance phenotype, leading to recurrence [17]. CSCs are resistant to chemotherapy and radiotherapy, which can play a significant role in cancer recurrence. Therefore, it is essential to develop therapies that bypass the resistance of CSCs to drugs and radiotherapy. Currently, an effective strategy for anti-cancer treatment is to apply two or more treatment methods to induce an additive or synergistic effect on the anti-cancer effect. Previous research has demonstrated that the combination of exercise and isoflavone daidzein can induce apoptosis by activating the mitochondrial apoptotic signaling pathway [23]. Another study has confirmed that berberine, one of the active ingredients in Coptis chinensis, can improve the immune system in synergy with exercise and enhance anti-cancer effects by activating the mitochondrial and Fas death receptor apoptotic pathway [30]. The present study is significant owing to its utilization of two therapeutic approaches, using myokines and Gen, which have anti-cancer effects known for previous studies, and found that their combination produced a greater effect than each individual treatment. However, the results of this study were based on *in vitro* cell culture assays rather than *in vivo* experiments. Therefore, additional *in vivo* animal studies using animals should be conducted to support and confirm the current results.

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The study showed that myokines and Gen or their combination decreased the self-renewal ability of CSCs and expression of CSCs markers SOX2 and OCT4 in MCF-7 breast cancer cells. Combining myokines and Gen had an additive effect on the anti-cancer effect by inhibiting cancer stemness in MCF-7 breast cancer cells. These findings suggest that a combination treatment of myokines and Gen positively impact breast cancer prevention and treatment. Furthermore, it indicates that it could potentially help to prevent and treat any side effects from breast cancer chemotherapy by reducing the dosage of the administered drugs.

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