Abstract. As a novel discovered myokine, irisin is considered to be a promising candidate for the treatment of metabolic disorders and cancer. However, little is known about the anti-metastasic effect of irisin on osteosarcoma cells and its underlying mechanisms. In the present study, we aimed to explore the effect of irisin on the migration and invasion of osteosarcoma cells and the underlying mechanisms involved. Viability and proliferation of osteosarcoma cells were examined by MTT assay. Then, by using scratch wound healing assay and Transwell assays, we evaluated migratory and invasive ability of the cells, respectively. Moreover, the expression of epithelial-to-mesenchymal transition (EMT) markers were determined by qPCR, western blot and immunofluorescence staining after treatment with IL-6 and irisin. Furthermore, the expression of ERK, p38, STAT3 and Snail were detected by western blot analysis. Finally, an inhibitor of STAT3, WP1066 was applied to testify the effect of irisin on the expression of EMT markers and Snail. It was found that irisin treatment significantly suppressed the proliferation, migration and invasion of osteosarcoma cells. Furthermore, irisin reversed the IL-6-induced epithelial-mesenchymal transition (EMT) in osteosarcoma cells by regulating the expression of E-cadherin, N-cadherin, vimentin, fibronectin, MMP-2, MMP-7 and MMP-9. In addition, irisin suppressed the IL-6-activated phosphorylation of STAT3 and the expression of Snail in osteosarcoma cells. Finally, blockade of STAT3 by WP1066 (a STAT3 inhibitor) further enhanced the effect of irisin on the EMT and Snail expression in osteosarcoma cells. Collectively, our findings revealed that irisin may play a critical role in the IL-6-induced EMT of osteosarcoma cells via the STAT3/Snail signaling pathway.

Introduction

It has been well documented that exercise has many potential benefits in chronic metabolic diseases including type II diabetes mellitus and metabolic syndrome, cardiovascular disease and cancer (1-4). Recently, studies have focused on the benefits of exercise in various types of cancers, such as breast, lung and gynaecological cancer (5-7). However the potential underlying mechanisms of exercise on cancer treatment and improved quality of life are not well established.

In recent years, skeletal muscle is gaining increased attention as an endocrine organ (8). Various myokines which regulate beneficial effects have been identified, such as irisin, a newly discovered myokine, which is secreted from the skeletal muscles following exercise (9). Irisin is believed to be a bridge that links exercise with increased energy expenditure. Studies have documented that irisin is released upon cleavage of the membrane of fibronectin type III domain-containing protein 5 (FNDC5) and is increased with exercise (9,10). Previous studies have demonstrated the role of irisin in body energy expenditure and in insulin sensitivity (9,10). Furthermore, irisin has been demonstrated to play a role in the mediation of H19-7 hippocampal neuronal cell proliferation (11) and in the differentiation of adipocytes and osteoblasts (12-14). In addition to these studies, irisin has been demonstrated to have a protective role in vascular pathology by regulating endothelial cell proliferation, apoptosis and migration, as well as smooth muscle cell phenotype modulation (15-18). Notably, recent studies have focused on the relationship between irisin and cancer. A recent study found that serum irisin levels were lower in patients with breast cancer (19), while other studies revealed that irisin was significantly increased in gastrointestinal cancer tissues (20,21). In addition, studies have revealed increased irisin immunoreactivity in tissues which were obtained from ovary, cervix and breast carcinomas and endometrial hyperplasias (22).
Moreover, a recent study demonstrated that irisin significantly inhibited the viability and migration and enhanced the tumor sensitivity to doxorubicin (DOX) in malignant breast cancer cells (23). Additionally another study revealed the suppressive effect of irisin in lung cancer cell migration and invasion (24). These studies underlined the critical role of irisin in carcinogenesis. Furthermore, these findings may offer therapeutic benefits for cancer prevention and treatment.

At present, osteosarcoma, the most common primary bone malignant tumor, has a poor prognosis due to distal metastases (25). Although chemotherapy is combined with surgery to improve the prognosis, the survival rate is still low (26). Therefore it is urgent to identify novel drugs for a better treatment outcome in osteosarcoma therapy.

Osteosarcoma cells possess highly invasive properties by undergoing a unique phenotypic switch, epithelial-mesenchymal transition (EMT) (27). EMT is thought to be a highly conserved cellular process which is characterized by inhibition of epithelial molecule E-cadherin and gaining of mesenchymal markers, such as N-cadherin, vimentin and fibronectin (28). Studies have revealed the critical role of EMT in carcinoma metastasis (28,29).

It has been well established that IL-6 promotes the proliferation, metastasis and angiogenesis of osteosarcoma (30,31). Studies further demonstrated that IL-6 induced EMT in various types of cancer cells, such as pancreatic (32), lung (33), hepatocellular (34) and colorectal cancer cells (35). The IL-6 downstream signals included STAT3, Akt and ERK1/2 MAPK (30,36,37). Among them, STAT3 has been demonstrated to exhibit an important role in IL-6-modulated EMT (33,38). Previous studies have revealed the critical role of STAT3 in modulating angiogenesis and metastasis of cancer (39,40). Furthermore, studies have revealed that the Snail family members, including Snail, ZEB1, Twist, Slug and SIP1, played a crucial role in regulating EMT (41).

Thus, the present study evaluated the effect of irisin on osteosarcoma cell migration, invasion and EMT and explored the mechanisms involved.

Materials and methods

Cell lines and reagents. The U2OS and MG-63 osteosarcoma cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10% FBS (Invitrogen Life Technologies), 100 u/ml penicillin, and 100 µg/ml streptomycin in 5% CO2. These cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10% FBS (Invitrogen Life Technologies), 100 u/ml penicillin, and 100 µg/ml streptomycin in 5% CO2. The u2OS and MG-63 osteosarcoma cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) containing 10% FBS (Invitrogen Life Technologies), 100 u/ml penicillin, and 100 µg/ml streptomycin in 5% CO2.

Cell viability assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess the cell proliferation and viability. The U2OS and MG-63 osteosarcoma cells (5,000 cells/well) in 100 µl medium were seeded into 96-well plates. After being stimulated with irisin at different doses (0, 25, 50, 100 and 200 ng/ml) for different time-points (12, 24 and 48 h), 20 µl MTT solution (5 mg/ml) was added into each well. After incubation for 4 h, 100 µl of dimethyl sulfoxide (DMSO) was added to each well for another 15 min. Then, the absorbance values were determined using a microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 490 nm.

Scratch wound healing assay. To evaluate the migration of the U2OS and MG-63 osteosarcoma cells, a scratch wound healing assay was used. Briefly, the U2OS and MG-63 cells (1x10^5/well) were seeded in 6-well plates and cultured with DMEM supplemented with 10% FBS. When reaching confluency, each well was directly scratched with a 200 µl pipette tip. To detect the effect of irisin on the migration of U2OS and MG-63 cells, 100 ng/ml irisin was added to each well. After 24 h of incubation, the wound healing areas were photographed and the distance between the two cell edges was analyzed by ImageJ software.

In vitro invasion assay. The Transwell system was used to evaluate the effect of irisin on the invasion of U2OS and MG-63 osteosarcoma cells. The U2OS and MG-63 cells were cultured in Boyden chambers, with 8-µm pore filter inserts, in 24-well plates (Corning Costar, Corning, NY, USA). The pore inserts were pre-coated with Matrigel (BD Biosciences, San Jose, CA, USA) overnight. The U2OS and MG-63 cells (1x10^5 cells/well) were suspended in 100 µl DMEM supplemented with 1% FBS and were added to the upper chamber. DMEM supplemented with 10% FBS and irisin (100 ng/ml) were added to the lower chamber. After 24 h of incubation, the cells that had attached to the lower surface were fixed with methanol and stained with 0.1% crystal violet. Then, 5 random high-power fields (magnification, x200) of each sample were chosen and counted to evaluate the average number of invasive cells.
Irisin inhibits IL-6-induced STAT3 phosphorylation in osteosarcoma cells. The MAPK and STAT3 signaling pathways play a crucial role in IL-6-induced cancer cell EMT (44-46). To further explore the potential mechanism of irisin on osteosarcoma cell EMT, the STAT3, ERK1/2 and p38 signaling pathways were detected. As shown in Fig. 3A, STAT3, ERK1/2 and p38 phosphorylation was upregulated when being treated with IL-6 (100 ng/ml). STAT3 phosphorylation was suppressed when
KONG et al: A NOVEL ROLE FOR IRISIN ON OSTEOSARCOMA CELLS

The STAT3 pathway is involved in irisin-modulated EMT in osteosarcoma cells. To further demonstrate whether STAT3 is involved in irisin-regulated osteosarcoma cell EMT, we selected WI1066 (a STAT3 inhibitor) to block STAT3 phosphorylation (Fig. 4A). As shown in Fig. 4B and C, at the mRNA and protein level, the suppression of STAT3 phosphorylation with WI1066 (10 µM) enhanced the expression of E-cadherin and further downregulated the expression of some EMT markers (N-cadherin, fibronectin, MMP-2 and MMP-7) which were induced by irisin. These results revealed that irisin-mediated...
Irisin inhibits IL-6-induced Snail upregulation via the STAT3 pathway in osteosarcoma cells. Evidence has established the expression and activation of Snail transcriptional factor during EMT in E-cadherin suppression and cancer progression (47). In the present study we further explored the effects of irisin on the IL-6-induced Snail expression and the relationship between STAT3 and Snail. Firstly, U2OS and MG-63 osteosarcoma cells were treated with IL-6 (100 ng/ml) and irisin (0, 50 and 100 ng/ml) for 24 h and the expression of Snail was detected using qPCR and western blot analysis. The U2OS and MG-63 osteosarcoma cells were treated with or without IL-6 (100 ng/ml) and/or irisin at the indicated concentrations (0, 50 and 100 ng/ml) for 24 h and the expression level of E-cadherin, N-cadherin, vimentin, fibronectin, MMP-2, MMP-7 and MMP-9 mRNA and the protein expression was examined by qPCR analysis and western blot analysis. The U2OS osteosarcoma cells were treated with or without IL-6 (100 ng/ml) and/or irisin at the indicated concentrations (100 ng/ml) for 24 h and the expression of E-cadherin and vimentin was detected by immunofluorescence staining.

Figure 2. Irisin treatment reverses IL-6-mediated EMT in U2OS and MG-63 osteosarcoma cells. (A and B) The U2OS and MG-63 osteosarcoma cells were treated with or without IL-6 (100 ng/ml) and/or irisin at the indicated concentrations (0, 50 and 100 ng/ml) for 24 h and the expression level of E-cadherin, N-cadherin, vimentin, fibronectin, MMP-2, MMP-7 and MMP-9 mRNA and the protein expression was examined by qPCR analysis and western blot analysis. (C) The U2OS osteosarcoma cells were treated with or without IL-6 (100 ng/ml) and/or irisin at the indicated concentrations (100 ng/ml) for 24 h and the expression of E-cadherin and vimentin was detected by immunofluorescence staining. *P<0.05, **P<0.01 vs. the control group; #P<0.05, ##P<0.01, compared with the cells treated with IL-6. Data shown are expressed as the means ± SD from three independent experiments. EMT, epithelial-mesenchymal transition.
 blot analysis. As shown in Fig. 5A and B, IL-6 induced Snail mRNA and protein expression and irisin inhibited the effect in a dose-dependent manner. Then, to further evaluate whether STAT3 participated in irisin-regulated Snail expression, U2OS and MG-63 osteosarcoma cells were also pretreated with WP1066 for 30 min before IL-6 and irisin were added. As displayed in Fig. 5C and D, inhibition of STAT3 phosphorylation with WP1066 further suppressed the mRNA and protein expression of Snail which was induced by irisin. These results indicated the crucial role of STAT3 in regulating Snail expression and irisin inhibition of the expression of Snail via the STAT3 pathway. Collectively, these results demonstrated that the suppression of metastasis by irisin was mediated by the inhibition of EMT via the STAT3/Snail signaling pathway in osteosarcoma cells.

Discussion

The current study revealed several new findings about irisin. Irisin can inhibit the proliferation, migration and invasion of osteosarcoma cells. Irisin can also suppress IL-6-induced EMT in osteosarcoma cells. The beneficial effects of irisin in the EMT of osteosarcoma cells were observed through the STAT3/Snail signaling pathway.

Recently, much attention has been paid on the potential benefits of exercise in chronic metabolic diseases,
cardiovascular diseases and cancer (1-4). As a novel discovered myokine, irisin is released from the skeletal muscles following exercise (9). Previous studies have demonstrated that irisin modulated body energy expenditure by promoting brown adipocyte thermogenesis in mice (9,10). Meanwhile, recent studies have revealed the beneficial effect of irisin in neuronal cells (11), adipocytes (12), osteoblasts (13,14), as well as vascular cells, such as endothelial cells (15,18) and smooth muscle cells (17). Furthermore, recent studies revealed the direct suppressive effect of irisin on malignant breast and lung cancer cell proliferation and migration (23,24). In addition with other findings concerning the expression of irisin in various cancer tissues (20-22), these findings revealed the close association between irisin and cancer. The present study is the first to investigate the role of irisin on osteosarcoma cell migration and invasion, as well as its detailed molecular mechanisms. Firstly, we evaluated the effect of irisin on the proliferation of osteosarcoma cells. We found that 100 ng/ml of irisin significantly suppressed cell proliferation after 24 h. (A) Western blot analysis of the phosphorylated STAT3 and total STAT3 protein expression. (B) qPCR analysis of the mRNA expression of E-cadherin, N-cadherin, fibronectin, MMP-2, and MMP-7. (C) Western blot analysis of the protein expression of E-cadherin, N-cadherin, fibronectin and MMP-2. *P<0.05, **P<0.01, compared with the cells treated with IL-6 and irisin. Data shown are expressed as the means ± SD from three independent experiments. EMT, epithelial-mesenchymal transition.
suppressed the migration and invasion of osteosarcoma cells. These findings demonstrated the anti-metastatic property of irisin.

Metastasis is considered as the primary cause of mortality in most cancer patients. Studies have well established that EMT is a distinctive phenotypic switch by which epithelial cells lose their polarity and acquire the invasive properties of mesenchymal cells in the process of metastasis (48). Furthermore, studies have demonstrated that IL-6 could induce EMT in pancreatic, lung, hepatocellular and colorectal cancer cells (32-35). In the present study, we revealed the role of irisin in IL-6-induced EMT in osteosarcoma cells. Our study found that irisin could significantly reverse the IL-6-induced downregulation of the epithelial marker (E-cadherin) and upregulation of mesenchymal markers (N-cadherin, vimentin, fibronectin, MMP-2, MMP-7 and MMP-9) in osteosarcoma cells. Therefore, for the first time, our study demonstrated that irisin could effectively reverse the IL-6-induced EMT of osteosarcoma cells.

IL-6, a major cytokine in the tumor microenvironment, has long been documented in tumorigenesis in the regulation of proliferation, apoptosis, angiogenesis and metastasis of various types of cancer cells (49). Studies have demonstrated that the MAPK and STAT3 pathways were involved in IL-6-regulation of various cancer cell functions, such as proliferation and metastasis (30,36-38). Notably, STAT3 has been documented to play a crucial role in IL-6-induced EMT (33,38). In the present study, the expression of STAT3, ERK1/2 and p38 in osteosarcoma cells was evaluated. We found that the expression of phosphor-STAT3, phosphor-ERK1/2 and phosphor-p38 was increased when treated with IL-6, while only the phosphorylation of STAT3 was inhibited by irisin treatment. To further examine whether the STAT3 pathway was involved in irisin-regulated EMT in osteosarcoma cells induced by IL-6, STAT3 was blocked using WP1066 (a STAT3 inhibitor). We found that inhibition of the STAT3 pathway significantly further enhanced irisin-mediated EMT of osteosarcoma cells. These results demonstrated that STAT3 is essential for IL-6-induced EMT in osteosarcoma cells. These results also revealed that the effect of irisin may be STAT3-dependent.

Finally, we explored the effect of irisin and STAT3 on the transcription factor Snail. Snail, a zinc finger transcription factor, has been demonstrated to modulate the transcriptional suppression of E-cadherin (50). Previous studies revealed that the expression of Snail was modulated by STAT3 (51). Another study further demonstrated the crucial role of the
STAT3/Snail pathway in regulating EMT in hepatocellular carcinoma cells (52). In the current study, we found that irisin suppressed the upregulation of Snail which was induced by IL-6, while blockade of STAT3 further downregulated irisin-mediated Snail expression in osteosarcoma cells. These results revealed that STAT3 was upstream of Snail. Furthermore, the aforementioned results demonstrated that irisin suppressed the expression of Snail via the STAT3 pathway.

In conclusion, for the first time, our study revealed that irisin suppressed the migration and invasion of osteosarcoma cells. Moreover, irisin reversed the EMT induced by IL-6 in osteosarcoma cells. Finally, the inhibitory role of irisin in IL-6-induced EMT was modulated via the STAT3/Snail pathway. Collectively, irisin reversed IL-6-induced EMT of osteosarcoma cells through the STAT3/Snail signaling pathway. Thus, our research indicated that irisin is a promising agent in osteosarcoma treatment. Therefore further in vivo research needs to be performed.

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