Hesperedin promotes MyoD-induced myogenic differentiation in vitro and in vivo

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BACKGROUND AND PURPOSE
The bioflavonoid, hesperedin, promotes osteoblast differentiation in human mesenchymal stem cells, indicating an anabolic effect of hesperedin on bone metabolism. Murine bone marrow mesenchymal stem cells undergo myogenic differentiation as well as osteogenic differentiation. We therefore explored whether hesperedin modulates muscle cell differentiation.

EXPERIMENTAL APPROACH
Myoblast C2C12 cells were differentiated into muscle cells in the presence or absence of hesperedin. The effects of hesperedin on myogenic differentiation were determined by analysing specific muscle markers in vitro using reporter gene assays, immunoblotting, RT-PCR and DNA pull-down assays. In vivo, the effects of hesperedin were assessed using the freeze injury-induced muscle regeneration model in mice and daily injections of hesperedin for 6 days.

KEY RESULTS
Hesperedin promoted myogenic differentiation, in a dose-dependent manner, by increasing myogenin gene expression. MyoD-induced myogenin gene transcription was enhanced by hesperedin, as this bioflavonoid augmented the nuclear localization and myogenin promoter-binding of MyoD. In addition, hesperedin increased myogenin and muscle creatine kinase gene expression during myogenic differentiation from C3H10T1/2 mesenchymal stem cells in a MyoD-dependent manner and accelerated in vivo muscle regeneration induced by muscle injury.

CONCLUSIONS AND IMPLICATIONS
Our results demonstrate that hesperedin promoted myogenic differentiation in vitro and in vivo through activation of MyoD-mediated myogenin expression, suggesting a beneficial role in promoting muscle regeneration, following injury.

Abbreviations
MCK, muscle creatine kinase; MEF, myocyte enhancer factor; MHC, myosin heavy chain; DAPI, 4',6-diamidino-2-phenylindole

Introduction
Many bioflavonoids have been identified and exhibit biological activities such as antioxidant, antibacterial and anti-inflammatory properties (Huang et al., 2005; Amin et al., 2007; Cazarolli et al., 2008; Viuda-Martos et al., 2008). Hesperidin and its aglycone hesperedin are classified as citrus bioflavonoids and exert anti-atherogenic activity by reducing apolipoprotein B secretion (Borredale et al., 1999; Wilcox et al., 2001) and by suppressing the gene expression of microsomal triglyceride transfer protein and acyl CoA: cholesterol acyltransferase (ACAT) 2 (Wilcox et al., 2001;
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Boradaile et al., 2003). Orally administered hesperedin is converted to a de-glycosylated metabolite, hesperedin, by intestinal microflora in vivo. This aglycone suppresses histamine release from mast cells, exhibiting an anti-allergic activity (Ameer et al., 2003; Choi et al., 2004). In addition, hesperedin protects neuronal cells and osteoblasts from oxidative stress by functioning as a potent radical scavenger (Kim et al., 2004; Hirata et al., 2005; Cho, 2006; Choi and Kim, 2008) and suppresses proliferation of breast cancer cells by arresting the cell cycle at the G1 phase (Choi, 2007). More recently, hesperedin was reported to enhance osteoblast differentiation by increasing alkaline phosphatase activity and by inducing RUNX2 activation in the bone morphogenetic protein signalling pathway, suggesting an anabolic effect of hesperedin on bone metabolism (Trzeciaikiewicz et al., 2010a,b).

Mesenchymal stem cells in adult bone marrow are multipotent stem cells that can differentiate into osteoblasts, adipocytes, chondrocytes and myocytes (Harada et al., 2003; Gregory et al., 2005). Cell lineage commitment is accompanied by the activation of lineage-specific transcription factors such as RUNX2 (Koromi et al., 1997), peroxisome proliferation-activated receptor (PPAR) (Tontonoz et al., 1994), SOX9 (Bi et al., 1999) and MyoD (Davis et al., 1987). In particular, myocyte differentiation is regulated by various muscle regulatory factors (MRFs), including MyoD, Myf5, myogenin and MRF4 (Rudnicki et al., 1993). MyoD initiates the expression of muscle-specific genes and induces commitment of mesenchymal stem cells to the myogenic lineage (Berkes et al., 2005). Induction of myogenin expression is controlled by MyoD activity and is crucial for terminal differentiation (Hasty et al., 1993; Nabeshima et al., 1993) and induction of muscle-specific genes like myosin heavy chain (MHC) and muscle creatine kinase (MCK) (Charge et al., 2004). Members of the myocyte enhancer factor 2 (MEF2) family of transcription factors are also able to modulate muscle differentiation, via cooperative association with myogenic transcription factors of the bHLH family, such as MyoD and myogenin (Molkentin et al., 1996).

In this study, we have investigated the effects of hesperedin on myogenic differentiation. We found that this bioflavonoid increased myogenic differentiation and muscle gene expression by promoting nuclear localization of MyoD and its DNA binding activity to the myogenin gene promoter. Furthermore, injury-induced muscle regeneration in mice was accelerated by treatment with hesperedin in vivo.

Methods

Cell culture and differentiation

Myoblast C2C12 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). For myogenic differentiation, the cells were grown to confluence for 48 h and then induced to differentiate by replacing the growth medium with DMEM containing 2% horse serum every other day (Yaffe and Saxel, 1977). C3H10T1/2 mesenchymal stem cells and HEK 293T cells were maintained in DMEM supplemented with 10% FBS.

Reporter gene assay

C2C12 cells were transfected with MyoD or MEF2C expression vectors and the reporter genes, such as myogenin promoter-linked (pMyogenin-luc) and MCK promoter-linked luciferase gene (pMCK-luc), by calcium phosphate transfection (Jung et al., 2009). Three independent experiments were conducted and luciferase activity was assayed according to the manufacturer’s instructions (Promega, Madison, WI, USA). A relative luciferase unit was determined by normalization to the β-galactosidase activity of pCMVβ (Clontech, Palo Alto, CA, USA), which was co-transfected as an internal control for transfection efficiency.

Immunoblot analysis

Whole cell extracts were prepared from the cells and resolved by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (7.5% PAGE), followed by electrophoretic transfer to Immobilon-P membranes (Millipore Inc., Invitrogen). The blots were incubated with antibodies against myogenin, MyoD, MHC and β-actin (1:1000, Santa Cruz Biotech. Inc., Santa Cruz, CA, USA) and subsequently with secondary antibody (1:10 000, Zymed), followed by development using a luminal reagent (GE Healthcare, Buckinghamshire, UK).

Immunofluorescence analysis

C2C12 cells were differentiated in the absence or presence of hesperedin and harvested at the indicated time points. Cells were permeabilized with 2% paraformaldehyde containing 0.1% Triton X-100, and were subjected to staining with myogenin or MHC antibody (1:100) and subsequent incubation with Alexa Fluor 488-conjugated secondary antibody (1:5000) (Jeong et al., 2010). Immunofluorescence was observed under a Zeiss Axiovert 200 fluorescence microscope (Carl Zeiss, Göttingen, Germany).

Reverse transcription and real-time PCR analysis

Total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen) and subjected to reverse transcription for cDNA synthesis. Real-time PCR was performed using SYBR Green PCR mix (2X) and an ABI-Prism 7000 sequence detector (Applied Biosystems, San Mateo, CA, USA). The relative expression level of each mRNA was determined by the threshold cycle (Ct) values after normalization to the level of β-actin. Real-time PCR was performed with specific primers (0.3 μM): β-actin-FWD 5′-agagggaaatagtgcgtgac-3′, β-actin-REV 5′-caatgtgtgacctgggcct-3′; MCK-FWD 5′-cacttccacag acagacag-3′, MCK-REV 5′-accttgccatggattggt-3′; myogenin-FWD 5′-caacagggagccagacccctg-3′, myogenin-REV 5′-agg cgttggggtgattgattcactc-3′; MyoD-FWD 5′-tgtagatagccagcttctt cgc-3′, MyoD-REV 5′-gtgtagtccaacacgacctc-3′.

Chemiiluminescent EMSA and DNA pull-down assay

Nuclear proteins were prepared and incubated with biotinylated double-stranded myogenin promoter containing the MyoD-binding site according to the manufacturer’s instructions (Active Motif Inc., Carlsbad, CA, USA). Briefly, reaction mixtures were resolved by non-denaturing PAGE and trans-
ferred to nylon membranes. The blot was incubated with streptavidin-horseradish peroxidase and subjected to chemiluminescent enhanced chemiluminescence. For DNA pull-down assay, cells were lysed using HKMG buffer (10 mM HEPES, pH 7.9, 100 mM KCl, 5 mM MgCl₂, 10% glycerol, 0.1% NP-40, 1 mM dithiothreitol and protease inhibitor cocktail) and whole cell extracts were incubated with biotinylated myogenin promoter DNA, then incubated with streptavidin agarose beads. Precipitates were washed with HKMG buffer three times and analysed by SDS-PAGE and immunoblot assay using MyoD antibody.

Myogenic conversion assay
Phoenix cells were transfected with a retroviral vector that expresses MyoD using the standard calcium phosphate transfection method. Cells were then refreshed and transferred to a 32°C incubator for an additional 30 h. The viral supernatant was added to C3H10T1/2 cells with polybrene (8 μg·mL⁻¹) for 48 h. Cells were selected in the presence of puromycin (2 μg·mL⁻¹) and then induced to myogenic differentiation in the presence or absence of hesperedin.

Injury-induced muscle regeneration and histological analysis
All animal care and experimental procedures were approved by the Ewha Womans University Institutional Animal Care and Use Committee. C57BL/6 mice were purchased from the Jackson Laboratories (Bar Harbor, ME, USA) and housed in the Ewha Laboratory Animal Genomic Center under specific pathogen-free conditions and under the standard light/dark cycle. The model of muscle injury-induced regeneration following freeze injury described by Jeong et al. (2010) was used.

Figure 1
Enhanced myogenic differentiation by hesperedin. (A) Chemical structure of hesperedin. (B) Confluent C2C12 cells were induced to differentiate by replacing with DMEM supplemented with 2% HS with either vehicle (-Hsp) or hesperedin (+Hsp, 100 μM). Cells were fixed at the indicated differentiation time points and incubated with anti-MHC antibody, followed by incubation with Alexa Fluor 488-conjugated secondary antibody. (C) Fluorescence-positive cells as in Figure 1B were counted per field under a fluorescence microscope. The average and standard errors from 10 fields were calculated. (D) Cells were stained with anti-MHC antibody and subsequent Alexa Fluor 488-conjugated secondary antibody at differentiation day 4. The nuclei were stained with DAPI. (E) C2C12 cells during myogenic differentiation were incubated with anti-myogenin antibody and subsequent fluorescence-tagged secondary antibody. Cells were observed under low power magnification. (F) Myogenin-positive cells in Figure 1E were counted from 10 fields as described in Figure 1C. (G) Cells were differentiated for 4 days in the presence or absence of hesperedin (100 μM) and stained with anti-myogenin antibody as well as DAPI for nuclear staining. Scale bars in Figure 1B, D and E indicate 50 μm. *P < 0.05; **P < 0.005.
Briefly, C57BL/6 mice were subjected to freeze injury in the tibialis anterior (TA) muscles and subsequently injected with either PBS or hesperedin every day for 6 days. On day 7 after injury, mice were killed for the preparation of protein extracts and tissue sections. TA muscles were isolated and 7 mm sections prepared by microtome. Tissue sections were then stained with hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO, USA).

**Statistical analysis**
All data are given as the means ± SEM. Statistical significance was determined by unpaired Student’s t-test or one-way ANOVA. *P*-values less than 0.05 were considered statistically significant.

**Materials**
Hesperedin (purity 95%) was obtained from Sigma-Aldrich. All reagents for cell culture were purchased from Gibco-BRL (Invitrogen), and FBS and horse serum were from HyClone (Logan, UT, USA).

**Results**

**Hesperedin promotes myogenic cell differentiation**
To investigate the effect of hesperedin in myogenic differentiation, myoblast C2C12 cells (Lassar et al., 1994) were induced to differentiate into myocytes in low mitogen medium in the absence or presence of hesperedin (Figure 1A). The morphological changes indicative of cylinder-shaped myocytes were time-dependently increased during myogenic differentiation (Figure 1B). Hesperedin significantly enhanced the numbers of cylinder-shaped myocytes and MHC expression, as shown by immunofluorescence staining using anti-MHC antibodies (Figure 1B and C). In addition, multinucleated myotubes, which are formed by cell fusion of terminally differentiated myocytes, were prominently observed among hesperedin-treated cells (Figure 1D). Myogenin expression was also considerably augmented in the presence of hesperedin during differentiation (Figure 1E and F). Myogenin expression was prominent in the nucleus (Figure 1G), indicating that hesperedin promotes myogenic gene expression and terminal differentiation.
Myogenin gene transcription is promoted by hesperedin

Because myogenic differentiation was promoted by hesperedin, we next examined the regulatory mechanisms of this bioflavonoid in myogenic gene expression. The expression levels of early MRFs were determined on differentiation day 2. The expression levels of MyoD and MEF2C were not changed by hesperedin, whereas it dose-dependently increased myogenin expression on day 2 (Figure 2A). Quantitative real-time PCR confirmed that hesperedin induced myogenin expression at the level of gene transcription, but did not affect MyoD gene transcription (Figure 2B and C). Hesperedin also boosted expression of the muscle-specific marker, MCK (Figure 2D). These results suggest that hesperedin may selectively control gene transcription of myogenin and MCK.

Hesperedin enhances MyoD activity to induce gene transcription of myogenin and MCK

Increased mRNA levels of myogenin and MCK by hesperedin provoked us to examine the regulatory functions of hesperedin in gene transcription. In order to test whether hesperedin directly modulates gene transcription of myogenin and MCK, C2C12 cells were transfected with a luciferase reporter gene linked to a myogenin promoter (pMyogenin-luc) or a MCK promoter (pMCK-luc) and were cultured in low mitogen medium with hesperedin for 24 h. Hesperedin substantially induced both myogenin and MCK promoter activity (Figure 3A and B), indicating that hesperedin may increase gene transcription of myogenin and MCK through promoter activation. Because both myogenin and MCK promoters possess myogenic bHLH protein binding sites, we tested whether a myogenic bHLH protein, MyoD, could be activated by hesperedin (Wilson and Rotwein, 2006). Ectopic expression of MyoD in 293T cells increased myogenin and MCK promoter activity by 7- and 4-fold respectively (Figure 3C and D). Hesperedin further increased these MyoD-induced gene promoter activities, but had no stimulatory effect in the absence of MyoD (Figure 3C and D). These results suggest that hesperedin may enhance the transcriptional activity of MyoD and induce its target gene transcription.
Hesperedin selectively modulates MyoD activity

We also examined the effect of hesperedin on the activity of myogenin and other MRFs in the promoter activity of myogenin and MCK. Enforced myogenin expression activated its own promoter activity, but hesperedin did not further increase the transcriptional activity of myogenin (Figure 4A). While MEF2C expression had no significant effect on myogenin promoter activity, its co-expression with MyoD cooperatively increased myogenin promoter activity induced by MyoD (Figure 4B). Hesperedin amplified MyoD-induced promoter activity, but did not affect the cooperative activity of MyoD and MEF2C (Figure 4B). Moreover, the direct interaction between MEF2C and MyoD was not affected by hesperedin (Figure 4C), indicating specific transcriptional regulation by hesperedin through activation of MyoD, not other transcription factors.

Hesperedin facilitates nuclear expression and DNA binding activity of MyoD

To elucidate the regulatory mechanisms underlying MyoD activation by hesperedin, we first assessed the subcellular localization of MyoD in the presence of hesperedin. Interestingly, hesperedin dose-dependently enhanced nuclear localization of MyoD in C2C12 cells, but did not affect expression of a nuclear control protein, OCT1, as shown by immunoblotting and densitometric scanning (Figure 5A) and immunocytochemistry using anti-myogenin antibody (Figure 5B). In addition, the DNA–MyoD binding complex that was specifically inhibited by competitor DNA was increased by treatment with hesperedin (Figure 5C), reflecting the increased nuclear level of MyoD by hesperedin. To explore the direct effect of hesperedin on the association of MyoD with myogenin promoter, a DNA pull-down assay was conducted using whole cell extracts from cells overexpressing MyoD. Hesperedin increased the DNA binding affinity of MyoD but did not directly change MyoD expression levels (Figure 5D). This flavonoid may therefore affect both the nuclear localization and DNA binding activity of MyoD.

The pro-myogenic activity of hesperedin requires MyoD expression

To confirm that the pro-myogenic activity of hesperedin was not confined to myoblast C2C12 cells, C3H10T1/2 mesenchymal stem cells were treated with hesperedin in the absence or presence of MyoD expression. As MyoD is critical...
for myogenic conversion of non-muscle cell types (Lassar and Munsterberg, 1994), there was no induction of muscle gene expression by hesperedin in the absence of MyoD (Figure 6A). However, comparable expression of MyoD by viral transduction increased myogenin expression (Figure 6A and B). In addition, hesperedin dose-dependently elevated the gene expression of myogenin and MCK in the presence of MyoD expression (Figure 6C and D). These results strongly suggest that hesperedin has a potent pro-myogenic activity via regulation of MyoD activity in the myogenic differentiation of mesenchymal stem cells.

Hesperedin accelerates muscle regeneration induced by injury in vivo
We further examined whether hesperedin affects muscle differentiation and regeneration in vivo. Mouse TA muscles were subjected to a freeze injury, followed by i.p. injection of hesperedin, daily for 6 days. Injured muscle exhibited pronounced necrosis and extensive immune cell infiltration with a few regenerating myofibers on day 7 post-injury, whereas treatment with hesperedin (50 mg·kg⁻¹) accelerated the induction of centrally nucleated regenerating myofibers and successfully induced lesion repair in injured muscle (Figure 7A). In addition, myogenin expression was increased in injury-induced regenerating muscle and further enhanced by the hesperedin treatment. The muscle markers in regenerating muscle, desmin and vimentin, were also expressed in regenerating muscle while those expression levels were attenuated in successfully repaired muscle tissues after treatment with hesperedin (Figure 7B). These results indicate that treatment with hesperedin may enhance myogenin expression in muscle regeneration processes and improve injury-induced muscle differentiation and regeneration in vivo.

Discussion
In this study, we have identified a pro-myogenic function of hesperedin and its regulatory mechanisms, which include promoting nuclear localization of MyoD and its interaction with target gene promoters, as well as enhancement of
MyoD-mediated myogenic gene transcription and myogenic differentiation.

MyoD is an essential transcription factor for initiating skeletal muscle differentiation and MyoD deficiency impairs MHC expression (Tapscott et al., 1988; Rudnicki et al., 1993). MyoD associates with other transcription factors such as MEF2C (Zang et al., 2004), pRB (Gu et al., 1993) and E12/E47-like proteins (Lassar et al., 1991) to activate gene transcription of myogenin and muscle-specific genes by directly binding to gene promoters/enhancers in the nucleus (Polesskaya et al., 2002; Wilson and Rotwein, 2006). Our results reveal that hesperedin increased the nuclear localization of MyoD and the binding affinity of MyoD to the gene promoter, but did not affect the functional cooperation between MyoD and MEF2C. Hesperedin seems to selectively affect MyoD, not MEF2C or myogenin, but its role in the heterodimerization of MyoD with other transcription factors is unclear.

Cell cycle arrest is an important molecular mechanism for inducing cell differentiation. Two molecules controlling cell cycle exit, p21 and p57, modulate skeletal muscle differentiation, and both p21- and p57-deficient cells fail to form myotubes (Zhang et al., 1999c). Cell cycle progression, coupled with cyclin D1-dependent nuclear targeting of CDK4, negatively modulates myocyte differentiation (Zhang et al., 1999a). However, under low-mitogen conditions, MyoD directly interacts with CDK4 and inhibits pRB phosphorylation, resulting in exit from the cell cycle and promotion of myogenic differentiation (Zhang et al., 1999b; Wei and Paterson, 2001). Interestingly, hesperedin inhibits cell proliferation of rat vascular smooth muscle cells, by suppressing pRB phosphorylation and lowering cyclin D expression (Jin et al., 2008). Collectively, hesperedin activates MyoD expression in the nucleus and attenuates the level of cyclin D and phosphorylated pRB, providing the molecular mechanisms underlying the pro-myogenic effects of hesperedin.

A high dietary intake of hesperedin has been implicated in lowering serum cholesterol and the risk of cardiovascular disease (Bok et al., 1999; Wilcox et al., 2001; Kanaze et al., 2007; Jin et al., 2008) and inhibiting breast cancer (Choi, 2007) and protecting cells from oxidative stresses (Kim et al., 2004; Cho, 2006; Choi and Ahn, 2008). Our results additionally suggest that hesperedin may have a beneficial role in promoting muscle cell differentiation and regeneration after muscle injury in vivo. Muscle injuries, which can be caused by a variety of genetic and environmental factors such as surgery, cancer, inflammation, infections and oxidative stresses, undergo various phases during healing such as myofiber degeneration, inflammation, regeneration and fibrosis at injured sites (Huard et al., 2002; Charge et al., 2004; Greffet et al., 2007; Ten Broek et al., 2010). During myoblast differentiation or regeneration, MyoD is essential for skeletal muscle repair of damaged tissue (Ferrari et al., 1998; Cooper et al., 1999; Charge et al., 2004). Hesperedin administration...
may activate MyoD to induce gene transcription of the muscle genes myogenin and MCK.

In conclusion, the ability of the dietary flavonoid, hesperedin, to activate MyoD and subsequent myogenin expression and to promote muscle differentiation and regeneration may indicate beneficial therapeutic potential for recovery from athletic injury and treating muscle diseases, including myopathy and muscular dystrophy.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure 7

In vivo muscle regeneration enhanced by hesperedin treatment. TA muscles of C57BL6 mice (n = 6) were damaged by freeze injury. Mice were intraperitoneally injected with either PBS or hesperedin (Hsp; 10 and 50 mg·kg⁻¹, i.p.) every day and killed on day 7. (A) TA muscles were sectioned and stained with haematoxylin and eosin. Representative images are presented; scale bar indicates 100 μm. (B) Whole cell extracts were harvested from TA muscles and resolved by SDS-PAGE with subsequent immunoblotting with antibodies against myogenin, vimentin, desmin or β-actin.
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