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Effect of resveratrol on proliferation and differentiation of embryonic cardiomyoblasts

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Abstract

Resveratrol (trans-3,5,4′-trihydroxystilbene), a polyphenolic compound found largely in the skins of red grapes, has been used as a nutritional supplement or an investigational new drug for prevention of cardiovascular diseases. Previous reports showed that resveratrol had a protective effect against oxidative agent-induced cell injury. Our studies indicate that resveratrol plays a role in the differentiation of cardiomyoblasts. The cardiomyoblast cell line, H9c2, was exposed to 30–120 μM resveratrol for up to 5 days. Resveratrol inhibits cardiomyoblast proliferation without causing cells injury. Moreover, resveratrol treatment modulated the differentiation of morphological characteristics including elongation and cell fusion in cardiomyoblasts. Proliferation and differentiation of H9c2 cells were further revealed by measurement of the mRNA expression of a cell cycle marker (CDK2), a differentiation marker (myogenin), and a contractile apparatus protein (MLC-2). Gene expression analysis revealed that resveratrol promoted entry into cell cycle arrest but extended the myogenic differentiation progress. These results have implications for the role of resveratrol in modulating cell cycle control and differentiation in cardiomyoblasts.

Keywords: Resveratrol; Cardiomyoblast; Differentiation

The triphenolic phytochemical resveratrol is present in various plant species and is particularly abundant in red grape skins, wine, peanuts, and mulberries [1–4]. Initially, resveratrol, characterized as a phytoalexin, attracted little interest until 1992, when it was postulated to explain some of the cardioprotective effects of red wine [5]. Since then, increasing evidence has suggested that resveratrol exhibits beneficial health effects, such as anti-cancer, cardioprotective, neuroprotective, anti-aging, and anti-inflammatory effects. Resveratrol not only exerts such a broad range of beneficial effects across disease types, it also has been shown to extend the lifespan of evolutionarily distant species [6–8]. In recent years, the effects of resveratrol on reduction in myocardial damage during ischemia–reperfusion [9–11] modulation of vascular cell functions [12,13], inhibition of LDL oxidation [14,15], suppression of platelet aggregation [16] have been largely described. Although substantial evidence indicates that resveratrol has beneficial effects in cardiovascular diseases, the underlying mechanism of the actions of resveratrol is not yet clear.

In the past, adult cardiac muscle was thought to lack the ability to regenerate after ischemic injury, and death of

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cardiomyocytes involved activation of an irreversible cascade leading to heart failure. Over the past decade, more evidence has shown that the generation of new myocytes plays a crucial role in the myocardial response to ischemic and non-ischemic injury. Studies have showed that by either implanting of endogenous resident cardiac stem cells [17,18], or exogenous donor-derived or allogeneic cells such as bone marrow stem cells [19,20], or mesenchymal stem cells [21,22], in heart could improve cardiac function. These results indicate that undifferentiated endogenous or exogenous precursors can contribute to the formation of new viable cardiac tissue. This process is accompanied by complex changes in the gene expression of the precursors upon cell cycle regulation and differentiation. As resveratrol exhibits a remarkable ability to reduce damage in myocardial injury, we hypothesized that resveratrol contributes to modulating the differentiation of cardiomyocyte precursors during heart injury and attempted to study the differentiation modulating effect of resveratrol on an embryonic cardiomyoblast cell line, H9c2. H9c2 is a subclone of the original clonal cell line derived from embryonic BDIX rat heart tissue by selective serial passage [23]. Myoblastic cells in this line will fuse to form multinucleated myotubes when the serum concentration in the medium is reduced to 1% [23]. It has been reported that H9c2 cells depolarize in response to ACh, yet they can exhibit rapidly activating cardiac L-type Ca^{2+} currents ($I_{Ca,L}$) [24]. The H9c2 cell line has been being used as an *in vitro* model of cardiac muscle in a variety of biochemical and pathophysiological studies, such as cardiac hypertrophy, [25] ischemia/reperfusion-induced injury, [26] oxidative stress, [27], and differentiation [28,29]. Results in this study have demonstrated that resveratrol definitely modulates the differentiation of cardiomyoblasts, which presents a new theory of the possible action of resveratrol on cardiac cells.

Materials and methods

Cell culture. H9c2 cells obtained from the were grown as a stock of cells in culture flasks in Growth Medium (GM) in which DMEM was supplemented with 10% fetal calf serum (FCS) (Gibco) and 1% penicillin/streptomycin (Gibco). Myogenic differentiation was promoted by changing subconfluent (80%) cells to Differentiation-enhancing medium (DM) in which the DMEM contained 1% FCS. Resveratrol was supplied by Sigma (St. Louis, MO) and the stock solution was prepared in ethanol. In GM group, cells receiving ethanol (0.25%) served as a vehicle control.

Cell proliferation assay. The Cell Proliferation Kit II (XTT) (Roche) was used to determine the effect of resveratrol on proliferation of H9c2 cells. It was conducted in accordance with the manufacturer's protocols. Briefly, after drug treatment for a specific period of time, cells of each well in the 96-well plate received 100 μL of XTT test solution and were then incubated for 4 h at 37 °C. Absorbance of the sample was determined using a microplate reader (Perkin-Elmer, Singapore) at 490 nm with a reference wavelength at 690 nm.

Determination of cell damage. Cytotoxicity was evaluated by the amount of lactate dehydrogenase (LDH) released from cells. The Cytotoxicity Detection Kit (LDH) (Roche) was used to determine the total LDH activity. One hundred microliters of cell culture mediums were transferred to a new 96-well plate at specific time points after exposure of cells to resveratrol. 100 μl reaction mixture was added to the sample medium collected before and incubated for 30 min at room temperature. Absorbance of the sample was determined using a microplate reader (Perkin-Elmer, Singapore) at 490 nm with a reference wavelength at 690 nm. The total amount of LDH release was normalized by the total amount of viable cells, indicated by XTT. LDH absorbance data was divided by the percentage of cell viability obtained from the XTT assay. The percentage of cell damage was calculated according to the equations below:

LDH/XTT(sample) = [sample OD(LDH) − blank OD(LDH)]/[sample OD(XTT) − blank OD(XTT)] % cell damage = [LDH/XTT(sample) − LDH/XTT(control)]/[LDH/XTT(high control) − LDH/XTT (control)] × 100%.

Cellular morphology observation. The effects of resveratrol on the differentiation morphology of H9c2 cells were observed with staining of the F-actin and the nucleus under a fluorescent microscope. Cells were fixed with 3.7% paraformaldehyde for 30 min at room temperature. After fixation, cells were washed twice with PBS and then co-stained with Rhodamine Phalloidin (5 U/ml, Molecular Probe) and Hoechst 33342 (5 μg/ml, Molecular Probe) in PBS at room temperature for 30 min. After washing three times, cells were observed under the fluorescent microscope (Axiovert 200, Carl Zeiss) and the same view was observed under bright-field illumination. Images of cells were captured at 200× (20× objective and 10× eyepiece) magnification using a CCD camera (AxioCam HRC, Carl Zeiss).

Cell cycle analysis. The cell cycles of H9c2 cells were examined by flow cytometry. After 9 h of incubation in different concentrations of resveratrol, cells were detached and fixed with 70% ethanol overnight at −20 °C. Then, cells were collected by centrifugation at 270*g* for 5 min, then re-suspended in 0.8 ml of PBS containing Triton X-100 (0.5%, Bio-Rad), RNase (4 μg/mL, Sigma) and propidium iodide (10 μg/ml, Molecular Probe), and incubated in a water bath at 37 °C for 30 min. Cell cycle analysis was performed by a flow cytometry apparatus (FACS, BD, USA).

RNA isolation and analysis of mRNA expression by real-time PCR. The total RNA of H9c2 cells was isolated using 6100 Nucleic Acid Prepstation (Applied Biosystems, Singapore). The extracted RNA (0.8 μg) was converted to single-strand cDNA using the SuperScriptTM III First-Strand Synthesis System (Invitrogen). All real-time PCRs were performed using TaqMan® Universal PCR Master Mix and primers (CDK2, myogenin, MLC, and GAPDH, TaqMan®). The real-time PCR was performed in the ABI 7500 Real-Time PCR system (Applied Biosystems, Singapore). The reaction without cDNA product served as a negative control. Each TaqMan assay was run in duplicate for each RNA sample. The relative expression was determined using the relative quantification method as described by the manufacturer. GM group at day 0 acted as the calibrator. Each of the normalized target values is divided by the calibrator-normalized target value to generate the relative expression levels.

Statistical analysis. Each experiment was performed at least three times. All values are presented as means ± SEM. Student's *t*-test was used to analyze the statistical significance of the results. $P < 0.05$ was considered as statistically significant.

Results

Fig. 1A shows a schematic illustration of the study design.

Growth inhibitory and cell damaging effects of resveratrol on H9c2 cells

H9c2 cells were seeded on a 96-well plate with a density of 4.5×10^3 cells/well in 100 μl growth medium (GM). After 24 h of incubation, GM containing different concentrations of resveratrol (30–120 μM) were added to the cells and incubated for 3 and 5 days. GM group serving as a vehicle control, was equivalent to no treatment. Medium

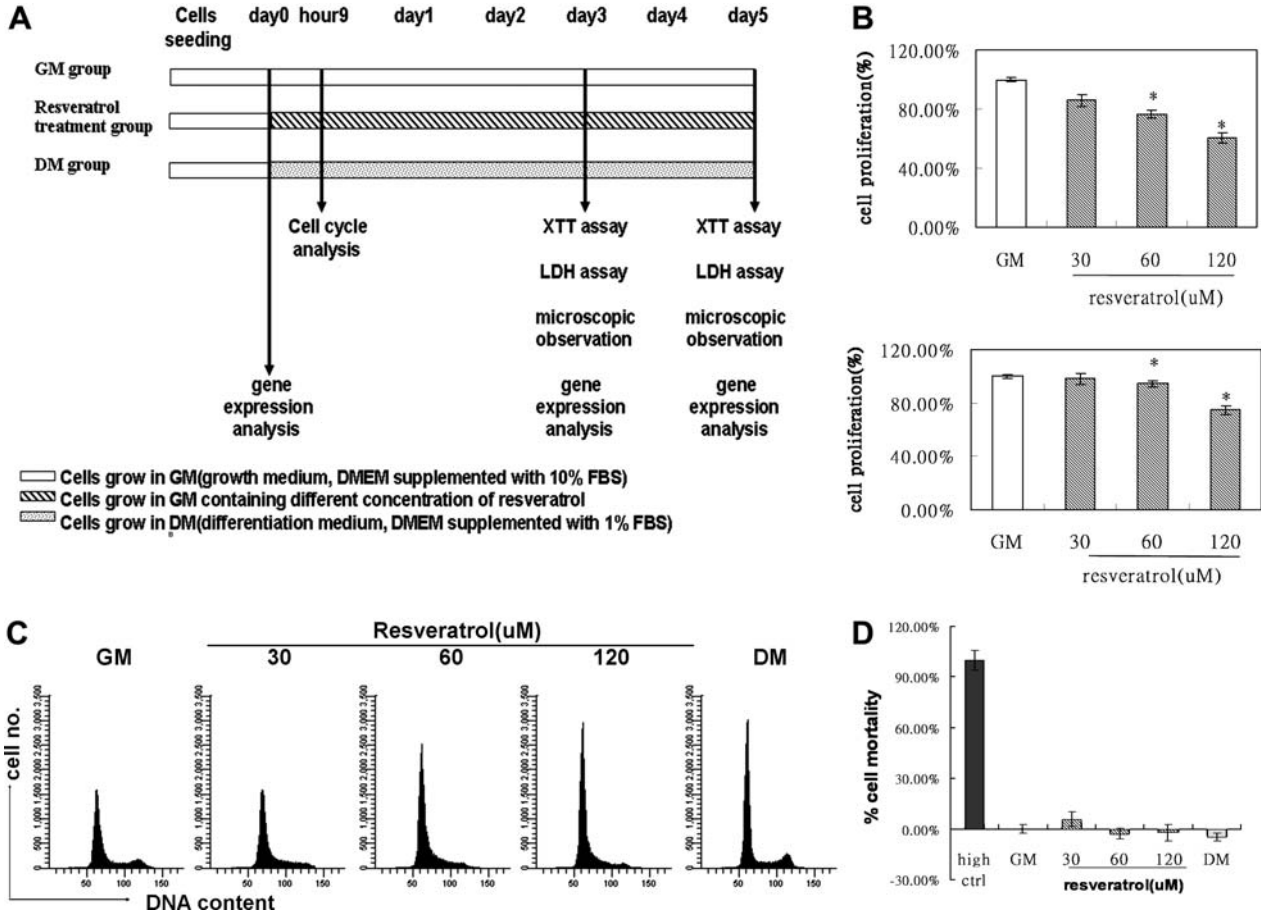

Fig. 1. (A) Schematic illustration of the study design. (B) Growth inhibitory effect of resveratrol on H9c2 cells. (C) Evaluation of cell damage. The results in (B) and (C) represent the means of triplicate experiments ± SEM, $^{*}P < 0.05$ versus GM group. (D) Cell cycle analysis of H9c2 cells by flow cytometry.

was changed every other day. The effect of resveratrol on proliferation of H9c2 cells was determined by XTT at day 3 and day 5. The XTT results showed that resveratrol inhibits cell proliferation in a dose-dependent manner. (Fig. 1B, upper panel) Resveratrol at a concentration of 60 μM began to show statistically significant proliferation inhibitory effects on H9c2 cells at day 3 and day 5. Reductions in proliferation up to 23.2% ($P < 0.03$) and 39.7% ($P < 0.01$) were detected in the 60 and 120 μM resveratrol groups, respectively, at day 3. However, this effect was less pronounced at day 5; only 5.7% ($P < 0.03$) and 25.3% ($P < 0.01$) of reduction in the 60 and 120 μM resveratrol groups were detected, respectively, which may be due to the density of cells reaching a plateau level after 5 days culture (Fig. 1B, lower panel).

We further investigated whether the proliferation inhibitory effect of resveratrol was due to cytotoxicity and cellular damage in H9c2 cells. Twenty-four after plating in a 96-well plate, the medium in the plate was changed to fresh GM containing different concentrations of resveratrol for up to 5 days. GM group served as a vehicle control while cells cultured in DM served as a myogenic differentiation-enhancing control. Medium was changed every other day. Cell damage was evaluated by the amount of lactate dehydrogenase (LDH) released from cells into the medium at day 3 and day 5 (Fig. 1C). The total amount of LDH release was normalized to the percentage of cell viability. The maximum amount of releasable LDH enzyme activity (high control) was determined by lysis of all cells with Triton X-100 (1%, V/V). Our results showed that the cell damage (%) of all groups did not show statistically significant differences versus GM group at day 3 (data not shown) and day 5 (Fig. 3). This demonstrated that resveratrol (30–120 μM) did not cause cellular cytoplasmic membrane damage to H9c2 cells [14,15]. Therefore, the results of Fig. 1B and C suggest that resveratrol can inhibit the proliferation of the cardiomyobast cell line, H9c2 in a dose-dependent manner, without causing cellular cytoplasmic membrane damage.

Cellular morphology observations

During muscle differentiation, it is thought that most of the cell types undergo cell cycle arrest and morphological changes including myoblast elongation and fusion of mono-nucleated myoblasts into multinucleated myotubes [28]. To determine the effect of resveratrol on cardiac differentiation, the differences of cellular morphology between GM, resveratrol and DM groups were compared (Fig. 2). When cells reached 70% confluence, the GM containing

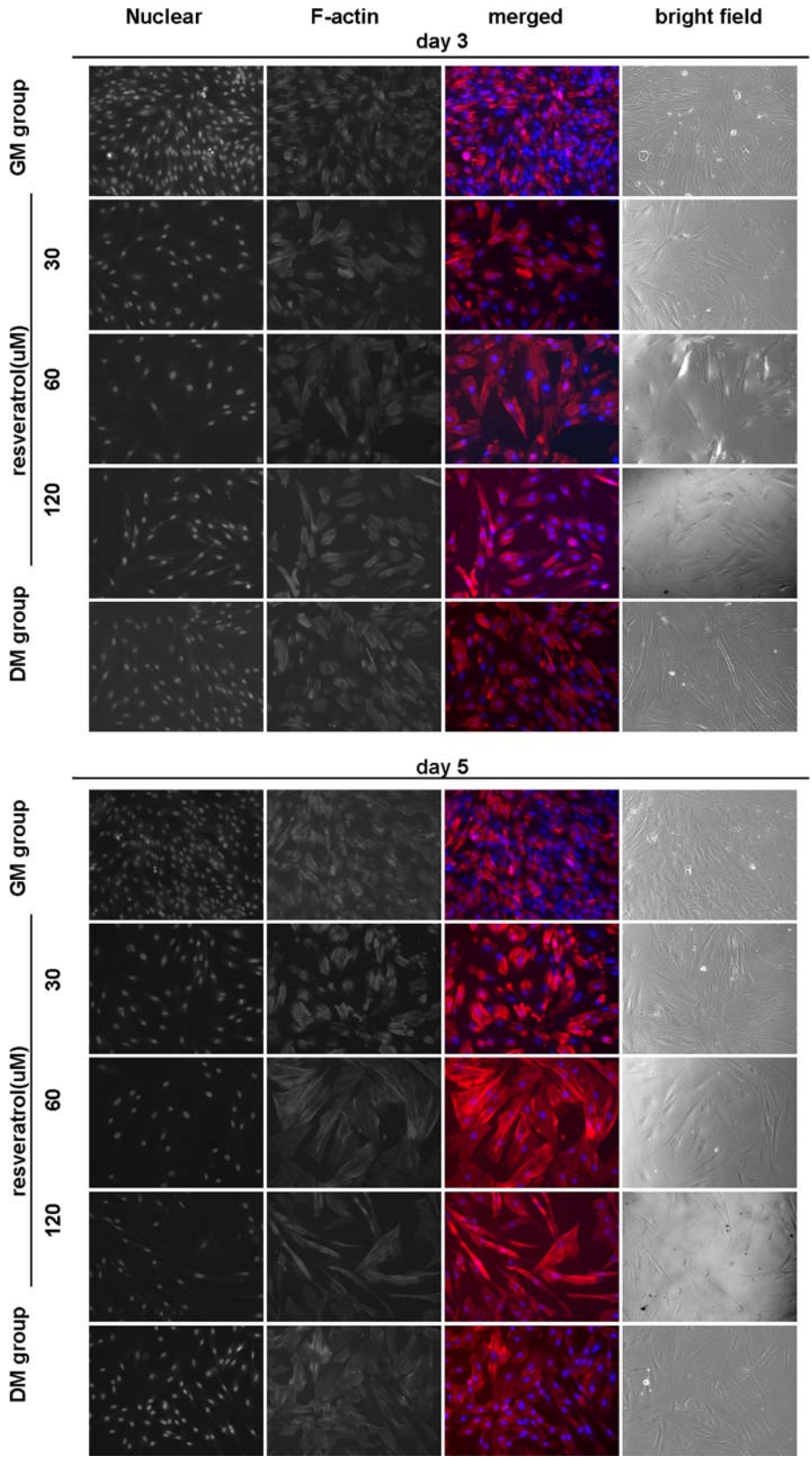

Fig. 2. Observation of F-actin and nuclei under a fluorescent microscope. Magnification 20×.

different concentrations of resveratrol was used to treat the cells for up to 5 days. GM group served as a vehicle control while cells cultured in DM served as a myogenic differentiation-enhancing control. Medium was changed every other day. As shown in Fig. 2 (nuclear), after 3 days of incubation, the number of nuclei is apparently less in the

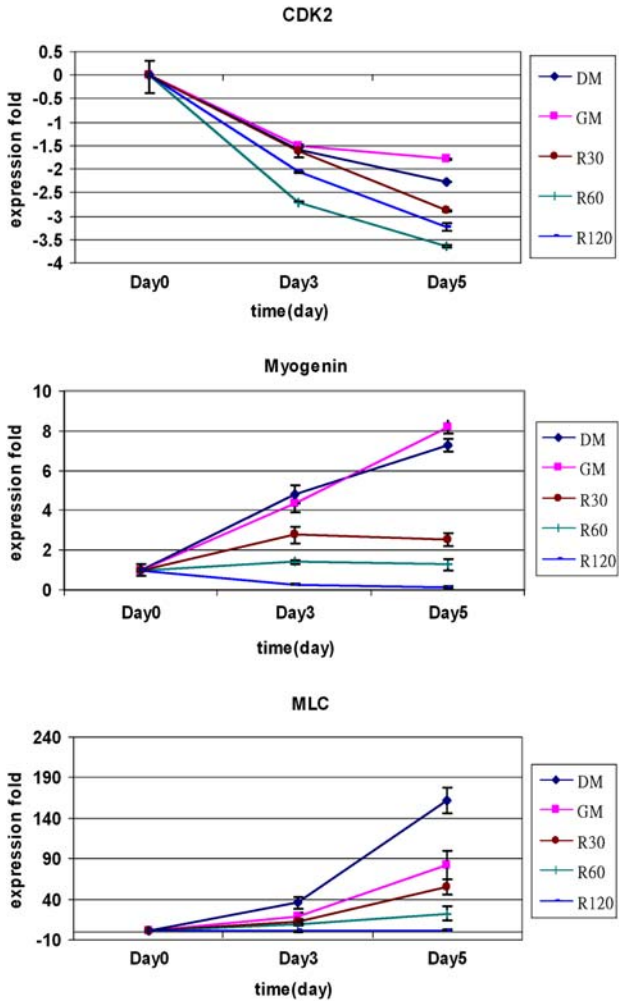

Fig. 3. Gene expression analysis.

resveratrol groups and the DM group compared with the GM group under the same magnification view. This indicated that cell proliferation was suppressed in the resveratrol groups and the DM group. This phenomenon of a cell proliferation suppressive effect was most obvious in the 120 μM resveratrol group and showed a dose-dependent trend among the resveratrol groups. The effect was more dramatic at day 5. In addition, the cell morphology of the DM group displayed the typical characteristic of differentiation, the cells exhibiting an elongated shape and fusing with each other to form multinucleated myotubes at day 3 (Fig. 2, merged) and also at day 5. In GM group, cells grew to a more confluent state, but did not show elongated morphology at day 3 and day 5. Nevertheless, the degree of cell fusion appearing in GM group could not be clearly observed as the cells grew to a high confluence. Compared with GM group, more elongated cells were seen in the resveratrol group, even though 10% serum is present in the medium. Cell elongation started to occur at day1 (data not shown), and exhibited change in a dose-dependent and time-dependent manner (Fig. 2, F-actin). Moreover, the morphology of 60 and 120 μM resveratrol-treated cells tended to show a "branching" phenotype and form di-nucleated cells or tri-nucleated cells (Fig. 2, merged), while multinucleated myotubes were commonly seen in the DM group. These results suggested that resveratrol not only suppresses the proliferation of but also affects the elongation and fusion of cardiomyoblasts.

Cell cycle analysis

To better understand the proliferation and cell cycle regulation of cardiomyoblasts in response to resveratrol, cell cycle analysis was performed as described in Materials and methods. When cells reached 80% confluence, GM containing different concentrations of resveratrol was added to the cells. GM group served as a vehicle control while cells cultured in DM served as a myogenic differentiation-enhancing control. Data from 30,000 cells of each sample were collected for evaluation. A two-parameter dot-plot of forward light scatter versus side scatter was constructed, along with a two-parameter dot-plot of PE (PI) area versus width. In addition, a single-parameter PE (area) histogram was constructed to illustrate the relative DNA content in each cell cycle phase. The cell cycle analysis (Fig. 1D) has revealed that resveratrol causes a dose-dependent accumulation of cells in the G1 phase and a corresponding decrease of cells in the G2–M phases after 9 h incubation. Accumulation of cells in the G1 phase was clearly observed in 60 μM resveratrol-treated cells and increased at a concentration of 120 μM resveratrol. Cells of the DM group also appeared to show a sharp peak in the G1-phase but no decrease in the G2-M phases. Therefore, cell cycle analysis suggested that resveratrol causes G1 phase arrest dose-dependently within 9 h of incubation.

Analysis of mRNA expression by real-time PCR

To further address how resveratrol regulates the cell cycle control and differentiation in cardiomyoblasts, mRNAs from different groups were isolated and converted to cDNA; the relative gene expression was determined at 3 different time points (day 0: before treatment, day 3: 3 days after treatment, day 5: 5 days after treatment) by real-time PCR (Fig. 1). The data was exported to the relative quantification software for calculation of the fold change relative to GM group at day 0.

Regulation of cell cycle G1/S phase transition gene—CDK2

Cyclin-dependent kinase 2 (CDK2) is a catalytic subunit of the cyclin-dependent protein kinase complex, with its activity tightly controlled to the G1–S phase, and essential for cell cycle G1/S phase transition. Our results found that the CDK2 gene expression of all groups exhibited a decreasing trend during 5 days of incubation (Fig. 3). Among all groups, GM group showed a modest down-regulation of CDK2 mRNA expression by 1.5- and 1.8-folds at day 3 and day 5, respectively, compared to day 0. Resveratrol groups at concentrations of 60 and 120 μM showed 2.7- and 2.1-folds of down-regulation, respectively,

at day 3 as well as 3.7- and 3.2-folds, respectively, at day 5. The DM group also showed a higher magnitude of reduction in CDK2 expression compared with GM group at day 3 and day 5. This suggested that the down-regulation of the positive-acting cell cycle regulator CDK2 caused by resveratrol should be a contributing factor for growth inhibitory effect and cell cycle arrest in the G1–S phase.

Regulation of muscle-specific transcription factor gene expression—myogenin

Myogenin is a muscle-specific, basic helix–loop–helix transcription factor that is important during early muscle differentiation. The expression of myogenin is determined as a key step of the differentiation of myoblast cell lines into skeletal myocytes/myotubes [29]. Our results showed that expression of myogenin increased markedly in GM and DM groups at day 3 (4.8-fold) and day 5 (7.2-fold) (Fig. 3). Interestingly, even though serum was present in GM group, the increase of myogenin expression in GM group was very obvious and similar to the DM group. One possible reason is that the high confluence of cells in GM group contributes to initiate transcriptional activation of the intrinsic differentiation program, although we could not clearly observe the phenotype of myogenic differentiation during the 5 days of incubation. Interestingly, resveratrol seemed to significantly and dose-dependently reduce the rate of transcriptional up-regulation of myogenin. This suggests that resveratrol may extend the progression of myogenic differentiation in cardiomyoblasts.

Regulation of contractile apparatus protein gene expression—myosin light chain-2

MLC-2 is one of the contractile proteins in the regulation of myosin ATPase activity in smooth muscle. Also, an increase of mRNA expression of MLC-2 was reported to be observed in the hypertrophic myocardium; thus, MLC-2 is commonly regarded as a useful marker for pathological cardiac hypertrophy [30,31]. Also, MLC-2 could serve as a marker of maturation upon differentiation. The gene expression of MLC-2 was reported to be increased in differentiated cultures of myogenic cell lines [32,33]. Our results showed that MLC-2 mRNA expression displayed a dramatic increase in fold change in GM group (81.6-fold at day 5) and the DM group (163-fold at day5) during the 5 days of incubation. In GM group, the significant up-regulation of MLC-2 mRNA expression was in line with the results of the up-regulation of myogenin mRNA expression; both markers suggest that the intrinsic differentiation program of cells in GM group was transcriptionally activated but the rate of differentiation in GM group should be slower than in DM group, based on the overall assessment of the morphological changes, the higher expression level of CDK2, lower expression level of MLC-2 and the reduced amount of cell cycle alternation in GM group compared with DM group. The resveratrol groups appeared to significantly suppress the increase of MLC-2 mRNA expression, dose-dependently, at both day 3 and day 5.

Discussion

Increasing evidence suggests that resveratrol has an excellent cardioprotective effect [11–13]. However, the mechanisms underlying how resveratrol contributes to gross cardiac function have not been completely elucidated. Over the past decade, more studies have shown that the generation of new myocytes plays a crucial role in the myocardial response to ischemic and non-ischemic injury [17–22]. Although it is a general contention that cardiac myocytes are terminally differentiated cells and regeneration of muscle cells is impossible in adult heart, [34] several reports have indicated that severe ongoing necrotic and apoptotic myocyte death point to the possibility that not all myocytes are terminally differentiated and cell proliferation can possibly be reactivated in the pathologic heart.

Other reports have shown that cardiac myocytes have the ability to proliferate after myocardial infarction, indicating the existence of cardiac stem cells [35,36]. Kajstura et al. have demonstrated that the human heart possesses a cardiac stem cell (CSC) pool that promotes regeneration after infarction, and concluded that the loss of functionally competent CSCs in chronic ischemic cardiomyopathy may underlie the progressive functional deterioration and the onset of terminal failure [37]. All of these studies indicate that the existence of these cardiac stem cells opens new opportunities for myocardial repair. The participation of these cardiac stem cells in myocardial performance recovery is accompanied by a complex process of potential cells recruitment, homing, growth and differentiation. As resveratrol exhibits a remarkable ability to reduce damage in myocardial injury, we hypothesize that resveratrol contributes to modulate the differentiation of cardiomyocyte precursors during heart injury. To demonstrate this, the rat H9c2 embryonic cardiomyoblast cell line, which maintains substantial molecular markers of cardiac myocytes and has the ability to differentiate into multinucleated myotubes, was used for studying the effect of resveratrol on cardiac myogenic proliferation and differentiation.

In our present study, the results of the XTT assay showed that cell proliferation was inhibited significantly after resveratrol treatment (Fig. 1B). Meanwhile, cell elongation occurred and showed a ‘‘branching’’ phenotype, which gave rise to di-nucleated cells or tri-nucleated cells over the 3–5 days of incubation (Fig. 2). Cell cycle analysis revealed that resveratrol caused a G1 cell cycle arrest, evidenced by the obvious accumulation of cells in the G1 phase, dose-dependently (Fig. 1D). During muscle differentiation, it is thought that most of the cell types undergo cell cycle arrest, morphological changes including myoblast elongation and fusion of mono-nucleated myoblasts into multinucleated myotubes [28]. Resveratrol-treated cardiomyoblasts appeared to show some of the above-mentioned differentiation phenotypes. Resveratrol-treated cells tended to enter cell cycle arrest earlier and show bi-nucleated or tri-nucleated forms, which is the hallmark of the ventricular myocytes phenotype [38], but did not reach the status of

multinucleated myotubes, which is a more mature and differentiated phenotype, during 5 days of incubation. This finding inspires our great interest in studying how resveratrol modulates the differentiation markers in cardiomyoblasts. The results of real-time PCR analysis showed that the gene expression of cyclin-dependent kinase 2 (CDK2) among all the groups was down-regulated during the 5 days' culture. Resveratrol groups exhibited stronger down-regulation in a dose-dependent manner compared with GM and DM groups (Fig. 3). As the activity of CDK2 is tightly controlled at the G1–S phase, and essential for cell cycle G1/S phase transition, the significant down-regulation of CDK2 in the resveratrol groups could explain the accumulation of cell populations in the G1 phase in the flow cytometry analysis. All of these results suggest that down-regulation of CDK2 contributed to the inhibitory effect of resveratrol on cell proliferation.

Surprisingly, for the gene expression of myogenin, a marker for the entry of myoblasts into the differentiation pathway, resveratrol treatment was found to significantly and dose-dependently delay its up-regulation (Fig. 3). Moreover, gene expression of contractile apparatus proteins such as MLC-2 has been reported to be transcriptionally upregulated during myogenic differentiation, and also serves as a marker for the maturation phenotype of differentiated myogenic cells. In contrast to the intrinsic up-regulation during the muscle differentiation program in the DM and GM groups, the rate of increasing mRNA expression of MLC-2 in the resveratrol groups was retarded dose-dependently (Fig. 3). In the cellular phenotype observations, unlike in the DM group, resveratrol-treated cardiomyoblasts showed merely bi-nucleated or tri-nucleated forms, but not multinucleated myotubes over 5 days incubation. Therefore, these results provide evidence that resveratrol possesses the capability to extend the duration of the myogenic differentiation program in cardiomyoblasts and to delay the time for reaching the maturation of differentiation. Other reports have also identified that resveratrol modulates proliferation and differentiation in cardiac fibroblasts [39]. This finding provides a new insight into the effects of resveratrol on the gene expression of the muscle-specific markers in cardiomyoblasts, which play important roles in myogenic growth and differentiation. The mechanism underlying the protective effects of resveratrol on myocardium injury is probably accompanied by complex changes in gene expression in myocyte regeneration and differentiation. Investigation of the molecular pathways underlying this process remains a challenge for cardiovascular research. Taking our results together, our study found that resveratrol inhibited proliferation without causing cell injury and modulated the differentiation of morphological characteristics including elongation and cell fusion in cardiomyoblasts *in vitro*. Gene expression analysis revealed that it forces the entry of cell cycle arrest but extended the myogenic differentiation progress. These findings should enhance our understanding of the effects of resveratrol on the modulation of the proliferation and differentiation program in cardiomyoblasts, and facilitate further study on the novel mechanisms underlying the cardioprotective effect of resveratrol.

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