

# High Glucose Causes Human Cardiac Progenitor Cell Dysfunction by Promoting Mitochondrial Fission: Role of a GLUT1 Blocker

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## Abstract

Cardiovascular disease is the most common cause of death in diabetic patients. Hyperglycemia is the primary characteristic of diabetes and is associated with many complications. The role of hyperglycemia in the dysfunction of human cardiac progenitor cells that can regenerate damaged cardiac tissue has been investigated, but the exact mechanism underlying this association is not clear. Thus, we examined whether hyperglycemia could regulate mitochondrial dynamics and lead to cardiac progenitor cell dysfunction, and whether blocking glucose uptake could rescue this dysfunction. High glucose in cardiac progenitor cells results in reduced cell viability and decreased expression of cell cycle-related molecules, including CDK2 and cyclin E. A tube formation assay revealed that hyperglycemia led to a significant decrease in the tube-forming ability of cardiac progenitor cells. Fluorescent labeling of cardiac progenitor cell mitochondria revealed that hyperglycemia alters mitochondrial dynamics and increases expression of fission-related proteins, including Fis1 and Drp1. Moreover, we showed that specific blockage of GLUT1 improved cell viability, tube formation, and regulation of mitochondrial dynamics in cardiac progenitor cells. To our knowledge, this study is the first to demonstrate that high glucose leads to cardiac progenitor cell dysfunction through an increase in mitochondrial fission, and that a GLUT1 blocker can rescue cardiac progenitor cell dysfunction and downregulation of mitochondrial fission. Combined therapy with cardiac progenitor cells and a GLUT1 blocker may provide a novel strategy for cardiac progenitor cell therapy in cardiovascular disease patients with diabetes.

**Key Words:** Cardiac progenitor cell, Hyperglycemia, Fasentin, Mitochondrial dynamics, Diabetic cardiomyopathy

## INTRODUCTION

Diabetes mellitus (DM) is one of the main threats to human health in the 21<sup>st</sup> century: it is regarded as the fifth leading cause of death worldwide. Environmental and lifestyle changes are the main reasons for an increase in this disease, and the number of studies to better understand DM is growing (Zimmet *et al.*, 2001; Roglic *et al.*, 2005). There are two types of DM: type 1 DM (T1DM) and type 2 DM (T2DM). T1DM patients do not secrete insulin, which is the hormone that decreases blood sugar, because of autoimmune destruction of beta cells in the pancreas (Di Lorenzo *et al.*, 2007). In contrast, T2DM patients are insulin resistant; although they se-

crete insulin, its function is diminished in the body. Patients of both DM types are hyperglycemic. Hyperglycemia, the major characteristic of DM, leads to complications, including stroke, cardiovascular disease, and neuropathy. Cardiovascular disease accounts for the largest proportion of deaths in diabetic patients (Bell, 2003; Davidson and Parkin, 2009). Nevertheless, studies on diabetic cardiomyopathy have only recently been undertaken, and the relationship between diabetic cardiomyopathy and hyperglycemia is still not well understood.

Human cardiac progenitor cells (hCPCs) have the ability to regenerate damaged cardiac tissue. These cells self-renew and differentiate into endothelial cells, alpha-smooth muscle actin, and cardiomyocytes (Leri *et al.*, 2005). Therefore, there

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is potential for the use of hCPCs in cell therapies. In a previous study, a population of hCPCs was shown to decrease with hyperglycemia, indicating chronic dysfunction of the cells (Molgat *et al.*, 2014). Although the effects of hyperglycemia on hCPCs have been studied, the mechanism underlying the association has not been fully established.

The heart requires more energy than other organs; thus, studies on mitochondria, which are the critical intracellular organelles for energy metabolism, have been a focus of research. In their normal state, mitochondria establish an active network within cells. They continuously join by the process of fusion and divide by the process of fission (Archer, 2013). However, in abnormal states, such as those associated with hyperglycemia, hyperinsulinemia, and hypertension, mitochondrial dynamics are out of balance (Lowell and Shulman, 2005; Yu *et al.*, 2008). This imbalance leads to cellular dysfunction (van der Blik *et al.*, 2013). In recent studies, the association between mitochondrial dynamics and cardiovascular disease has been explored (Knowlton and Liu, 2015), and mitochondrial dysfunction as a major cause of various diseases has been postulated (Archer, 2013).

In this study, we established an *in vitro* model of hyperglycemia. To mimic hyperglycemic conditions, we treated hCPCs with glucose above the physiological concentration. We hypothesized that high glucose affects hCPCs and alters mitochondrial dynamics. Furthermore, we examined whether blocking glucose uptake could rescue hCPC function.

## MATERIALS AND METHODS

### Isolation of c-kit positive human cardiac progenitor cells (hCPC<sup>c-kit+</sup>)

We used protocols modified from a previously described method (Choi *et al.*, 2013) to isolate hCPC<sup>c-kit+</sup> from human infant-derived heart tissue. The Ethical Review Board of the Pusan National University Yang San Hospital, Gyongsangnam-do, Republic of Korea, approved the protocols (IRB 05-2015-133). First, biopsy specimens from the atria of human hearts were digested to obtain a suspension of single cardiac cells, and then these cells were incubated in cardiac expansion media. The expanded cells were conjugated with c-kit primary antibody (Santa Cruz, Santa Cruz, CA, USA) and sorted by magnetic activated cell sorting (MACS). In the current study, all experiments were conducted with hCPCs from passage numbers 7 and 8.

### Drugs and CPC culture

hCPCs were maintained in Ham's F12 medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS, Gibco, CA, USA), 1× penicillin/streptomycin (PS, Welgene, Daegu, Republic of Korea), 2.5 U of human erythropoietin (hEPO, R&D Systems, Minneapolis, MN, USA), 10 ng/mL of basic human recombinant fibroblast growth factor (bFGF, Peprotech, Rocky Hill, NJ, USA), and 0.2 mM/L glutathione (Sigma-Aldrich, St. Louis, CA, USA). hCPCs cultured in growth medium containing D-glucose (Sigma-Aldrich) and Fasentin (Sigma-Aldrich) was used as a GLUT1 blocker (Wood *et al.*, 2008), and dapagliflozin (Selleckchem, Houston, TX, USA) was used as an SGLT2 blocker. Both blockers were used to co-treat hCPCs cultured in medium supplemented with 25 mM D-glucose.

### Cell viability assay

A cell viability assay (WST kit, Ez-Cytox, Daillab, Seoul, Republic of Korea) was performed to compare the viability of the control and high glucose-exposed hCPCs. hCPCs were seeded on 96-well plates, and then the maintenance medium was changed to hCPC culture medium containing 5 mM, 15 mM, or 25 mM D-glucose. Culture plates were incubated for 24 h or 72 h. Following incubation, the D-glucose-containing culture medium was removed and replaced with WST solution. The plates were incubated for another 2 h, and the absorbance of each sample was measured at a wavelength of 450 nm using an absorbance reader (Tecan, Grodig, Austria).

### Annexin V/Propidium Iodide (PI) staining

For apoptosis analysis, hCPCs were cultured in hCPC medium containing 5 mM, 10 mM, 15 mM, or 25 mM D-glucose. Cells were stained using an Annexin V/PI staining Kit (Becton Dickinson, San Jose, CA, USA) according to the manufacturer's protocol. After staining, data were analyzed by flow cytometry (BD Accuri, Becton Dickinson).

### Western blot analysis

Antibodies against CDK2 (Santa Cruz), cyclin E (Santa Cruz), Fis1 (Abcam, Cambridge, UK), Drp1 (BD Biosciences, Franklin Lakes, NJ, USA), Mfn1 (Santa Cruz), Mfn2 (Abcam), and OPA1 (Abcam) were used. In general, 10-25 µg of total protein was loaded, and the blots were visualized by chemiluminescence (ECL solution, Bionote, Hwaseong, Republic of Korea) using an LAS-3000 Imaging System (Fuji Film, Tokyo, Japan). A quantitative analysis was performed using Statview software® (Version 5.0.1, SAS Institute Inc, Cary, NC, USA), and the respective protein expression levels were normalized to that of β-actin (Santa Cruz).

### Tube formation assay

hCPCs were cultured with high glucose (5 mM, 15 mM, or 25 mM) for 72 h. The 96-well plates were coated with 60 µL of Matrigel (BD Biosciences), and then the plates were incubated for 30 min. hCPCs were harvested, seeded on the 96-well plates coated with Matrigel, and incubated for another 8 h. After incubation, the lengths of mitochondria were determined using ImageJ software (NIH, Bethesda, MD, USA).

### Immunofluorescence

Mitochondrial morphology was determined by staining hCPCs with 200 nM MitoTracker Red CMXRos (Molecular Probes, Eugene, OR, USA) and visualizing cells under a fluorescence microscope (Olympus, Tokyo, Japan). To determine changes in mitochondrial morphology, we measured the total length of mitochondria using ImageJ software.

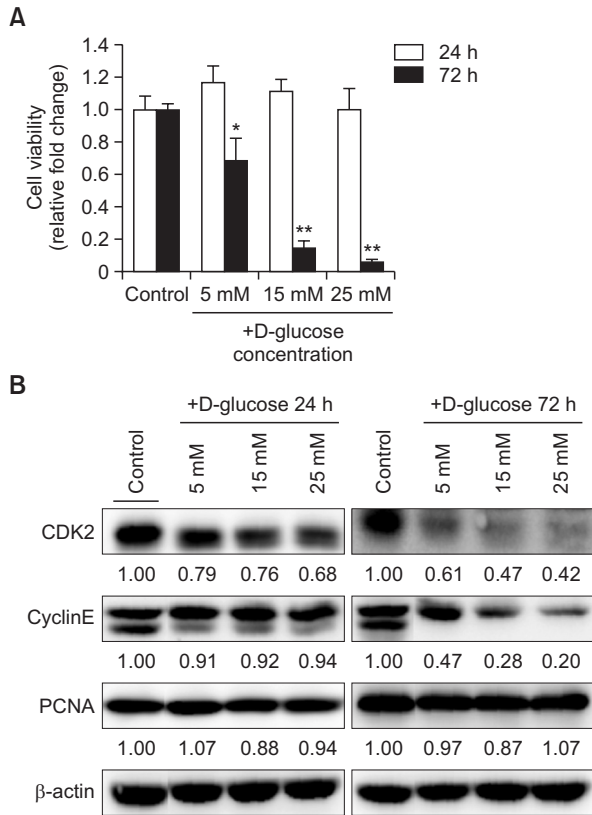
### Statistical analysis

All values are reported as the mean ± standard deviation (SD). Results were compared by unpaired Student's *t*-test and considered statistically significant at *p*-values <0.05.

## RESULTS

### High-dose D-glucose decreased viability and progression of the cell cycle in hCPCs

hCPCs were treated with doses of D-glucose (5 mM, 15 mM,

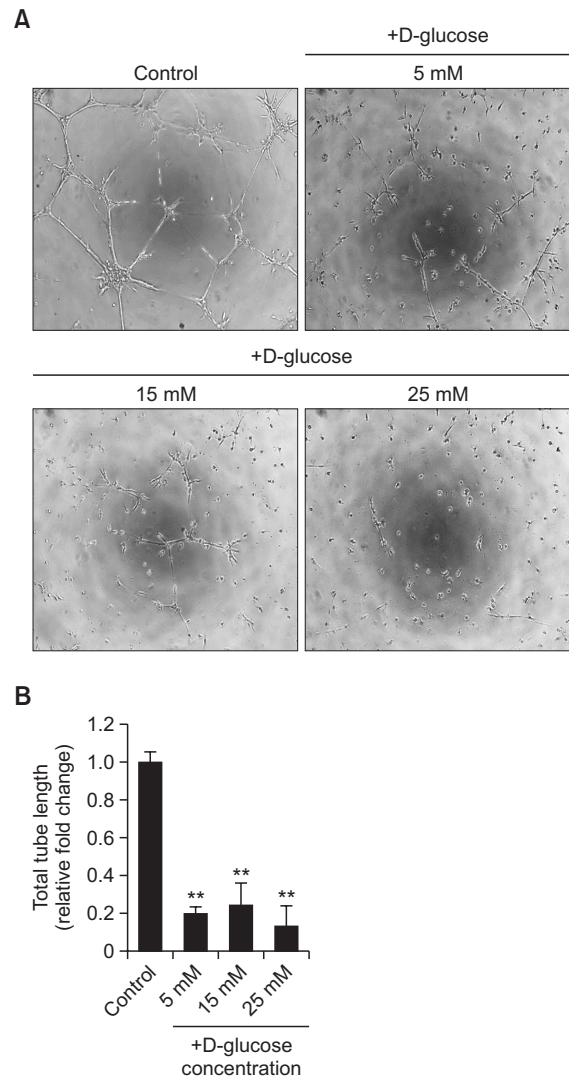


**Fig. 1.** Viability and cell cycling of c-kit<sup>+</sup> hCPCs treated with different doses of D-glucose and different exposure times. (A) Percentage of viable hCPCs following treatment with different doses of D-glucose (5 mM, 15 mM, or 25 mM) for 24 h or 72 h. hCPC viability was significantly and dose-dependently reduced following treatment with D-glucose for 72 h. Results are presented as means  $\pm$  SD. \* $p$ <0.05 vs. control, \*\* $p$ <0.01 vs. control. (B) After treatment with D-glucose, hCPCs were harvested and cell cycle-related proteins were analyzed by western blotting. Western blots of hCPCs exposed for 72 h indicated decreased expression of CDK2 and cyclin E.

or 25 mM) above that of the normal culture medium (D-glucose, 5 mM). To examine whether treatment with high doses of D-glucose could affect hCPCs, a WST assay was conducted (Fig. 1A). Treatment of hCPCs with high doses of D-glucose for 24 h did not show any effect. However, hCPCs exposed to high doses of D-glucose for 72 h showed significant dose-dependent decreases. Western blot analysis demonstrated that expression of cell cycle-related proteins decreased when hCPCs were treated with high doses of D-glucose for 72 h (Fig. 1B). These data suggest that high doses of D-glucose lead to a decrease in hCPC viability and cell cycle progression.

### High-dose D-glucose decreased the tube-forming capacity of hCPCs

To determine whether high doses of D-glucose are associated with hCPC dysfunction, we treated cells with different doses of D-glucose (5 mM, 15 mM, or 25 mM) for 72 h, and then the tube-forming capacity of hCPCs was assessed by viewing the formation of capillary networks on Matrigel (Fig. 2A, 2B). Tube formation decreased significantly relative to that of the control, when hCPCs were treated with high doses of

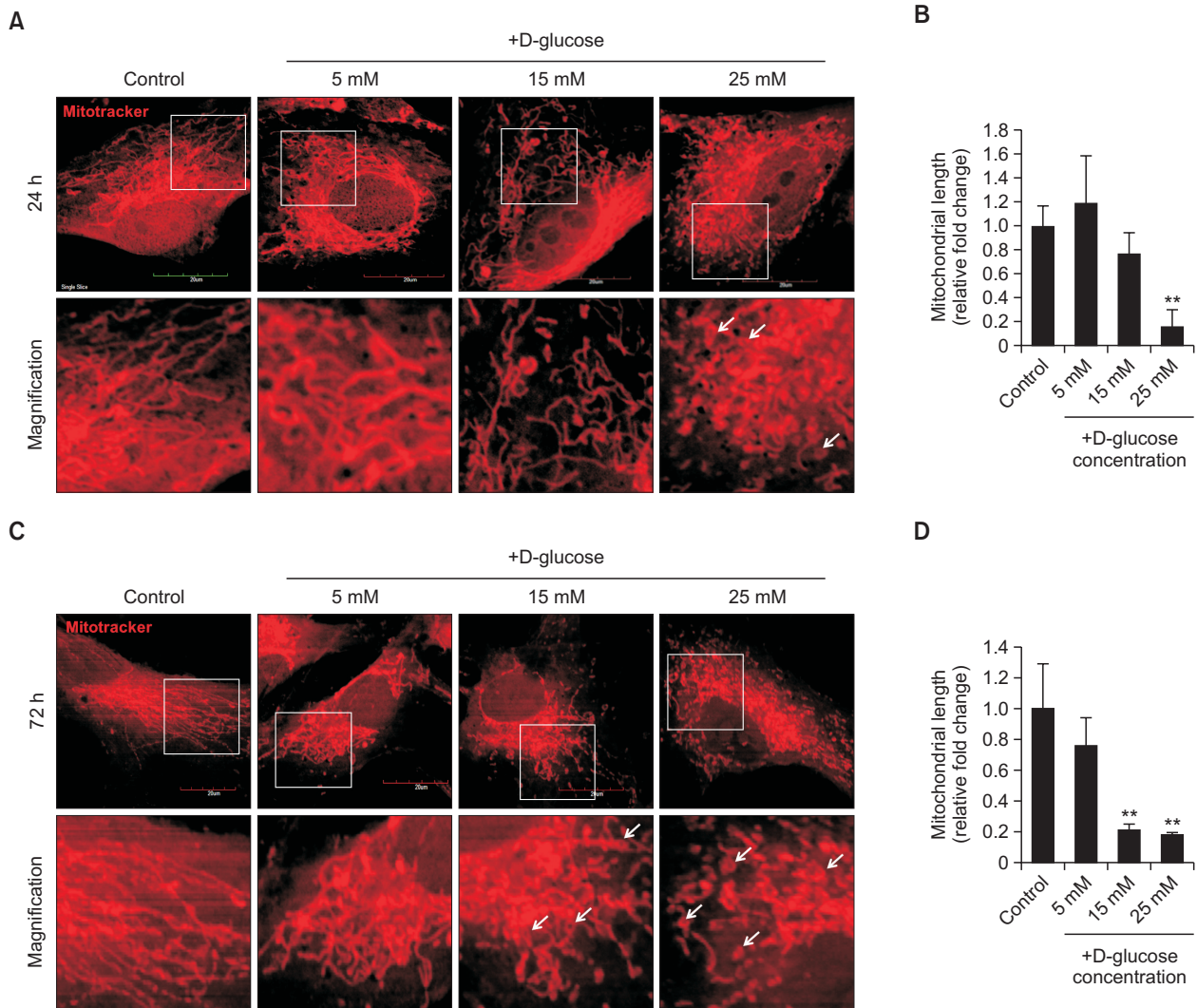


**Fig. 2.** The effect of high-dose D-glucose on the tube-forming capacity of hCPCs. (A) The capacity of hCPCs exposed to high doses of D-glucose for 72 h to form angiogenesis-like networks *in vitro* was analyzed using a Matrigel tube formation assay. hCPCs treated with high doses of D-glucose for 72 h showed markedly diminished tube formation capacity. (B) Total mitochondrial tube lengths. Results are presented as means  $\pm$  SD. \*\* $p$ <0.01 vs. control.

D-glucose. These data indicate that the differentiation capacity of hCPCs was impaired by prolonged exposure to high D-glucose.

### High-dose D-glucose altered mitochondrial morphology

Alterations in the extracellular environment, including the initiation of hyperglycemia, hyperinsulinemia, or hyperlipidemia, cause mitochondrial dysfunction in T2DM (Guilherme *et al.*, 2008; Muoio and Newgard, 2008). To investigate whether hyperglycemia is involved in modifying mitochondrial dynamics, we treated hCPCs with a high dose of D-glucose for 24 h and 72 h, and used MitoTracker Red CMXRos to stain mitochondria and obtained images under a fluorescence microscope. Mitochondria appeared as fragmented, discontinuous



**Fig. 3.** High doses of D-glucose alter the morphologies of mitochondria in hCPCs. (A, C) Images of hCPCs stained with MitoTracker Red CMXRos after of 24 h (A) and 72 h (C) of incubation in medium containing 5 mM, 15 mM, or 25 mM D-glucose. The arrows indicate tubular mitochondria in hCPCs treated with high doses of D-glucose. (B, D) Mitochondrial length in hCPCs incubated in media with high doses of D-glucose for 24 h (B) or 72 h (D). Results are presented as means  $\pm$  SD. \*\* $p < 0.01$  vs. control.

networks after hCPCs were treated with 25 mM D-glucose for 24 h or with 15 mM or 25 mM D-glucose for 72 h (Fig. 3A, 3C). Determination of the total lengths of mitochondria revealed that treatment with high doses of D-glucose shifted mitochondrial morphology toward a fission type in a time- and dose-dependent manner (Fig. 3B, 3D).

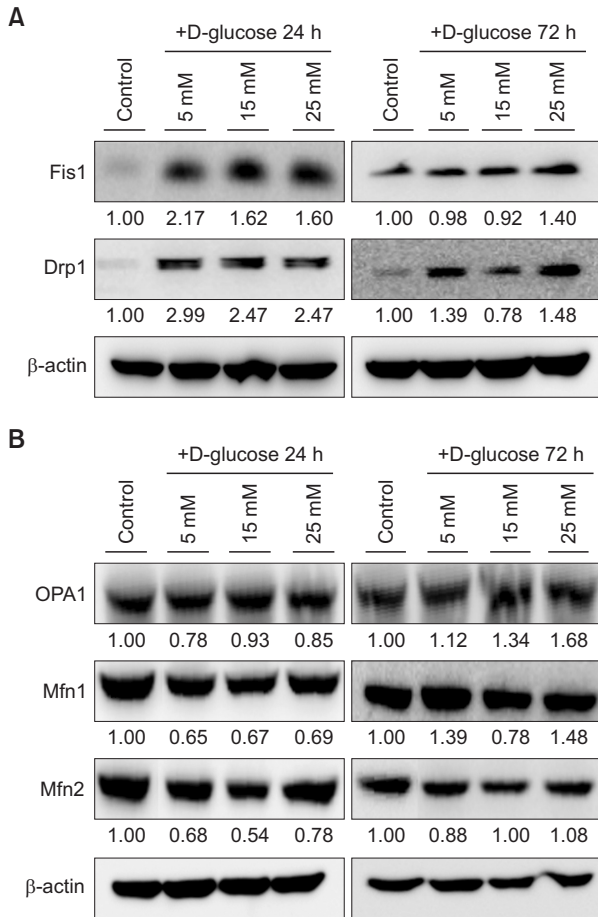
**High-dose D-glucose increased mitochondrial fission-associated Drp1 and Fis1**

To examine which molecules, those associated with mitochondrial fusion or mitochondrial fission, mediated mitochondrial fragmentation in a high D-glucose environment, we treated hCPCs with a high dose of D-glucose for 24 h and 72 h and performed a western blot analysis. While no significant difference in the levels of mitochondrial fusion-related proteins Mfn1, Mfn2, and OPA1 was detected between D-glucose- and vehicle-treated hCPCs (Fig. 4B), the mitochondrial fission-related proteins Drp1 and Fis1 were greatly increased in the

high-dose D-glucose treatment groups (Fig. 4A). These results indicate that the molecules that regulate mitochondrial fission increase with high doses of D-glucose, contributing to an imbalance in mitochondrial dynamics that favors fission.

**Inhibition of D-glucose uptake recovered hCPC dysfunction caused by high dose of D-glucose**

We next asked whether inhibition of D-glucose uptake by blocking the glucose transporter could rescue high-dose D-glucose-induced decreases in hCPC viability and cell cycling. We co-treated hCPCs with 25 mM D-glucose and 1  $\mu$ M Fasentin or dapagliflozin, which block GLUT1 and SGLT2, respectively. Following co-treatment for 72 h, we performed WST and tube formation assays. First, we observed that co-treatment with 1  $\mu$ M Fasentin and 25 mM D-glucose for 72 h significantly increased hCPC viability relative to that of the high-dose D-glucose treatment group (Fig. 5A). However, cells co-treated with 1  $\mu$ M dapagliflozin and 25 mM D-glucose for 72 h did not



**Fig. 4.** Proteins related to mitochondrial dynamics in hCPC culture medium containing high doses of D-glucose. (A) Western blot analysis of fission-related proteins (Fis1 and Drp1) in high dose-exposed hCPCs and control. hCPCs treated with high doses of D-glucose showed significantly higher Fis1 and Drp1 expression than untreated hCPCs. (B) Western blot analysis of fusion-related proteins (Mfn1, Mfn2, and OPA1) in high dose-exposed hCPCs and control. Treatment with high doses of D-glucose showed no effects on Mfn1, Mfn2, and OPA1 expression in hCPCs.

differ from control cells (Fig. 5B). Thus, our data indicated that blocking glucose uptake through GLUT1-specific inhibition recovered hCPC viability that decreased with high doses of D-glucose. Amelioration of 25 mM D-glucose-induced dysfunction of hCPCs by co-treatment with 1  $\mu$ M Fasentin was also associated with recovery of hCPC tube-forming ability (Fig. 5C). Therefore, the inhibition of glucose uptake by a specific inhibitor of GLUT1 improved the differentiation capacity of hCPCs.

#### Inhibition of D-glucose uptake decreased mitochondrial fission caused by high doses of D-glucose

We further examined whether the GLUT1 inhibitor Fasentin rescued hCPC dysfunction and regulated mitochondria dynamics, and found that 1  $\mu$ M Fasentin inhibited mitochondrial fission caused by high doses of D-glucose. As shown in Fig. 6A, treatment with 1  $\mu$ M Fasentin mitigated the fragmented and discontinuous network. The total lengths of mitochondria were significantly greater in hCPCs treated with 1  $\mu$ M Fasentin

for 72 h than in untreated hCPCs. We next demonstrated that the levels of mitochondrial fission-related proteins Fis1 and Drp1 decreased when glucose uptake was blocked in hCPCs. Thus, excessive mitochondrial fission resulting from high doses of D-glucose was moderated by blockage of glucose uptake through specific inhibition of GLUT1 in hCPCs (Fig. 6B).

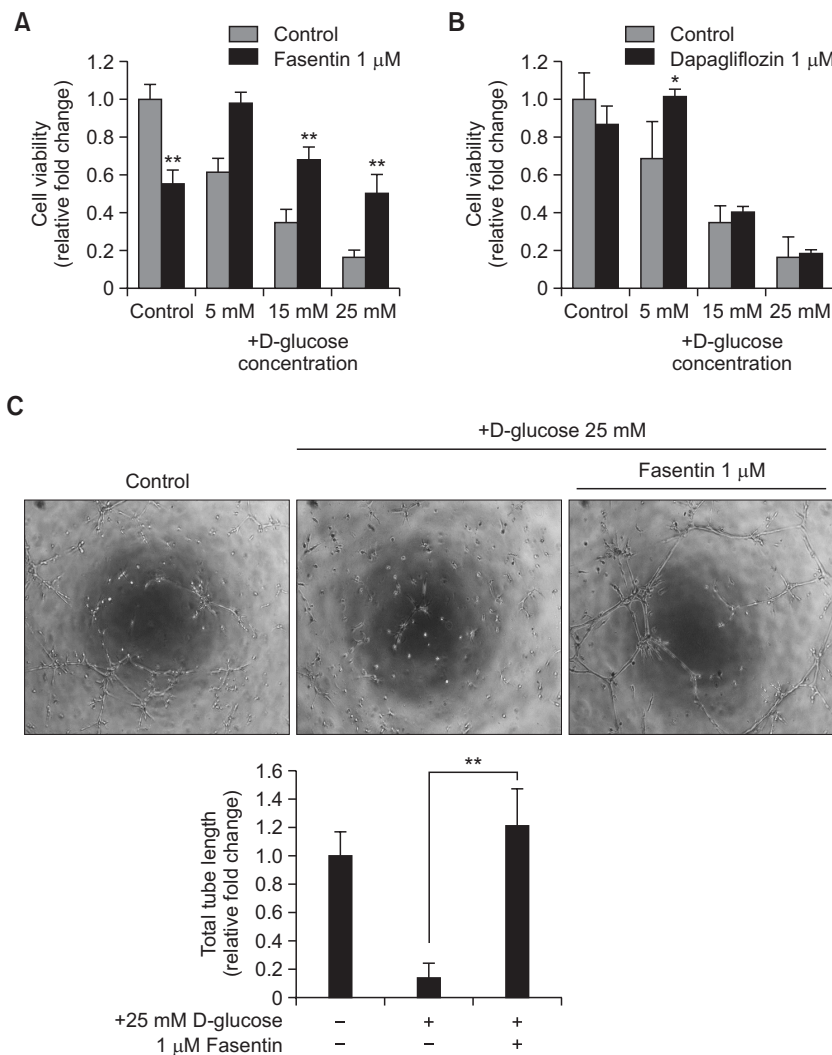
## DISCUSSION

The idea that an imbalance in mitochondrial dynamics underlies the pathogenesis of cardiovascular disease is gaining support. For example, a previous study showed that mitochondrial fragmentation in HL-1 cardiac cells favoring Drp1-dependent processes plays a critical role in myocardial ischemia (Ong *et al.*, 2010). Another study revealed changes in mitochondrial dynamics during simulated ischemia and reperfusion in H9c2 cells (Liu and Hajnoczky, 2011). In addition, there have been several studies on diabetic conditions using cardiac progenitor cells (Rota *et al.*, 2006; Molgat *et al.*, 2014). These studies all support the notion that DM is a clear cause of hCPC dysfunction. However, it is not clear which specific factors in DM are related to hCPC dysfunction and mitochondrial dynamics.

Our results suggest that the mitochondrial fission process is likely to be a main regulating process involved in the alteration of mitochondrial dynamics. In agreement with our hypothesis, high doses of D-glucose resulted in hCPC dysfunction, and fission-related proteins Drp1 and Fis1 were increased in hCPCs treated with high doses of D-glucose. Treatment of hCPCs with doses of D-glucose above that of normal culture medium decreased cell viability, as shown by the MTS assay. Interestingly, we found that reduced cell viability seems to be not related cell apoptosis, demonstrated by Annexin V/PI staining (Supplemental figure). Cell cycle-related proteins CDK2 and cyclin E, involved in progression from the late G1 phase to the early S phase, were evaluated by western blot analysis, and the results indicated an interruption in cell cycle progression. Thus, high doses of D-glucose correlate with decreased viability and function of hCPCs. We next investigated whether high doses of D-glucose impaired the tube-forming capacity of hCPCs, because hCPCs differentiate into endothelial cells, as well as smooth muscle actin and cardiomyocytes following cardiac injury.

Mitochondrial dynamics in hCPCs are imbalanced by high doses of D-glucose. This was demonstrated by images of mitochondrial morphologies, showing a change from a continuous network to a fragmented and discontinuous network. Western blot analysis revealed increases in mitochondrial fission-related proteins Drp1 and Fis1 mediated by high doses of D-glucose. Interestingly, we found that the levels of mitochondrial fusion-related proteins (Mfn1, Mfn2, and OPA1) did not differ from those of the vehicle control. These data indicate that high doses of D-glucose in hCPCs induce excessive fission, or fragmentation, in mitochondria, but it still remains to be resolved how high D-glucose upregulates mitochondrial fission-related proteins Drp-1 and Fis-1 and causes mitochondrial fragmentation.

In high D-glucose conditions, increases in intracellular glucose follow increases in glucose uptake. Fasentin, a GLUT1 blocker, was used in the co-treatment of hCPCs with 25 mM D-glucose for 72 h. In agreement with our hypothesis, specifi-



**Fig. 5.** GLUT1 blocker rescues CPCs dysfunction in hyperglycemic conditions. (A) Percentage of viable hCPCs following treatment with Fasentin and 25 mM D-glucose for 72 h. hCPC viability was significantly increased with exposure to 1 μM Fasentin and 25 mM D-glucose for 72 h relative to that of the control. Results are presented as means ± SD. \*\**p*<0.01 vs. control. (B) Percentage of viable hCPCs following treatment with dapagliflozin and 25 mM D-glucose for 72 h. Viability was not significant different in hCPCs exposed to 1 μM dapagliflozin and 25 mM D-glucose for 72 h and in the control. Results are presented as means ± SD. \**p*<0.05 vs. control. \*\**p*<0.01 vs. control. (C) The tube formation ability of hCPCs treated with 1 μM Fasentin. Total tube length is presented in the lower panel. Results are presented as means ± SD. \*\**p*<0.01 vs. + 25 mM D-glucose.

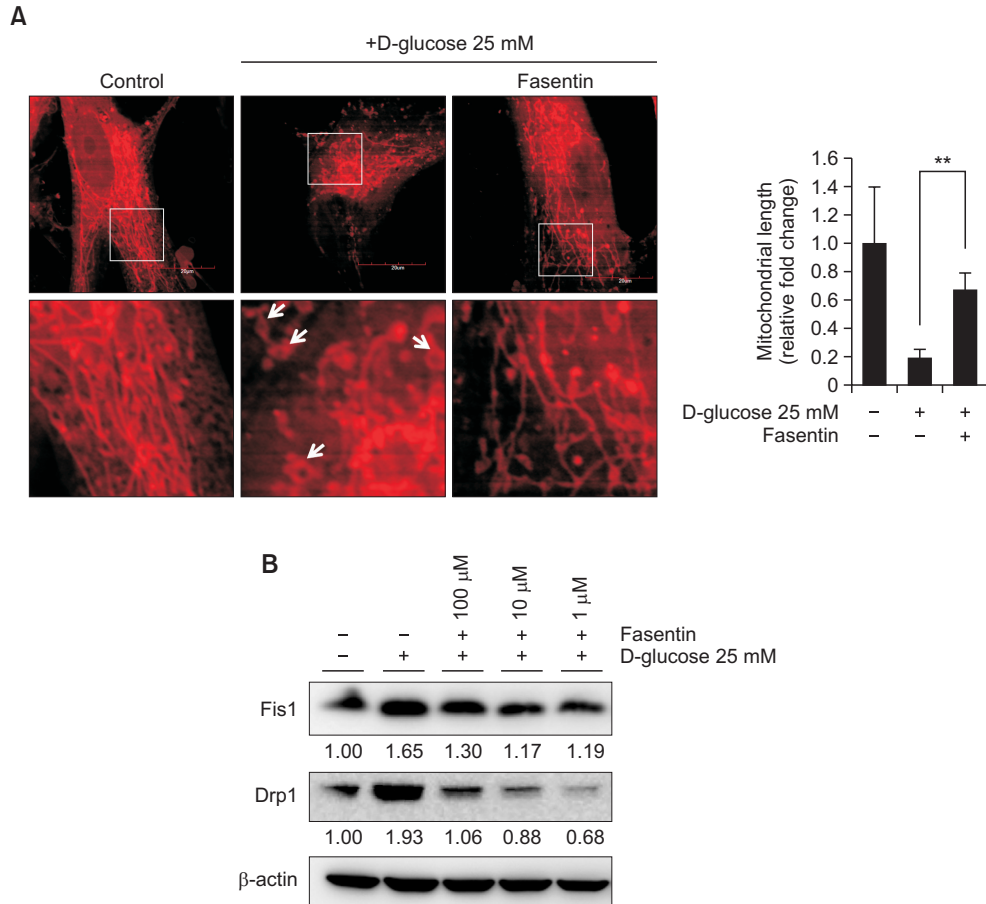
cally blocking glucose uptake rescued hCPC viability and tube formation. We suggest that GLUT1 is the major protein for glucose uptake in hCPCs. Furthermore, Fasentin attenuated mitochondrial fragmentation by selectively inhibiting glucose uptake. We demonstrated the recovery of mitochondrial length and mitochondrial fission-related proteins by co-treatment of hCPCs with Fasentin and 25 mM D-glucose for 72 h. Thus, our results provide evidence that specific inhibition of glucose uptake in hCPCs can prevent mitochondrial fragmentation in hCPCs.

In conclusion, we established hyperglycemic conditions *in vitro* by adding D-glucose to normal culture medium. We provide evidence that high-dose D-glucose leads to hCPC dysfunction and the promotion of mitochondrial fission. These effects were ameliorated by specifically blocking GLUT1. When hCPCs are exposed to hyperglycemic conditions, the intracel-

lular glucose concentration in hCPCs increases with glucose uptake. High doses of glucose within hCPCs causes abnormal metabolism in mitochondria, leading to imbalanced mitochondrial dynamics and dysfunction in hCPCs. For this reason, it is important that the glucose concentration in hCPCs is decreased when glucose uptake is blocked. Therefore, blocking glucose uptake might provide a novel therapeutic strategy for DM.

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**Fig. 6.** GLUT-1 blockade rescues abnormal mitochondrial morphology and downregulates mitochondrial fission related to protein level. (A) Images of hCPCs stained with MitoTracker Red CMXRos after co-treatment with 25 mM D-glucose and 1  $\mu$ M Fasentin for 72 h. The arrows indicate tubular mitochondria. Mitochondrial length is presentation in the right panel. Results are presented as means  $\pm$  SD. \*\* $p$ <0.01 vs. + 25 mM D-glucose. (B) Expression of mitochondrial fission-related markers Fis1 and Drp1 in hCPCs exposed to 1  $\mu$ M Fasentin and 25 mM D-glucose. Value were normalized with those of  $\beta$ -actin.

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