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β-Catenin Mediates Cyclic Strain-Stimulated Cardiomyogenesis in Mouse Embryonic Stem Cells Through ROS-Dependent and Integrin-Mediated PI3K/Akt Pathways

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ABSTRACT

Wnt/β-catenin signaling regulates various cellular events involved in the proliferation and differentiation and these events are affected sensitively by applying to mechanical stimuli. However, the mechanisms by which mechanical force stimulates cardiomyogenesis are not extensively explored. In this study we investigated the cellular mechanisms by which β-catenin signaling regulates cardiac differentiation of strain-subjected embryonic stem (ES) cells. The application of cells to cyclic strain increased beating cardiomyocyte foci with the attendant increases of Cx 43 and Nkx 2.5 proteins. Anti-oxidants such as vitamin C or *N*-acetyl cysteine (NAC) blocked the strain-mediated increases of Cx 43, Nkx 2.5, and α5/β1 integrins. These anti-oxidants also suppressed the activation of phosphoinositide 3-kinase (PI3K) and Akt in cyclic strain-subjected cells. Western blot analysis revealed that PI3K is a critical downstream effector of β1 integrin signaling and mediates Cx 43 and Nkx 2.5 expression in cyclic strain-applied ES cells. Cyclic strain increased the expression of β-catenin and stimulated its nuclear translocation from the cytosol, which was prevented by anti-oxidant treatment. In addition, the application to cyclic strain increased mRNA expression of β-catenin target genes, Axin2 and c-myc, as well as the phosphorylation of glycogen synthase kinase-3β. Furthermore, the blockage of β-catenin by its specific siRNA transfection diminished the cellular levels of Cx 43 and Nkx 2.5 proteins and the number of beating cardiomyocyte foci. Collectively, these results suggest that β-catenin-mediated signaling is required for cyclic strain-stimulated cardiomyogenesis through ROS-dependent and integrin-mediated PI3K–Akt signaling cascades. J. Cell. Biochem. 112: 1880–1889, 2011.

KEY WORDS: EMBRYONIC STEM CELLS; CYCLIC STRAIN; CARDIOMYOGENESIS; INTEGRINS; PI3K/AKT; β-CATENIN

Stem cells have self-renewal capacity with the ability to differentiate into multiple types of cells. Thus, they provide a source of uncommitted progenitor cells for therapeutic applications by means of cell transplantation or tissue engineering [Smith, 2001; Weissman et al., 2001]. Controlling the steps required for differentiation is one of the most important events in stem cell therapy. The process of cell differentiation is influenced by various biochemical factors, such as growth factors and hormones. Mechanical stimuli also affect cellular events, such as metabolism, proliferation, and differentiation [Kook et al., 2009; Obi et al., 2009]. Indeed, numerous in vivo and in vitro studies have provided evidence that mechanical stimuli including strain, fluid shear stress, and hydrostatic compression, modulate cellular differentiation, particularly of vascular smooth muscle cells, endothelial cells, and chondrocytes [Shimizu et al., 2008; Bougault et al., 2009; Obi et al., 2009]. It was recently reported that mechanical strain induces cardiovascular differentiation from embryonic stem (ES) cells through the production of intracellular reactive oxygen species (ROS) [Schmelter et al., 2006]. In addition, the application of cyclic strain enhanced cardiomyocyte differentiation of ES cells cultured

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in an elastic polymer scaffold [Gwak et al., 2008]. These data indicate that mechanical stress affects the signal transduction pathways involved cellular differentiation.

Integrins are implicated as critical transducers of mechanical signals into cellular signals [Katsumi et al., 2004]. Integrins also play an important role in regulating cardiac gene expression in response to mechanical strain in the myocardium [Liang et al., 2000]. In addition, stretch-induced increases in adhesion junction proteins, including connexin (Cx) 43, are initiated via integrin signaling in cardiomyocytes [Yamada et al., 2005]. β1 Integrin-deficiency in ES cells delays cardiac differentiation, suggesting that β1 integrin plays a crucial role in normal cardiogenesis [Fassler et al., 1996].

Among the signal transduction activated by the integrin protein family [Mitra et al., 2005; Walker and Assoian, 2005], it is believed that there is a functional interaction between the phosphoinositide 3-kinase (PI3K)/Akt pathway and Wnt/β-catenin signaling through the regulation of glycogen synthase kinase-3β (GSK3β). In particular, β-catenin plays a central role in Wnt signaling through its nuclear translocation and association with T-cell factor (TCF) and lymphoid enhancer factor (LEF) [Schneider and Mercola, 2001]. Moreover, Wnt/β-catenin signaling regulates the tissue interaction for cardiomyocyte specification. Inactivation of β-catenin in the node, notochord, and endoderm of the developing mouse causes the formation of ectopic hearts, thereby suggesting a key role of Wnt/β-catenin signaling in cardiomyogenesis [Lickert et al., 2002]. Furthermore, activation of Wnt/β-catenin signaling is essential for mammalian cardiac differentiation in the pluripotent model system [Nakamura et al., 2003]. However, the precise role of β-catenin-mediated signaling in cardiac differentiation of ES cells is still unclear. The cellular mechanism by which mechanical stress stimulates cardiomyogenesis in ES cells is not well explored.

Here we demonstrate that the β-catenin pathway plays a critical role in cyclic strain-mediated commitment of cardiomyocytes derived from ES cells, where ROS-dependent activation of integrins and subsequent induction of PI3K/Akt signaling are closely involved.

MATERIALS AND METHODS

MATERIALS

The mouse ES cell line D3 was obtained from the American Type Culture Collection (Rockville, MD). Fetal bovine serum (FBS) was purchased from Gibco-BRL (Gaithersburg, MD). Unless otherwise specified, chemicals and laboratory wares were purchased from Sigma Chemical Co. (St. Louis, MO), Calbiochem (San Diego, CA), and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ), respectively.

ES CELL CULTURE AND EMBRYOID BODY FORMATION

The mouse ES cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) supplemented with 1.7 mM L-glutamine, 0.1 mM β-mercaptoethanol, 5 ng/ml mouse leukemia inhibitory factor (LIF), 15% FBS, and 1% penicillin and streptomycin, without a feeder layer at 37°C in an atmosphere containing 5% CO_2. To form embryoid bodies (EBs), the cells were dissociated by 0.05% trypsin/EDTA and then 2,000 cells were hung from the lids of 100-mm culture dishes for 2 days in 20 μl DMEM without LIF. EBs were then suspended in additional medium for 2 days. EBs were plated onto type I collagen-coated flexible-bottom six-well plates (BioFlex1 plates, Flexcell International Corporation, Hillsborough, NC) and outgrown in DMEM without LIF for 1 day (10 EBs per well) before the application of mechanical strain.

APPLICATION TO CYCLIC STRAIN

EBs were subjected to mechanical strain on day 6 by stretching the flexible bottomed culture plates by 5% or 10% with 1 Hz for 1 h using a computer-controlled vacuum stretch apparatus (FX-4000T Tension Plus System, Flexcell International Corporation). Strain and frequency were controlled with a computer by regulating the rate of evacuation (vacuum concentration) and rate of air influx to the bottom of the culture plates. As controls, EBs were plated on the same culture plates and induced to differentiate without mechanical stress. All experiments were performed at 37°C in an atmosphere containing 5% CO_2.

MEASUREMENT OF INTRACELLULAR ROS

Cellular levels of ROS were measured using 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate (CM-H_2DCF-DA; Molecular Probes, Eugene, OR) according to the method described previously [Ali et al., 2006]. EBs were incubated with serum-free medium containing vitamin C (10^{-3} M) or NAC (10^{-5} M). Ten micromolars of CM-H_2DCF-DA was added prior to the application of mechanical strain. DCF fluorescence was determined by flow cytometry (Partec CyFlow® SL, Germany) at an excitation wavelength of 488 nm and an emission wavelength of 530 nm (FL-1). Mean fluorescence intensities were obtained by histogram statistics using the Partec software.

RNA ISOLATION AND REAL-TIME RT-PCR

The total RNA was extracted from the cells treated with each of the designated agents using STAT-60, which is a monophasic solution of phenol and guanidine isothiocyanate (Tel-Test, Inc., Friendwood, TX). The real-time quantification of RNA targets was then performed in the Rotor-Gene 2000 real-time thermal cycling system (Corbett Research, NSW, Australia) using a QuantiTect SYBR Green RT-PCR kit (QIAGEN, CA). The reaction mixture (20 μl) contained 200 ng of the total RNA, 0.5 μM of each primer, the appropriate amounts of enzymes, and fluorescent dyes, as recommended by the supplier. The Rotor-Gene 2000 cycler was programmed as follows: 30 min at 50°C for reverse transcription; 15 min at 95°C for DNA polymerase activation; 15 s at 95°C for denaturing; and 45 cycles of 15 s at 94°C, 30 s at 55°C, 30 s at 72°C. Data collection was carried out during the extension step (30 s at 72°C). The PCR reaction was followed by melting cure analysis to verify the specificity and identity of the RT-PCR products, which can distinguish the specific PCR products from the non-specific PCR product resulting from primer–dimer formation. The primers used were 5′-TCACAGCCCTTGTGGTTCAAG-3′ (sense), 5′-GGTAGATTCCTGATGGCCGTAGT-3′ (antisense) for Axin2, and 5′-TCTCCATCCTATGTTGCGGTC-3′ (sense), 5′-TCCAAG-TAACTCGGTCATCATCT-3′ (antisense) for c-myc. The temperature of the PCR products was increased from 65 to 99°C at a rate of

1°C/5 s, and the resulting data was analyzed using the software provided by the manufacturer.

CELL FRACTIONATION

Nuclear and cytosolic extraction was performed according to the method described elsewhere [Hsieh et al., 2006]. Briefly, the cells were resuspended in a buffer A (20 mM Tris (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonylfluoride, and 0.5 mM sodium orthovanadate). The suspension was sonicated for 10 s at output 4 and then centrifuged. The pellet contained the nuclear fraction. The supernatant was centrifuged at 15,000 rpm for 60 min at 4°C and contained the cytosolic fraction.

IMMUNOPRECIPITATION

β1 Integrin was subjected to immunoprecipitation. In brief, 10 μl of 200 μg/ml anti-β1 integrin antibody was added to protein lysates with equivalent volume and kept overnight at 4°C with occasional shaking. We then added 25 μl of Protein G sepharose beads, and the samples were incubated for another 2 h. The sepharose beads were collected by centrifugation, washed three times with TSA buffer (0.01 M Tris, 0.14 M NaCl, pH 8.0) before electrophoresis by SDS–PAGE.

siRNA TRANSFECTION

Cells were transfected for 24 h with either a stealth small interference RNA (siRNA) specific to β-catenin (5′-CCC UCA GAU GGU GUC UGC CAU UGU A-3′, 200 pmol/L; Invitrogen, Carlsbad, CA) or a non-related control siRNA targeting the green fluorescent protein (GFP) (5′-CCA CTA CCT GAG CAC CCA GTT-3′) using LipofectAMINE 2000 according to the manufacturer's instructions before being subjected to 10% cyclic strain.

WESTERN BLOT ANALYSIS

Proteins (20 μg) were separated by 8–10% SDS–PAGE and blotted onto polyvinylidene difluoride (PVDF) membranes. The blots were washed with TBST [10 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20], blocked with 5% skim milk for 1 h, and incubated with the appropriate primary antibody at the dilutions recommended by the supplier. The membrane was then washed, and the primary antibodies were detected with goat anti-rabbit IgG or goat anti-mouse IgG conjugated to horseradish peroxidase. The blots were developed with enhanced chemiluminescence (ECL) (Santa Cruz Biotechnology, CA) and exposed to X-ray film (Eastman-Kodak, Rochester, NY).

FLOW CYTOMETRIC ANALYSIS

Cellular levels of various cell lineage marker proteins were measured by flow cytometer. Briefly, strain-applied EBs were collected and resuspended at approximately 10^6 cells/ml in PBS containing 0.1% BSA. Cells were then incubated with each primary antibody on ice for 30 min before exposure to goat anti-rabbit IgG-fluorescein isothicyanate (FITC; Santa Cruz Biotechnology). These samples were analyzed with a flow cytometer (Partec CyFlow® SL) and approximately 20,000 events were recorded for each sample.

STATISTICAL ANALYSIS

Unless otherwise specified, all data are expressed as the means ± standard deviation (SD) of triplicate experiments. One-way ANOVA was used for multiple comparisons (Scheffe's multiple range test) using SPSS version 18.0 software. A P-value <0.05 was considered statistically significant.

RESULTS

STIMULATING EFFECTS OF CYCLIC STRAIN ON CARDIOMYOGENESIS

Initially, EBs were subjected to strain by stretching the bottom of flexible plate by 5% or 10% strain with 1 Hz for 1 h and then EBs differentiated toward cardiomyocytes up to day 14. As shown in Figure 1A, the application to strain increased the number of beating EBs in a time- and strain-dependent manner (n = 5) (Fig. 1A). Western blot analysis showed that the protein levels of cardiac muscle cell markers, Cx 43 and Nkx 2.5, dramatically increased in EBs incubated for 7 days after the application to strain (Fig. 1B). Similarly, flow cytometric data revealed that the fluorescence signals corresponding to the cardiac markers were apparently right-shifted compared to that of unstrained EBs (Fig. 1C).

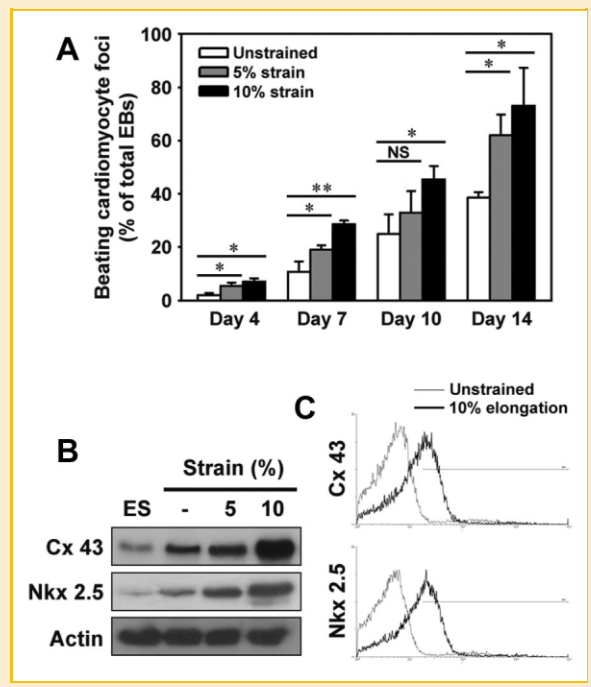

Fig. 1. Effects of cyclic strain on cardiomyogenesis and cellular protein levels of Cx 43 and Nkx 2.5. A: EBs were subjected to 5% or 10% strain for 1 h on day 6 and the cells differentiated into contracting cardiomyocytes. On days 4 through 14 of cultures after strain application, beating cardiomyocyte foci were counted by microscopic inspection. The values reported are the mean ± SD of five independent experiments. $^{*}P<0.05$ and $^{**}P<0.01$ versus the experiments. NS, not significant. EBs were also subjected to 5% or 10% strain for 1 h, and after 7 days of incubation, Cx 43 and Nkx 2.5 levels were determined through Western blot (B) or flow cytometric analyses (C). A representative result from five independent experiments is shown. US, unstrained.

CYCLIC STRAIN INCREASES INTRACELLULAR ROS, WHICH CORRELATES TO CX 43 AND NKX 2.5 PROTEIN LEVELS

When EBs were subjected to 10% cyclic strain for 1 h, intracellular ROS levels increased up to 2.26-fold, compared with the unstrained EBs ($^{***}P < 0.001$) (Fig. 2A). This increase was significantly inhibited in the presence of vitamin C (10^{-3} M, $^{\#\#\#}P < 0.001$) or NAC (10^{-5} M, $^{\#\#}P < 0.01$) (n = 4). To verify whether intracellular ROS stimulated cardiomyocyte differentiation, we investigated the effects of anti-oxidants on the protein levels of Cx 43 and Nkx 2.5. Cyclic strain-mediated increases in Cx 43 and Nkx 2.5 levels were inhibited by adding the anti-oxidants, where the level of Nkx 2.5 was reduced to basal levels (n = 4) (Fig. 2B).

EFFECTS OF CYCLIC STRAIN ON α5/β1 INTEGRINS AND PI3K/Akt PATHWAYS

We next examined the effects of cyclic strain on the expression of α5 and β1 integrins, because integrins are known as the mechanical signal transducer. EBs expressed α5 and β1 integrins at higher levels than ES cells and the protein levels of α5/β1 integrins was further augmented ($P < 0.05$) by applying to cyclic strain (n = 3) (Fig. 3A,D). Cyclic strain also increased the levels of PI3K p110α and β isoforms in EBs, which was significantly ($P < 0.05$) elevated by the application to 10% strain (n = 3) (Fig. 3B,E). When the effects of cyclic strain on the phosphorylation of Akt were determined, a significant increase in the level of p-Akt (ser 473) was observed at 60 min and maintained up to 120 min after the application to 10% strain for 1 h (n = 3) (Fig. 3C,F).

ANTI-OXIDANTS DIMINISH CYCLIC STRAIN-INDUCED EXPRESSION OF α5/β1 INTEGRINS AND TOTAL PI3K AND AKT PROTEINS

Subsequently, we examined the effects of anti-oxidants on the induction of α5/β1 integrins and total PI3K and Akt in 10% strain-exposed EBs. Western blot analysis showed that the strain-mediated increases in the induction of integrins, PI3K, and Akt proteins were inhibited by treating them with vitamin C or NAC (n = 3) (Fig. 4A–C). Flow cytometric analysis revealed that vitamin C almost completely inhibited the strain-mediated expression of α5 and β1 integrins (n = 5) (Fig. 4D). Cyclic strain-mediated increase of PI3K was also observed via immunoprecipitates of β1 integrin (n = 3) (Fig. 4E). In addition, treatment of Wortmannin, a PI3K inhibitor, or Akt inhibitor attenuated the cyclic strain-induced increase of Cx 43 and Nkx 2.5 proteins (n = 3) (Fig. 4F,G).

APPLICATION TO CYCLIC STRAIN INDUCES THE ACTIVATION OF β-CATENIN SIGNALING IN EBS

In order to examine whether β-catenin signaling is involved in cyclic strain-stimulated cardiomyogenesis, EBs were subjected to 5% or 10% strain for 1 h and then processed for Western blot analysis (n = 5) (Fig. 5A). Cyclic strain application markedly increased the protein levels of β-catenin. Cyclic strain also induced translocation of β-catenin into the nucleus from the cytosol (n = 5) (Fig. 5B). To verify the strain-mediated activation of β-catenin signaling, the mRNA expression of β-catenin target genes, Axin2 and c-myc, was assessed by real-time RT-PCR. As shown in Figure 5C, mRNA levels of these genes were significantly increased

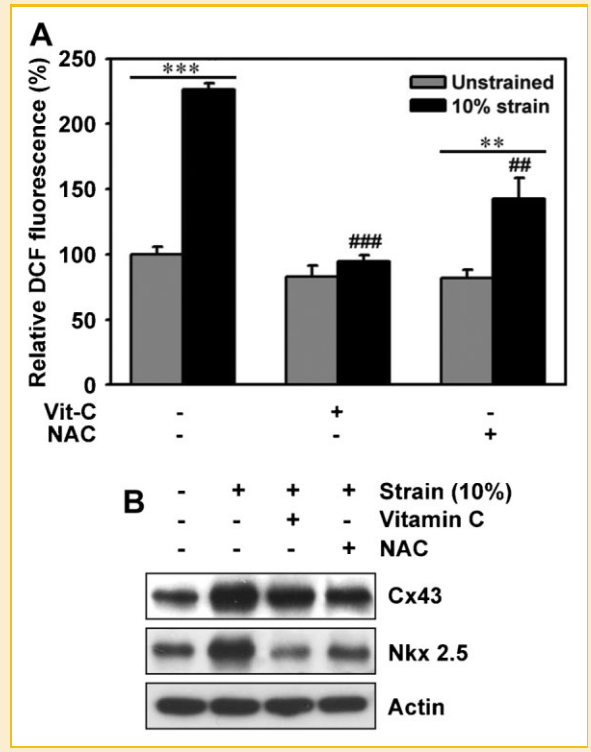

Fig. 2. Cyclic strain increases intracellular ROS levels in EBs. EBs were incubated in serum-free media containing vitamin C (10^{-3} M) or NAC (10^{-5} M) before the application to 10% strain for 1 h, and then DCF fluorescence and Cx 43 and Nkx 2.5 protein levels were measured by flow cytometry (A) and Western blotting (B), respectively. The values reported are the mean ± SD of four independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus the experiments. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus 10% strain alone.

by 10% strain, where Wnt3a was used as positive stimulator of β-catenin signaling (n = 6). Moreover, the phosphorylation of GSK3β was sensitively increased after application to strain, such that approximately 2.5-fold increase of p-GSK3β was found at 60 min and maintained to 120 min after the strain application (n = 5) (Fig. 5D).

REACTIVE OXYGEN SPECIES AND PI3K/Akt SIGNALING ARE INVOLVED IN THE STRAIN-STIMULATED INCREASE IN β-CATENIN PROTEIN LEVELS

In order to elucidate the relationship between intracellular ROS levels and β-catenin expression in the process of cardiomyogenesis, EBs were subjected to 10% cyclic strain in the presence of vitamin C or NAC (n = 4) (Fig. 6A). As shown in the figure, pretreatment of these anti-oxidants blocked apparently the strain-induced increase of β-catenin, where NAC inhibited more sensitively than NAC did. Wortmannin treatment also inhibited cyclic strain-induced increase of β-catenin level (n = 4) (Fig. 6B). Approximate threefold increase of β-catenin was detected in strain-exposed EBs compared to the unstrained control cells, whereas this increase was reduced to the basal level by the PI3K inhibitor. In parallel with this, treatment with Akt inhibitor almost completely diminished the increased levels of β-catenin in the strain-exposed cells (n = 4).

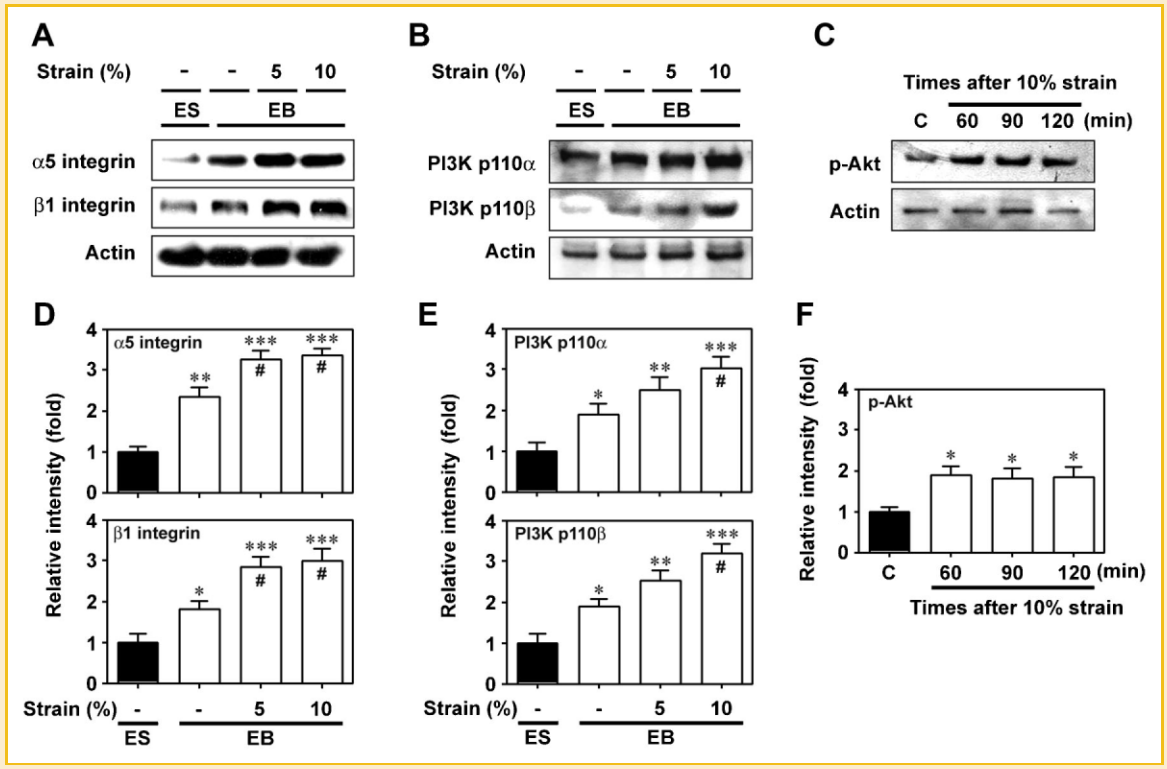

Fig. 3. Stimulating effects of cyclic strain on α5/β1 integrins and PI3K/Akt signaling. EBs were subjected to 5% or 10% strain for 1 h on day 6 and processed for Western blotting after 2 days of incubation. A: The protein levels of α5/β1 integrins or (B) PI3K p110α and PI3K p110β were shown. C: EBs were also subjected to 10% strain for 1 h and at various times (0–120 min) after the strain, then p-Akt levels were determined. These data obtained from triplicate experiments were quantified by densitometry after normalizing the bands to actin and the results were expressed in D, E, and F, respectively, as the relative intensity (fold) to the unstrained ESs. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ versus the unstrained ESs. $^{\#}P<0.05$ versus the unstrained EBs.

β-CATENIN SIGNALING IS INVOLVED IN THE INCREASES IN CX 43 AND NKX 2.5 PROTEIN LEVELS AND CARDIOMYOGENESIS

We further evaluated the roles of β-catenin signaling on cardiomyogenesis stimulated by cyclic strain. Figure 7A shows that the cellular levels of β-catenin protein were reduced markedly by its specific siRNA transfection, where control siRNA transfection did not affect the β-catenin protein levels (n = 4). The blockage of β-catenin expression by siRNA transfection diminished the cyclic strain-induced increases in Cx 43 and Nkx 2.5 protein levels (n = 4) (Fig. 7B). Finally, knockdown of β-catenin decreased the number of beating cardiomyocyte foci to that of the unstrained EBs, when the numbers were counted after 7 days of strain application, suggesting that β-catenin signaling is closely related to the stimulation of cardiomyogenesis in strain-subjected EBs (n = 6) (Fig. 7C).

DISCUSSION

The present study demonstrate that cyclic strain promotes ES cells-derived cardiomyogenesis through activation of ROS-dependent β1 integrin and PI3K/Akt-mediated β-catenin signaling pathways, as illustrated in Figure 8. Numerous studies have documented that mechanical strain stimulates production of intracellular ROS which functions as secondary signaling molecules in various types of cells including ES cells [Vanden Hoek et al., 1998; Chandel and Schumacker, 2000; Ali et al., 2006; Schmelter et al., 2006]. In parallel, our current data reveal that intracellular ROS is an important mediator of cardiomyogensis derived from cyclic strain in EBs. ROS and integrin signaling have emerged as potentially important elements contributing to cellular communication [Werner and Werb, 2002; Gregg et al., 2003]. We postulate that ROS may participate in inducing cardiomyogenesis from EBs by triggering integral membrane protein signaling. Indeed ROS may be an important modulator of integrins in various cellular events through both outside-in (integrins stimulating ROS production) and inside-out signaling (intracellular ROS altering integrin function) [Gregg et al., 2004]. This idea is in agreement with our results where EBs subjected to cyclic strain expressed high levels of α5 and β1 integrins, whereas such protein levels were inhibited by anti-oxidant treatment.

Integrins are key mediators of cellular signaling involved in mechanotransduction in cells. Our results show that up-regulation of integrins is required for cyclic strain-mediated cardiomyogenesis in EBs. Cardiomyocyte differentiation is severely impaired in β1-integrin null ES cells [Fassler et al., 1996]. Mechanical stress increased Cx 43 protein levels in cardiomyocytes, which was inhibited by adding β1 integrin antibody to the cultures [Shanker et al., 2005]. In addition, stretch-induced up-regulation of adhesion junction proteins may be related to mechanotransduction mediated

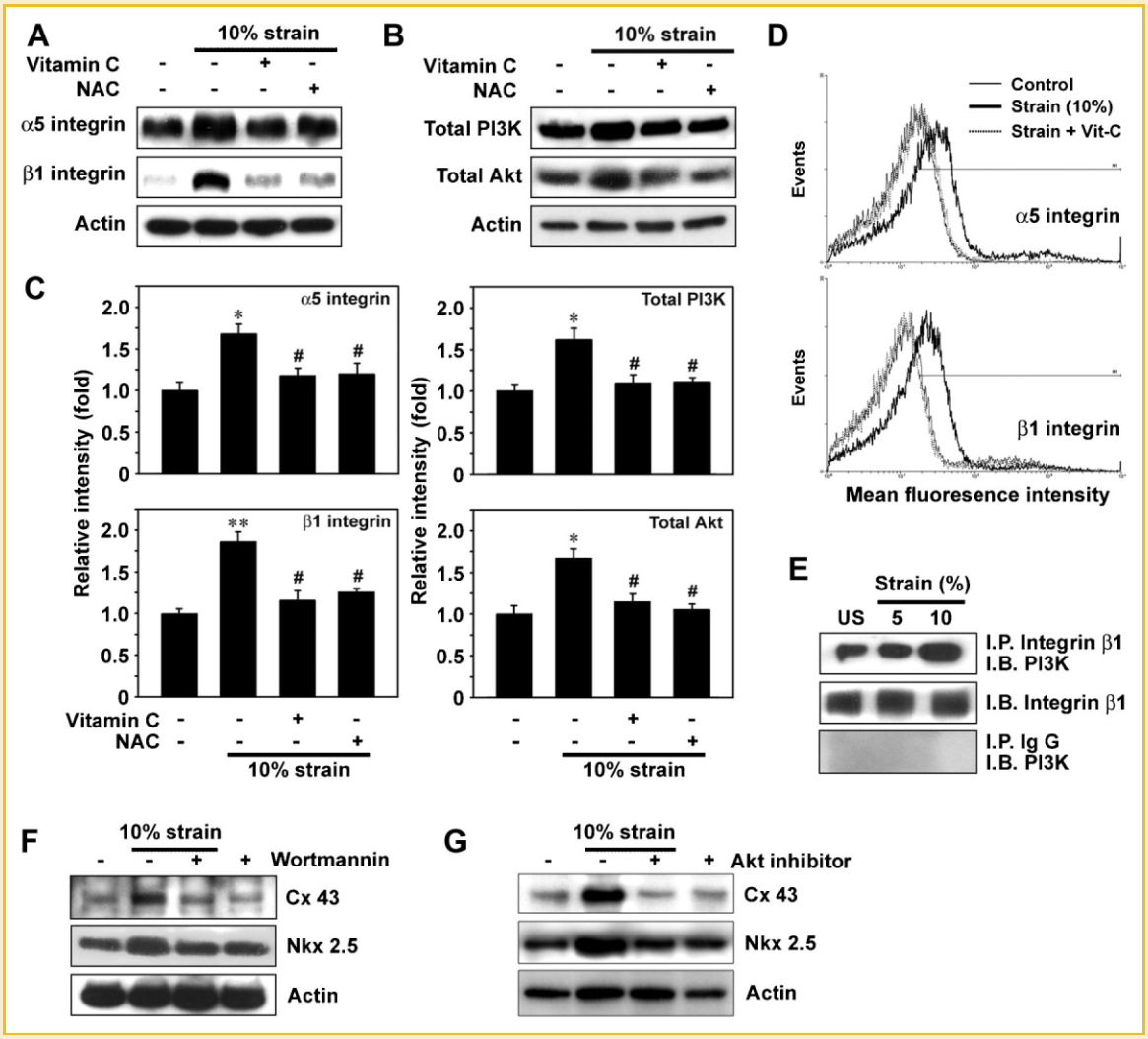

Fig. 4. Effects of anti-oxidants on cyclic strain-induced increases of α5/β1 integrins and PI3K/Akt levels. EBs were exposed to vitamin C (10^{-3} M) or NAC (10^{-5} M) 1 h before the application to 10% strain, and after 2 days of incubation, the levels of α5 and β1 integrins and total PI3K and Akt proteins were measured by immunoblotting (A,B) or flow cytometry (D). C: The panels (bars) denote the means ± SD of triplicate experiments for each condition determined by densitometry after normalizing the bands to actin. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the unstrained EBs. $^{\#}P < 0.05$ versus the stained control values. E: Total protein was immunoprecipitated with β1 integrin antibody or rabbit IgG (as negative control) and then processed for Western blot analysis against PI3K or β1 integrin. US, unstrained. EBs were pretreated with Wortmannin (10^{-6} M) (F) or Akt inhibitor (10^{-5} M) (G) before strain application and the levels of Cx 43 and Nkx 2.5 were assessed by Western blotting.

by integrins [Yamada et al., 2005]. Based on previous reports and our current data, integrins may be critical mediators for cyclic strain-stimulated cardiomyogenesis.

Integrin-mediated signaling signals the recruitment of intracellular signaling components such as kinases and phosphatases, linked to cytoplasmic tails of these proteins [Calautti et al., 2005]. With this regard, integrin activation may promote binding of PI3K to phosphorylated-focal adhesion kinase (p-FAK), resulting in PI3K activation and subsequent Akt stimulation [Chen et al., 1996]. In contrast, integrins also induce phosphorylation of Akt Ser473 in the absence of active FAK [Velling et al., 2004]. Our data obtained from immunoprecipitation analysis showed that there is a direct interaction between β1 integrin and PI3K/Akt signals in cyclic strain-subjected EBs. Thus, integrins may lead to FAK-dependent and/or -independent signaling required for PI3K/Akt activation, depending on the conditions applied. More detailed experiments will be needed to clarify the relationship between integrins and FAK signaling during cardiomyogenesis.

Accumulating evidence demonstrates that exposure of cells to mechanical stimuli such as stretch and shear stress can activate various signaling pathways including JAK/STAT [Pan et al., 1999] and PI3K/Akt signaling [Kim et al., 2002; Albinsson et al., 2008]. In addition to the regulation of mechanical signaling, these factors are involved in a broad range of cellular events, including migration, proliferation, and survival [Toker and Yoeli-Lerner, 2006]. Here we demonstrated that PI3K/Akt activation plays crucial roles as both down-stream effectors for integrin-mediated signaling and up-stream effectors for the induction of Cx 43 and Nkx 2.5 levels in EBs subjected to cyclic strain. Similarly, the PI3K/Akt pathway is involved in the differentiation of bone marrow-derived cells into

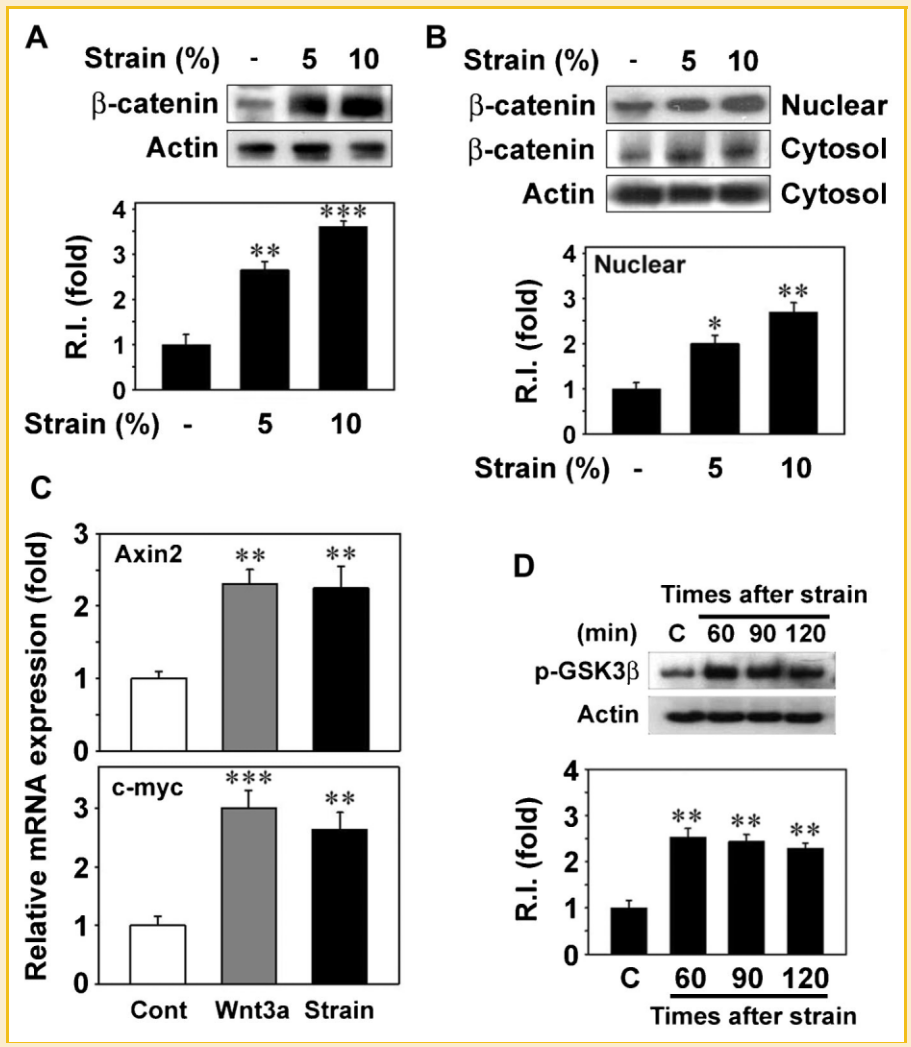

Fig. 5. Stimulating effects of cyclic strain on β-catenin signaling. EBs were subjected to 5% or 10% strain for 1 h, and the protein levels of β-catenin were determined by Western blotting using total protein lysates (A) and cytosolic or nuclear fractions (B). C: EBs were treated with 500 ng/ml Wnt3a or 10% strain, and after 2 days of incubation, the expression of Axin2 and c-myc was determined by real-time RT-PCR. D: In addition, EBs were subjected to 10% strain for 1 h and the levels of p-GSK3β were determined at various time (0–120 min) after the strain application. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus the unstrained control values.

cardiomyocytic phenotypes [Chen et al., 2008]. Akt signaling may regulate myocardial vascularization and expression of vascular endothelial growth factor and angiopoietin-2 [Shiojima et al., 2005; Walsh, 2006]. These data led us to assume that PI3K/Akt pathway is involved in cardiomyocyte differentiation as well as in cardiac development.

The most interesting finding in this study is the role of β-catenin on cyclic strain-mediated differentiation of ES cells into cardiomyocytes, which is consistent with several reports suggesting the involvement of Wnt/β-catenin genes in cellular response to mechanical stress [Robinson et al., 2006; Avvisato et al., 2007]. Our present study also verifies that β-catenin signaling requires PI3K/Akt signaling. β-Catenin signaling is stimulated by the activation of PI3K/Akt and subsequent inactivation of GSK3β which typically phosphorylates β-catenin, inducing rapid degradation via the ubiquitin/proteasome pathway [Götz 2008]. In addition, PI3K/Akt regulates cardiomyocyte differentiation by suppressing GSK3β activity and by maintaining Wnt/β-catenin activity in a murine embryonic carcinoma cell line [Naito et al., 2005]. It was recently reported that cyclic stretch activated Akt, on the other hand, GSK3β is inactivated, then, this process up-regulates Cx 43 in cardiomyocytes [Salameh et al., 2010]. Beyond the role of membrane associated-β-catenin in adherens junctions, therefore, soluble β-catenin might participate in cardiomyocyte differentiation through β1 integrin-mediated PI3K/Akt activation. Collectively, we suggest that Wnt/β-catenin signaling is critical for inducing cardiac differentiation, at least in a pluripotent cell system [Nakamura et al., 2003; Case et al., 2008].

In summary, this study demonstrates the stimulating effects of cyclic strain on cardiomyogenesis from ES cells through activation of ROS-dependent integrins and subsequent PI3K/Akt signaling, where β-catenin plays a central role. Our data are the first elucidating the distinctive mechanical pathways involved in cardiomyogenesis in ES cells. Understanding of the integration

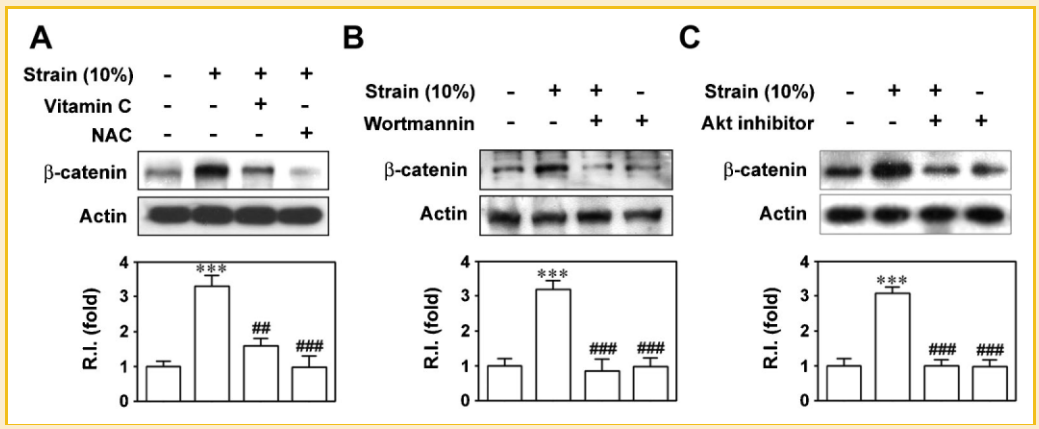

Fig. 6. Anti-oxidants, Wortmannin, and Akt inhibitor block the strain-induced increase in cellular β-catenin levels. EBs were pretreated with anti-oxidants (vitamin C and NAC) (A), Wortmannin (B), or Akt inhibitor (C) before the application to 10% cyclic strain, and β-catenin levels were determined by Western blotting after 2 days of incubation. The panels (bars) denote the means ± SD of four experiments for each condition determined by densitometry after normalizing the bands to actin. $^{***}P < 0.001$ versus the unstrained control values. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus 10% strain alone.

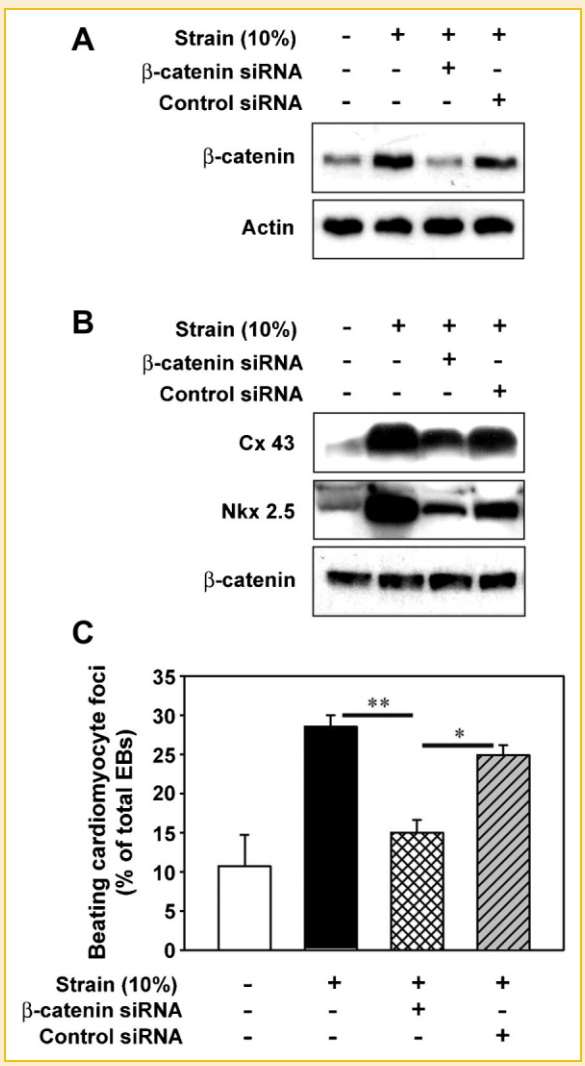

Fig. 7. Knockdown of β-catenin by siRNA transfection inhibits cyclic strain-stimulated cardiomyogenesis. EBs were transfected with either β-catenin- or GFP-specific siRNA using LipofectAMINE 2000 and after 24 h of transfection, these cells were subjected to 10% strain for 1 h. A: The change in β-catenin levels according to the transfection was determined by Western blotting after 2 days of transfection. B: The protein levels of Cx 43 and Nkx 2.5, and (C) the beating cardiomyocyte foci were also determined by immunoblotting and microscopic observation, respectively, after 7 days of incubation. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the unstrained control values.

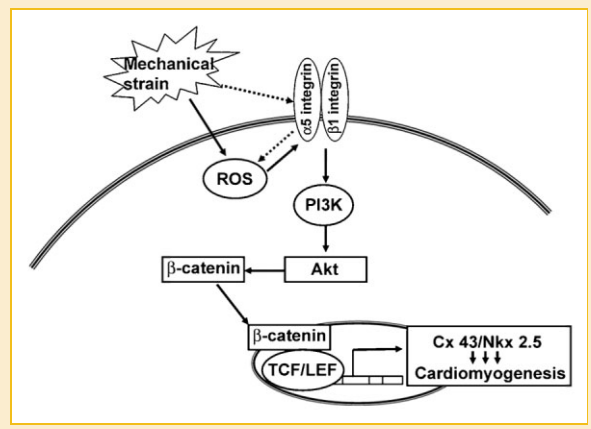

Fig. 8. The hypothesized model for the signal pathways involved in cyclic strain-stimulated cardiomyogenesis from ES cells. Mechanical strain increases ROS generation, which activates α5/β1 integrins and subsequent PI3K/Akt signaling. PI3K/Akt pathways induce the translocation of β-catenin into the nucleus, which leads to the increases of Cx 43 and Nkx 2.5 protein levels required for cardiomyocyte differentiation. In this scheme, solid lines are proposed pathways and dashed lines are a suspected pathway. TCF, T cell factor; LEF, lymphoid enhancer factor.

between biomechanical and biochemical pathways in stem cells could improve tissue engineering and stem-cell-based therapies.

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