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Small Molecule Induction of Neural-Like Cells From Bone Marrow-Mesenchymal Stem Cells

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ABSTRACT

Bone marrow-derived mesenchymal stem cells (MSCs) have been demonstrated to be able to differentiate into neuron-like cell, but the precise mechanisms controlling this process are unclear. We report here that LY294002, a small molecule inhibitor of PI3K/AKT signal pathway, can inhibit proliferation and promote neuronal differentiation of MSCs after MSCs incubated with LY294002 for 6 and 12 h. RT-PCR results indicated that mRNA expression of α5β1 integrin significantly increased in neuron-like cell from MSCs. Interestingly, neuron-like cells derived by this method adhere much more strongly than MSCs, which was related to the expression of α5β1 integrin and FAK phosphorylation. However, these effects could be attenuated by LiCL or GSK-3β–siRNA. Our results indicate that activation GSK-3β signaling may be involved in MSCs proliferation, differentiation, and adhesion. Furthermore, this study demonstrates that small molecule regulators of PI3K/AKT signaling may be valuable tools for stem cell research aimed at treatment of neurodegenerative disease. J. Cell. Biochem. 113: 1527–1536, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: MSCs; DIFFERENTIATION; ADHESION; LY294002; LiCL

Mesenchymal stem cells (MSCs) have been considered to be an ideal source of stem cells for tissue repair and therapy in regenerative medicine [Cuenca-Lopez et al., 2008; Hatzistergos et al., 2010]. MSCs can be induced to overcome their mesenchymal commitment and transdifferentiate toward nonmesenchymal cell lineages under certain conditions [Lu et al., 2004; Keilhoff et al., 2006]. Recent studies have demonstrated that MSCs have ability to repair injury in brain tissues by differentiating into neuron-like cell, generating neurotrophic factor, or more recently through antioxidant and neuroprotective actions [Barzilay et al., 2008].

Phosphatidylinositol 3-kinase (PI3K) is an enzyme implicated in signal transduction by associating with receptor and nonreceptor tyrosine kinases. Akt kinase, one of downstream transmitters of PI3K-dependent effects, has been implicated in the regulation of an array of physiological processes: the control of proliferation, differentiation, and apoptosis of MSCs [Sadidi et al., 2009]. In addition, there is a cross-talk between the PI3K/AKT and GSK-3β/β-catenin signaling pathways. For example, PI3K/AKT and GSK-3β/β-catenin signal pathway play an important role in the process of osteoblast differentiation of MSCs [Lee et al., 2010].

LY294002, as a cell-permeant pharmacological inhibitors of PI3K, has been proving to regulate differentiation in some cell lines, but its effectiveness in different cell are more controversial. For example, LY294002 could block cardiomyocyte differentiation of embryonic stem (ES) cells and effectively regulate cardiomyocyte differentiation [Klinz et al., 1999]. Kubota et al. [2001] found that LY294002 inhibited the erythroid differentiation of K562 cells. On the other hand, Llorens et al. [2002] found that LY294002 were unable to induce full differentiation in PC12 cells. In addition, previous results have demonstrated that LY294002 resulted in loss of pluripotency markers in mouse ES cells and induced cells differentiation [Liu et al., 2009].

Our experiments aimed to explore the relationship between PI3K signaling pathway and neuronal differentiation of MSCs. We observed the effect of LY294002, a specific inhibitor of PI3K pathway, on the expression of α5β1 integrins, FAK phosphorylation, and cell adhesion. To investigate the mechanism of LY294002, the anti-α5β1 integrin antibody and GSK-3β inhibitor LiCL were applied to MSCs before and after the induction of neural differentiation. In the current study, we describe a highly efficient method for deriving neurons-like cells from MSCs using the small molecule PI3K/Akt inhibitor LY294002.

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MATERIALS AND METHODS

CELL CULTURE

Sprague–Dawley rats were purchased from the animal facility of Nanchang University. MSCs were separated from the femurs and tibias taken from Sprague–Dawley rats. The quality of MSCs was ensured by flow cytometry, differentiation assays. For flow cytometry, MSCs were stained with CD29, CD44, CD45, CD71, and CD106. CD106-FITC as described previously [Li et al., 2011], adipose differentiation was analyzed by Oil Red O, osteogenic differentiation by mineralization nodule staining and chondrogenic differentiation by alcian blue staining or Western bloting for collagen II. Cells used in these experiments were harvested from passage 4.

Cerebral microvascular endothelial cells were cultured as previously described [Song and Pachter, 2004]. Briefly, the isolated cerebral gray matter was homogenized, and then passed through 200 and 74 μm mesh. The matter left on the mesh screen was digested by type II collagenase at 37°C for 20 min, centrifuged at room temperature for 5 min at 200*g*, and then the fragments of microvessels were collected. The fragments of microvessels were suspended in complete culture medium (DMEM supplemented with 20% FBS, 20 mM sodium bicarbonate, 2 mM L-glutamine, 75 mg/L ECGS, 1,000 U/L heparin, 200 U/L insulin, 1×10^5 U/L penicillin, 1×10^5 U/L streptomycin sulfate, pH 7.2) and the suspension was seeded in gelatin-coated Petri dishes. The cells at third passage were used for the experiments.

NEURONAL INDUCTION

For neuronal induction, MSCs were seeded at 2,500–5,000 cells/cm^2 in 35 mm dishes in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin, and 1 mg/ml streptomycin and cultured for 24 h, then cells were washed twice with PBS. Neuronal commitment was induced by exposing the cells to DMEM/25 μM LY294002 for 6 and 12 h. To investigate the role of LiCL on neuronal induction, MSCs were incubated with 10 mM LiCL for 30 min, then were washed twice with PBS and exposed to DMEM/25 μM LY294002 for 6 or 12 h.

GSK-3β–siRNA PREPARATION AND TRANSFECTION

Several siRNAs targeting either isoform of GSK-3 were designed according to GenBankTM accession numbers (NM_032080 for rat GSK-3β) and chemically synthesized. The target sequences for the rat-specific GSK-3–siRNAs are as follows: βP555, CCTCTTGC-TGGATCCTGAT; βP514, GGTCTACCTTAACCTGGTG; βP1093, GAACTGTCAAGTAACCCAC. siRNA transfection for GSK-3β was performed using the GSK-3β–siRNA Signal Silence Kit (Cell Signaling Technology) according to the manufacturer's instructions. Cells transfected with non-specific siRNA were used as a control.

CELL CYCLE ANALYSIS

Trypsinized cells were washed twice with PBS, fixed with 67% cold ethanol, and stored at −20°C until analysis. The cells were washed twice with PBS, resuspended in 250 U/ml RNase in PBS, and incubated at room temperature for 30 min. Propidium iodide was added to the suspension to give a final concentration of 50 μg/ml. The relative cellular DNA content in stained cells was measured using a FACSCalibur flow cytometer (Becton Dickenson). PI fluorescence emission was collected using a 585/42 band-pass filter. Cell cycle distributions were deconvoluted using the ModFit LT software program (Verity Software House, Topsham, ME) to determine the proportions of cells in the G0/G1, S, and G2/M phases.

ADIPOGENIC DIFFERENTIATION

MSCs were differentiated into adipocytes by culturing confluent cells with Adipogenic Differentiation Media kit (Cambrex) for approximately 4 weeks according to the manufacturer's instructions. Media were changed in regular intervals alternating from pre-differentiation media to post-differentiation media. Differentiated cells were fixed in 10% phosphate-buffered formalin and stained for lipid droplets using Oil Red O stain.

OSTEOGENIC AND CHONDROGENIC DIFFERENTIATION

MSCs were cultured in an osteogenic differentiation basal medium containing osteogenic supplement (Gibco BRL). After 2 weeks, osteogenic differentiation was evaluated with mineralization nodule staining. For chondrogenic differentiation, cells were cultured for 2 weeks in the presence of chondrogenic supplement in chondrogenic differentiation basal medium (Gibco BRL). This medium was replaced every 3–4 days for 3 weeks. The development of chondrogenic differentiation was determined by staining the medium with Alcian blue (Sigma Chemical Co.) or Western bloting for collagen II.

IMMUNOCYTOCHEMISTRY

Cells were fixed with 4% paraformaldehyde and processed for standard indirect immunofluorescent staining. Briefly, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was suppressed with 10% H_2O_2 for 20 min. Cells were then incubated with antibodies for Nestin (1:100; Sigma–Aldrich), Neurofilament (1:200; Chemicon) and β-tubulin III (1:200; Chemicon) overnight. This was followed by incubation with CY3-coupled anti-mouse (1:1,000; Immunological Sciences) and FITC-coupled anti-rabbit (1:500; Southern Biotechnology) secondary antibodies. Hoechst 33342 (1:5,000; Sigma–Aldrich) was used for nuclear staining. To quantify the percentage of cultured cells expressing neural markers, the number of positive cells in five randomly selected fields was scored and divided by the total number of cells.

RT-PCR AND REAL-TIME RT-PCR

For the semi-competitive RT-PCR, glyceraldehydes 3-phosphate dehydrogenase (GAPDH) gene was used as an internal control. The mRNA levels of cyclin D1, cyclin E1, α5 integrin, β1 integrin, Nestin, NF-200, β-tubulin-III, NSE, GAPDH in MSCs were determined by the standard RT-PCR method. Total RNA was isolated from 2×10^6 MSCs by using Trizol Reagent (Roche) following the manufacturer's instruction. First strand cDNA synthesis and amplification were performed using a MBI RevertAid First Strand cDNA Synthesis Kit (MBI, Lithuania). The Real-time PCR was done with an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) in 96-well plates, using SYBR-Green Master Mix

(Paragon Biotech Co., Ltd., Guangzhou) in 25 μl reaction mixtures. The thermal profile for the real-time PCR was 95°C for 3 min followed by 40 cycles of 95°C for 20 s, 52°C for 30 s, and 60°C for 30 s. Results were analyzed by the relative quantification method ($\Delta\Delta C_t$) using the iQ5 Optical System Software Version 2.0 (Bio-Rad Laboratories). Cycle threshold (C_t) values were obtained from the ABI 7000 software. Fold change of relative mRNA expression was determined using the $2^{-\Delta\Delta C_t}$ method [Livak and Schmittgen, 2001]. The primers were as follows: GCGTACCCTGACACCAATCT and GGCTCCAGAGACAAGAAACG for rat cyclin D1; CTCGTTGGAGTT-GATGCAGA and CTTTCTTTGCTTGGGCTTTG for rat cyclin E1; 5′-CAACCCTCACCACTCTATTT-3′ and 5′-GTGGCTGCTTCTTTCTTTA-3′ for rat Nestin; 5′-GCAGACATTGCCTCCTACC-3′ and 5′-TCA-CTCCTTCCGTCACCC-3′ for rat NF-200; 5′-CAAGGACAAGTATGG-CAAGG-3′ and 5′-CAGTGATGTATCGGGAAGG-3′ for rat NSE; 5′-CCTTCATCGGCAACAGCA-3′, 5′-CCTCGTCGTCATCTTCATACA-3′ for rat β-tubulin-III; GGTGACGGGACTCAACAACT and GGGCAT-TTCAGGACTTGTGT for rat α5 integrin; TCAGTGCCTCCATGT-CTCTG and CCCACAGTACAGCCCTTGAT for rat β1 integrin; 5′-TTGCCATCAATGACCCCTTCA-3′ and 5′-CGCCCCACTTGATTTTGG-A-3′ for GAPDH.

WESTERN BLOTING

For Western blotting, the cells were washed twice in ice cold phosphate-buffered saline and then scraped in cold lysis buffer [50 mM Tris–Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate and protease inhibitor mixture. Equal amounts of proteins (20–50 μg) were resolved on SDS–polyacrylamide gels and transferred to PVDF membranes, and the membranes were then probed with the specific primary antibodies. Membranes were washed extensively and then incubated for 1 h with anti-rabbit IgG or anti-goat IgG conjugated to HRP (1:4,000). Membranes were washed extensively again, and the protein bands were visualized using ECL. The sources of the primary antibodies used are as follows: Rabbit polyclonal antibody against GSK-3β, β-catenin were obtained from Chemicon, Rabbit polyclonal antibody against FAK was obtained from invitrogen, Goat polyclonal antibody against p-FAK (Tyr 407) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

ADHESION ASSAY

NC-like cells from MSCs to endothelial cells were stained with 4 mM Calcein AM (Invitrogen) for 30 min at 37°C. After staining, cells were washed twice with DMEM. One population was treated with LiCL or the antibody of α5β1 integrin for 30 min, whereas the other was not. Cells from different conditions were resuspended in DMEM, and co-cultured with 80% confluence endothelial cells for 1 h. Next, the medium was removed, and the remaining cells were washed three times with PBS. After washing, cells were incubated with 250 ml cooled methanol/acetone (1:1) at 22°C for 10 min. The solution was removed, and cells were allowed to dry for 10 min at room temperature. The MSCs that remained adhered to the endothelial cells were counted by an investigator who was blind to the type of serum in each preparation.

STATISTICS

All values are expressed as a mean ± standard deviation. One-way analysis of variance followed by Student's *t*-test was used to determine statistically significant differences between experimental and control samples. Differences were considered significant at $P < 0.05$.

RESULTS

BIOLOGICAL CHARACTERISTICS AND DIFFERENTIATION POTENTIAL OF MSCs

In this study, MSCs were analyzed for cell morphology immunophenotype. After removing nonadherent cells by replacing the medium (day 2 in culture), a small portion of attached nucleated cells was visualized in the MSCs culture dish. Under phase-contrast microscopy, these cells displayed fibroblast-like morphology with thin elongated processes around a central nucleus (Fig. 1A). When plated on adipogenic media, MSCs underwent morphological changes and produced vacuoles containing lipid that could be detected with Oil Red O staining (Fig. 1B). After 2 weeks of the osteogenic medium culture, the cells differentiated into osteoblasts, which were confirmed with mineralization nodule staining (Fig. 1C). Differentiation of MSCs into chondrocytes was determined by staining with Alcian blue (Fig. 1D) and Western bloting for collagen II (Fig. 1E).

LY294002 AFFECTS MSCs CELL CYCLE PROGRESSION

To assess whether PI3K/AKT inhibition mediated by LY294002 is associated with changes in cell cycle progression of MSCs, we employed flow cytometry and monitored their allocation to different cell cycle phases in the presence or absence of LY294002 at 6 and 12 h. As shown in Figure 1A and B, 13 ± 2.67% of MSCs were in S phase in control, 1.11 ± 0.41% of MSCs were in S phase in the presence of LY294002, which suggested that significantly fewer MSCs entered S phases in the presence of LY294002 (Fig. 2A,B). Differences in G2/M phase G0/G1 phase distribution between LY294002-treated and untreated MSCs were minimal.

LY294002 INHIBITS CYCLIN D1, CYCLIN E1 mRNA EXPRESSION

We further detected the change of both cyclin D1 and cyclin E1 after LY294002 treatment. To investigate whether LY294002 could directly regulate cyclin D1, cyclin E1 gene expression and cell cycle, MSCs were incubated in DMEM containing LY294002 (0 or 25 μM) for 12 h at 37°C in 5% CO_2. The mRNA expression of cyclin D1 and cyclin E1 significantly decreased after MSCs stimulated with 25 μM LY294002 for 6 and 12 h compared with control (Fig. 2C).

DERIVATION OF NEURAL-LIKE CELLS FROM MSCs

Neural-like cells can be efficiently derived from MSCs using LY294002 (PI3K inhibitor). After MSCs colonies treated with LY294002 for 12 h, MSCs showed neural morphological characteristics including retraction of the cytoplasm toward the nucleus, and formation of spherical and refractile cell bodies with neurite-like processes (Fig. 3A). Nevertheless, shape change may appear as coincidental and without links to a neural phenotype. We therefore

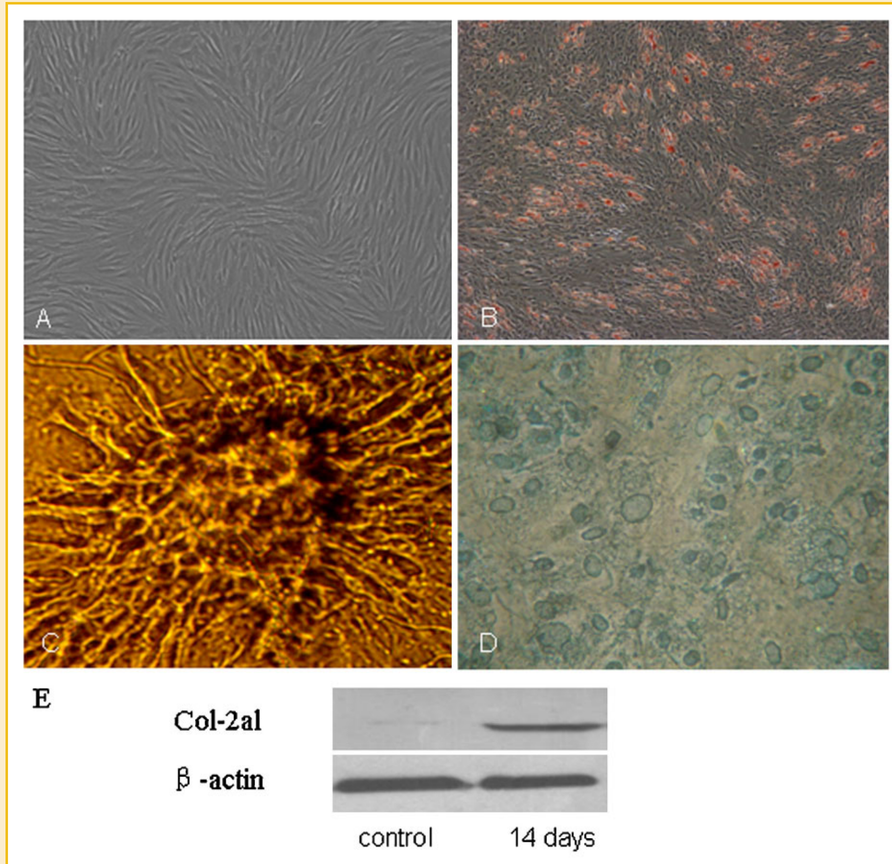

Fig. 1. MSCs and their multilineage differentiation staining. A: MSCs were observed under phase-contrast microscopy. B: Adipogenic differentiation was demonstrated by Oil Red O staining. C: Osteogenic was demonstrated by mineralization nodule staining. Chondrogenic differentiation was demonstrated by Alcian blue (D) or Western bloting for collagen II (E). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

attempted to correlate these results with the induction of neuronal marker expression. RT-PCR and Real-time RT-PCR data showed that mRNA levels of Nestin, NF-200, and β-tubulin III significantly increased in MSCs induced by LY294002, which were weakened by LiCL (Fig. 3B). Cells were fixed and stained for the neural markers, and differentiation rate was determined by counting β-tubulin III and Nestin positive cells as percentage of total number of neurons. Immunofluorescence analysis showed that β-tubulin III specific labeling was found widely distributed in the cell cytoplasm. The proportion of β-tubulin III-positive cells was also dramatically induced by the treatment with LY294002, from 3.33% to 76.45 in MSCs, then decrease to 51.77% by the treatment with LY294002 + LiCL. The proportion of Nestin -positive cells was also dramatically induced by the treatment with LY294002, from 41.96% to 85.57% in MSCs, then decrease to 62.66% by the treatment with LY294002 + LiCL (Fig. 3C).

THE ROLE OF GSK ON ADHESION-RELATED MOLECULES INDUCED BY LY29004 IN MSCs

Since it is well established that cell adhesion is mediated mainly by α5 integrin and the β1 integrin [Browe and Baumgarten, 2003; Kim et al., 2008], we evaluated the altered expressions of adhesion-related integrin molecules in LY294002-treated MSCs using RT-PCR and Western blot assay. As shown in Figure 4A, the mRNA level of integrin α5 was dramatically increased in LY294002-treated MSCs compared with control. Although this increase was significantly inhibited by LiCL or GSK-3β–siRNA, the level was still high than control. In the case of β1-integrin, the mRNA level was also significantly increased in LY294002-treated MSCs compared with control, and the increased mRNA levels were also partially inhibited by LiCL or GSK-3β–siRNA (Fig. 4A). It is well established that integrin activates the cellular focal adhesion-related focal adhesion kinase (FAK) at the adhesive stage. Therefore, we investigated the effect of LY294002 on the expression of Phospho-FAK and FAK at the early adhesive stage. LY294002 significantly increased the phosphorylation of FAK kinases, but it was weaken by LiCL or GSK-3β–siRNA (Fig. 4B). To investigate the role of GSK-3β on MSCs adhesion induced by LY294002, the expression change of GSK-3β Phosphorylation of GSK-3 was measured by Western blotting. We found that LY294002 significantly increased the expression levels of phospho-GSK-3β in MSCs compared with control, which was inhibited by LiCL or GSK-3β–siRNA (Fig. 5). These results suggested that GSK-3β signaling participates in MSCs adhesion.

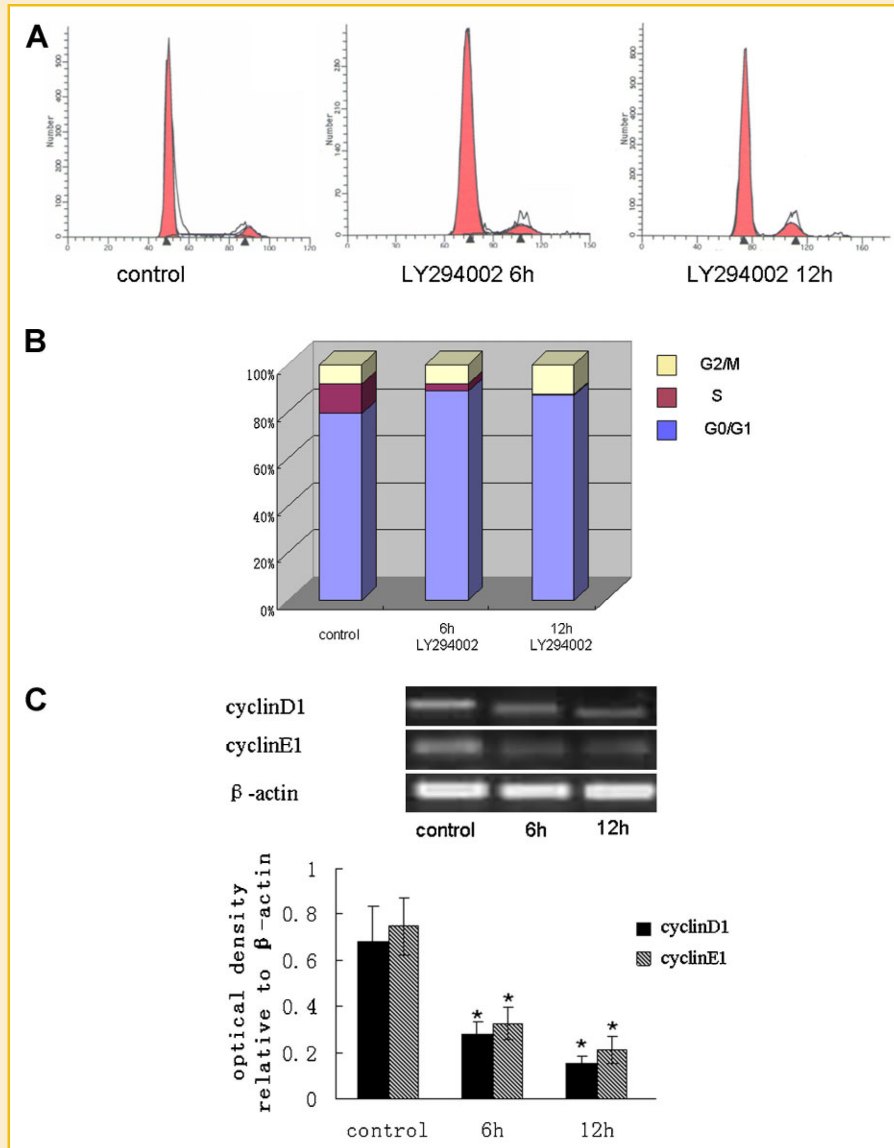

Fig. 2. LY294002 delayed cell cycle progression and inhibited cyclin D1, cyclin E1 mRNA expression. A: Analysis of MSCs cell cycle using flow cytometry. B: Percentage of cells in different phases of the cell cycle in MSCs cultures treated for 6 and 12 h with LY294002. Results are mean ± SD (n = 3). $^{*}P < 0.05$. C: Reverse transcription polymerase chain reaction analysis of cyclin D1, cyclin E1 mRNA expression in MSCs treated with LY294002 for 6 and 12 h. Results are expressed as mean ± SD, $^{*}P < 0.05$ compared to control group. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

LY294002 ENHANCES THE ADHESION OF MSCs IN VITRO

To investigate whether LY294002 facilitated the adhesion of MSCs, we co-cultured MSCs with endothelial cells and analyzed their capacity to adhere to rat endothelial cells EC52 by counting Calcein AM-labeled MSCs. A higher number of MSCs coupled with LY294002 could adhere to endothelial cells after 2 h of co-cultured than the control MSCs (MSCs-LY294002 vs. MSCs: 722 ± 55 vs. 263 ± 48, respectively, $P < 0.001$) (Fig. 5A). Furthermore, we incubated MSCs with LY294002 and LicCL or GSK-3β–siRNA, then co-cultured those cells with rat endothelial cells. In this setting, we found that LiCL or GSK-3β–siRNA significantly reduced numbers of adherent MSCs (Fig. 5B).

DISCUSSION

In this study, we have addressed the role of the LY294002 on MSCs proliferation, differentiation, and adhesion. LY294002 could inhibit MSCs proliferation, direct neuronal differentiation of MSCs, increase α5, β1 integrin mRNA expression, stimulate a rapid increase in FAK phosphorylation, and increase the ability of cell adhesion. By examining GSK-3β phosphorylation and β-catenin protein expression, we found that LiCL could inhibit α5β1 integrin mRNA expression and the adhesion differentiation of MSCs, which suggested that GSK-3β/β-catenin signaling pathway may play a role in the regulation of MSCs differentiation and adhesion.

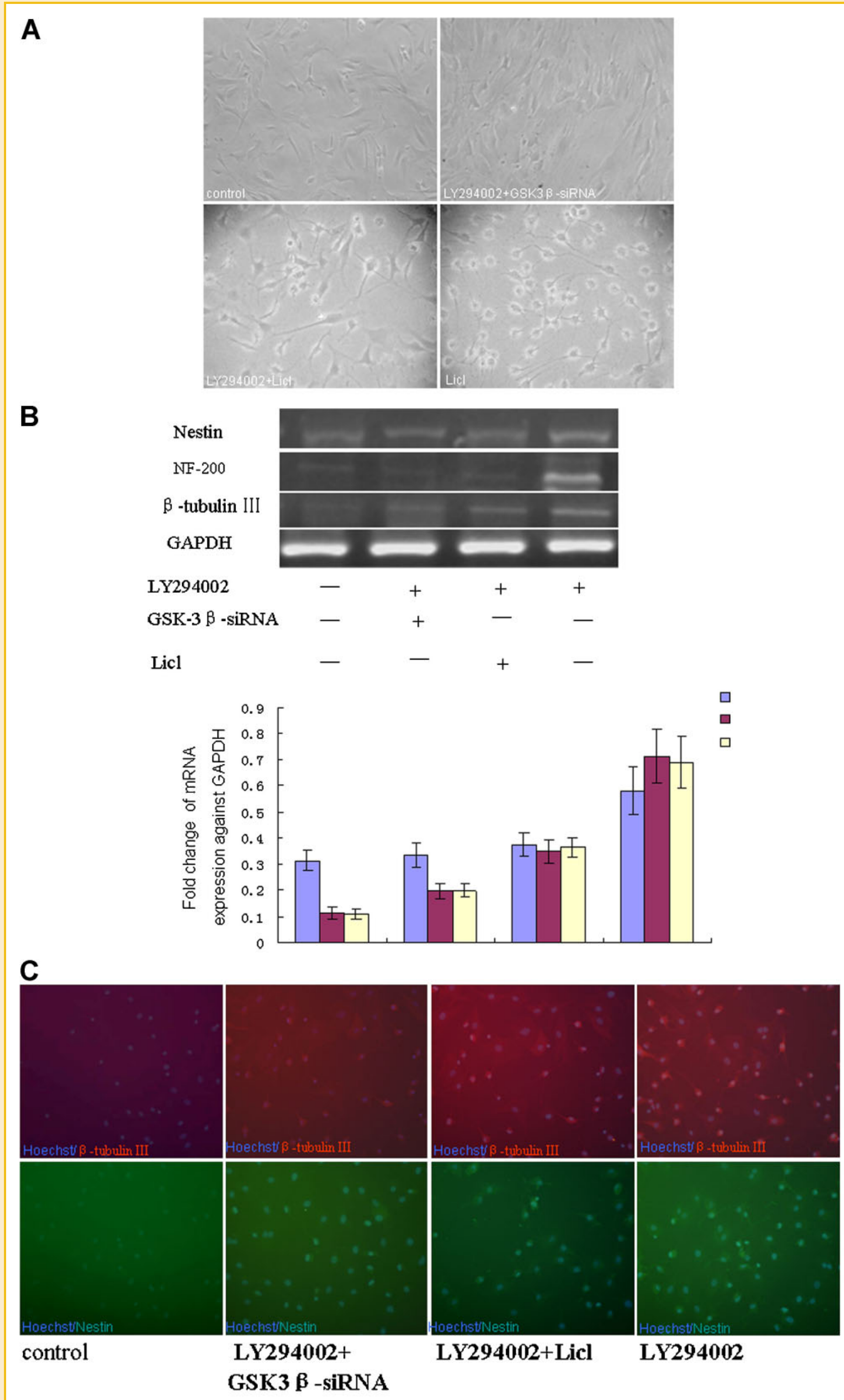

Fig. 3. Induction of major neuronal markers in MSCs treated with LY294002, which was attenuated by LiCL. A: Treatment MSCs with LY294002, LY294002 + GSK-3β–siRNA, or LY294002 + LiCL was observed under phase-contrast microscopy with. B,C: β-Tubulin III and Nestin were measured by immunofluorescent analysis. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

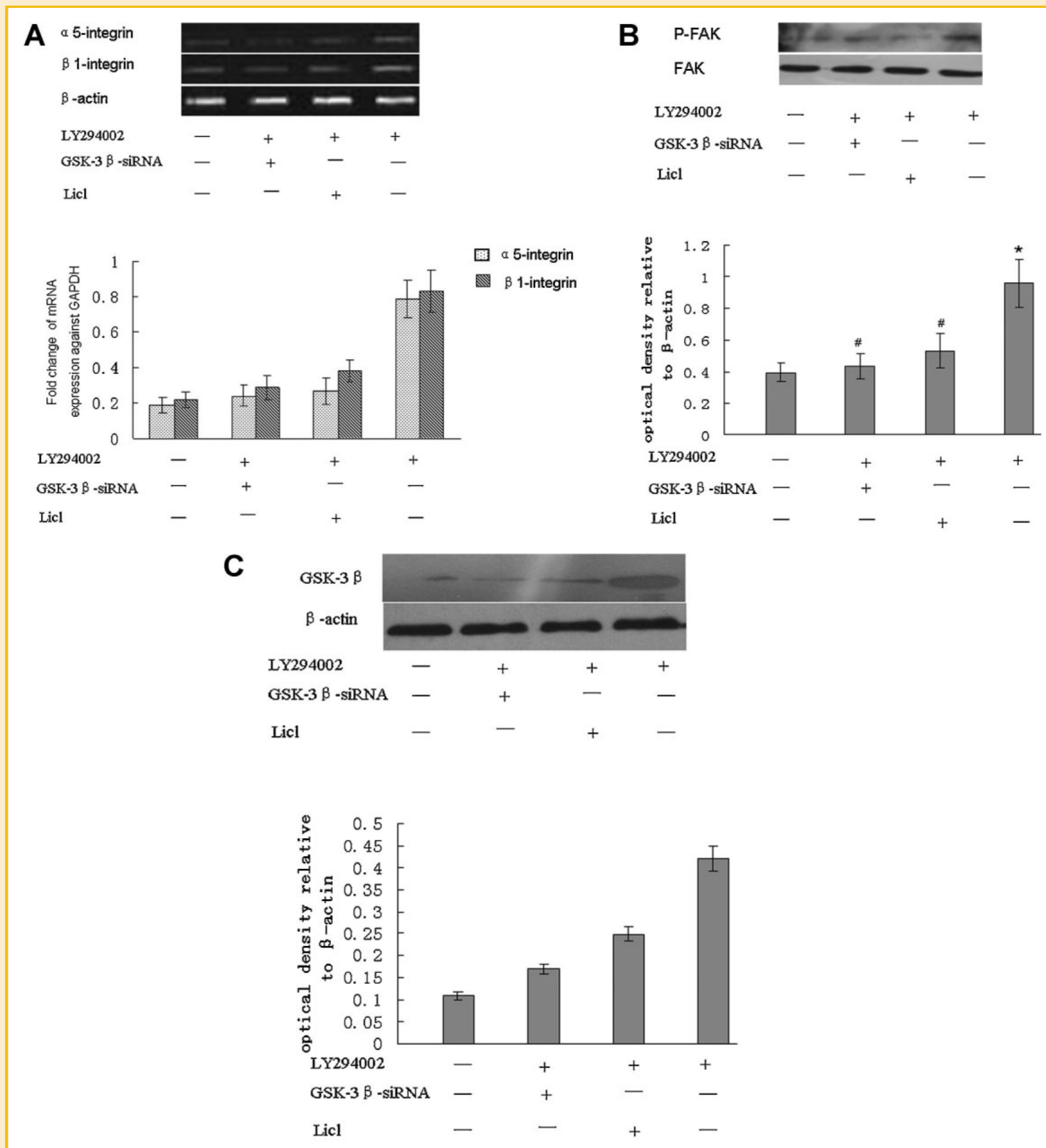

Fig. 4. Expression of adhesion molecules in MSCs. A: Reverse transcription-polymerase chain reaction analysis of the expression of α5 integrin and the β1 integrin. B: Western blot analysis of P-FAK and FAK expression in MSCs. C: The expression of GSK-3β were measured by western blot.

LY294002, as a synthetic compound [Walker et al., 2000], has been used widely as pharmaceutical tools for the involvement of the PI3K pathway in various biological systems [Romanelli et al., 2009; Tang et al., 2009]. For example, LY294002 has been shown to inhibit rat NPC proliferation [Hsu et al., 2005; Hu et al., 2010], which suggested that PI3K/AKT signal has been implicated in cell cycle control. Differentiation of MSCs coincides with a loss of cell cycling capacity, and inhibition of the G1/Stransition correlates with differentiation of MSCs [Alfaro et al., 2010]. Cyclin D1 and cyclin E1 are critical for entry of cells into cell cycle and mediate G1 to S phase progression [Lee et al., 2009]. Here, we found that LY294002 stimulated an increased number of rat MSCs to progress through G1/S, which was weaken significantly by LICL. LY294002 reduced the gene expression of D1 and E1 cyclins, whereas LiCL inhibited these events significantly. Our findings also indicate that the proliferation of cultured MSCs is negatively regulated by LY294002.

Chemical inducer is a key elements to impart fate choices of MSCs. Several laboratories have shown that MSCs are able to differentiate beyond tissues of mesodermal origin into neuron-like cells harboring a variety of neuronal markers and transcription factors [Woodbury et al., 2002; Hermann et al., 2004]. For example, MSCs can differentiate into neuron-like cells induced by beta-mercaptoethanol (BME), butyl hydroxyanisole (BHA) or gene expression, and express specific neural proteins [Woodbury et al.,

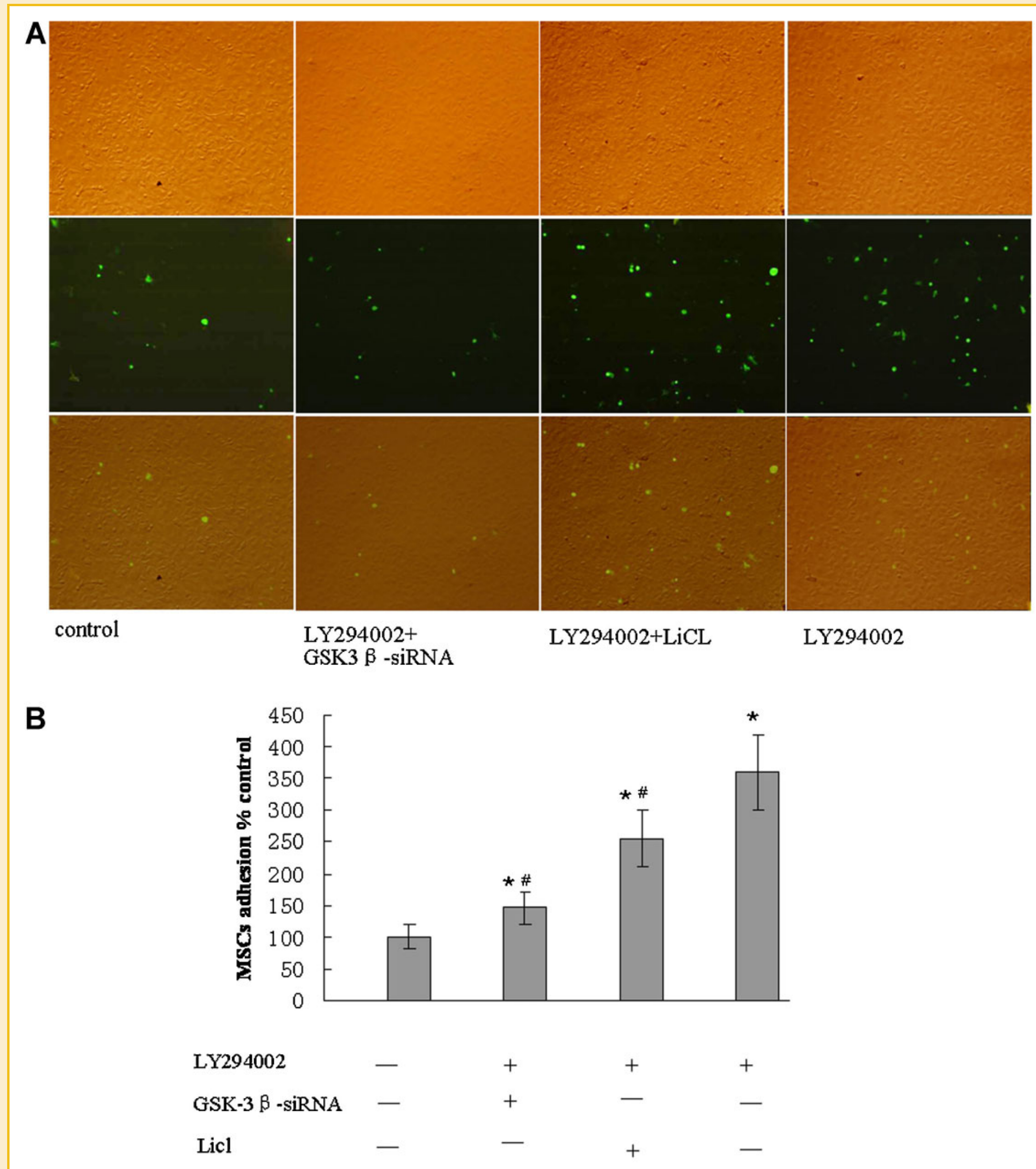

Fig. 5. MSCs-endothelial adhesion assay. A: MSCs were fluorescent labeled with Calcein AM, then were treated with LY294002, LY294002 + LiCL, or LY294002 + GSK-3β-siRNA and subsequently incubated on the endothelial cell monolayer for 2 h. B: In randomly selected in five vision to the number of representatives of Calcein AM labeled MSCs. Data are mean ± SD, $^{*}P < 0.05$ compared to control group. Data are mean ± SD, $^{*}P < 0.05$ compared to control group, $^{\#}P < 0.05$ compared to LY294002 treatment group. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

2000; Deng et al., 2001; Tondreau et al., 2008]. In the study, we found that LY294002 was sufficient to commit MSCs to a neural fate, but also to promote the terminal differentiation of these cells into mature neurons. LY294002 could increase the mRNA expression of nestin, NF-200 and β-tubulin III, and promoted MSCs differentiation toward the neuron-like cell, which could be inhibited by LiCL. This in vitro observation with rat MSCs demonstrated that LY294002 could also directly promote neuronal differentiation of MSCs.

Integrins comprise a large family of heterodimeric cell surface receptors that govern cell–extracellular matrix (ECM) interactions and mediate cellular adhesion and migration [Lorentz et al., 2002]. It has been reported that integrin could induce FAK phosphorylation, which play a role in cell survival and adhesion [Bezzi et al., 2003]. Recent studies have shown that over 80% of MSCs expressed integrin subunits β1, and 20–55% expressed α1 identified by flow cytometry and immunocytochemistry [Semon et al., 2010]. Our results extended these results and found that LY294002 significantly increased α5β1 integrin expression and FAK phosphorylation on neuron-like cell. To investigate the relationship between α5β1 integrin and adhesion ability on neuron-like cell, cell adhesion assay was performed. We found that the adhesion ability of neuron-like cell significantly increased compared with MSCs, which was blocked

by pretreatment endothelial cells with the antibody of α5β1 integrin. It suggested that this adhesion was α5β1 integrin dependent.

The glycogen synthase kinase 3β (GSK-3β) is a serine/threonine protein kinase that is involved in many physiological processes, playing important roles in glucose metabolism, cell cycle division, cell adhesion [Inoue et al., 2006; Holmes et al., 2008]. To investigate whether the role of GSK-3β on neural-like cells, GSK-3β expression was analyzed by Western blotting. We found that LY294002 could increase GSK-3β phosphorylation levels, which could be significantly weaken by LiCL or GSK-3β–siRNA. Further, α5β1 integrin expression and FAK phosphorylation were reduced by LiCL or GSK-3β–siRNA. Interestingly, the increased number of adherent cells induced by LY294002 could be also inhibited by LiCL or GSK-3β–siRNA. These data suggested that GSK-3β signaling pathway might be involved in neural-like cells adhesion to endothelial cells.

To conclude, LY294002 can trigger the expression of several neuronal markers of MSCs, which be inhibited by LiCL. LY294002 increase α5β1 integrins expression to activate FAK phosphorylation, and strengthen neuron-like cells adhesion force. Data presented here reveal a possible mechanism that GSK-3β/β-catenin signaling were involved in MSCs proliferation, differentiation and neuron-like cells adhesion force. At the same time, the data in this study provides a foundation for understanding the molecular and cell biological requirements for MSCs therapy.

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