Distinct Roles of Glycogen Synthase Kinase (GSK)-3α and GSK-3β in Mediating Cardiomyocyte Differentiation in Murine Bone Marrow-derived Mesenchymal Stem Cells**

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The signaling mechanisms facilitating cardiomyocyte (CM) differentiation from bone marrow (BM)-derived mesenchymal stem cells (MSCs) are not well understood. 5-Aza-cytidine (5-Aza), a DNA demethylating agent, induces expression of cardiac-specific genes, such as Nkx2.5 and α-MHC, in mouse BM-derived MSCs. 5-Aza treatment caused significant up-regulation of glycogen synthase kinase (GSK)-3β and down-regulation of β-catenin, whereas it stimulated GSK-3α expression only modestly. The promoter region of GSK-3β was heavily methylated in control MSCs, but was demethylated by 5-Aza. Although overexpression of GSK-3β potently induced CM differentiation, that of GSK-3α induced markers of neuronal and chondrocyte differentiation. GSK-3 inhibitors, including LiCl, SB 216743, and BIO, abolished 5-Aza-induced up-regulation of CM-specific genes, suggesting that GSK-3 is necessary and sufficient for CM differentiation in MSCs. Although specific knockdown of endogenous GSK-3β abolished 5-Aza-induced expression of cardiac specific genes, surprisingly, that of GSK-3α facilitated CM differentiation in MSCs. Although GSK-3β is found in both the cytosol and nucleus in MSCs, GSK-3α is localized primarily in the nucleus. Nuclear-specific overexpression of GSK-3β failed to stimulate CM differentiation. Down-regulation of β-catenin mediates GSK-3β-induced CM differentiation in MSCs, whereas up-regulation of c-Jun plays an important role in mediating CM differentiation induced by GSK-3α knockdown. These results suggest that GSK-3α and GSK-3β have distinct roles in regulating CM differentiation in BM-derived MSCs. GSK-3β in the cytosol induces CM differentiation of MSCs through down-regulation of β-catenin. In contrast, GSK-3α in the nucleus inhibits CM differentiation through down-regulation of c-Jun.

Ischemic cardiomyopathy and myocardial infarction are accompanied by an irreversible loss of cardiomyocytes, endothelial cells, and smooth muscle cells, essential components of the heart (1). Cell-based cardiac repair offers the promise of rebuilding the injured heart from its component parts (reviewed in Refs. 2 and 3). Although remarkable progress in the field has clearly proven the concept of “cell-based cardiac repair,” initial clinical studies using adult stem cells have shown that the salutary effects mediated by cell transplantsations are generally modest (4–7). A major challenge for cardiac regeneration therapy using adult stem cells may be to enhance stem cell differentiation into cardiomyocytes.

Among several important signaling mechanisms generally involved in cardiomyocyte differentiation, Wnt/β-catenin (canonical Wnt) and non-canonical Wnt signaling have been suggested to have a critical role in cardiogenesis (8). Glycogen synthase kinase (GSK)2−3 is a key component of the canonical Wnt signaling pathway. GSK-3 phosphorylates β-catenin, and phosphorylated β-catenin is then subjected to ubiquitin proteasome degradation. However, upon Wnt binding to its receptors, Frizzled and low-density lipoprotein receptor-related protein, β-catenin phosphorylation by GSK-3β is inhibited and β-catenin is stabilized. Stabilized β-catenin translocates into the nucleus and induces target gene expression. Although both the canonical and non-canonical Wnt pathways are important in mediating cardiomyocyte differentiation in stem cells and cardiac progenitor cells (9–15), the role of downstream components of the Wnt pathway, and, in particular, the role of GSK-3, in mediating cardiomyocyte differentiation is not yet fully understood.

GSK-3 is a serine/threonine kinase that has a wide variety of functions in cells. GSK-3 phosphorylates many known intracellular targets, including β-catenin, glycogen synthase, eIF2β, GATA4, myocardin, c-Jun, cyclin D1, and N-Myc, thereby regulating various cellular functions, including hypertrophy and apoptosis in cardiomyocytes (16). GSK-3 has two major isoforms, GSK-3α and GSK-3β, which have 97% identical amino acids in the catalytic domain but differ substantially in the N and C termini. Increasing lines of evidence suggest that GSK-3α and GSK-3β both have common and non-overlapping functions (17). For example, both GSK-3α and GSK-3β phosphorylate/degrade β-catenin in embryonic stem cells, but GSK-3α

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2 The abbreviations used are: GSK, glycogen synthase kinase; 5-Aza, 5-azacytidine; BIO, 6-bromo-indirubin-3’-oxime; MSC, mesenchymal stem cell; Dox, doxycycline; Tg, transgenic; NLS, nuclear localization signal; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription; α-MHC, α-myosin heavy chain; cTnI, cardiac troponin I; cTnC, cardiac troponin C; DN, dominant-negative; shRNA, short hairpin RNA; tTA, tetracycline-controlled transactivator; rtTA, reverse tetracycline controlled transactivator.

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and GSK-3β play distinct roles in cardiac development in mice (18, 19). Importantly, the isoform-specific functions of GSK-3α and GSK-3β during cardiomyocyte differentiation are not well understood in mesenchymal stem cells (MSCs).

5-Azacytidine (5-Aza) is a chemical analogue of cytidine that removes methyl groups from DNA, thereby inducing gene expression. 5-Aza is a potent inducer of cardiomyocyte differentiation in bone marrow-derived MSCs (20). MSCs show potential for clinical application with evidence of tissue regeneration, including myocardial regeneration (21). We reasoned that by studying the function of signaling molecules modulated during cardiac differentiation, we should be able to elucidate the signaling mechanisms involved in stimulating cardiomyocyte differentiation in adult stem cells. Through initial screening of signaling molecules modulated by 5-Aza during cardiomyocyte differentiation in MSCs, we found that GSK-3 plays an important role in regulating this process.

Thus, the goals in this study were to elucidate whether GSK-3α/β is affected during differentiation of MSCs into the cardiomyocyte lineage in response to 5-Aza treatment, and if so, to examine whether modulation of GSK-3 plays a causative role in mediating cardiomyocyte differentiation in MSCs. Furthermore, we evaluated whether GSK-3α and GSK-3β play distinct roles in mediating cardiomyocyte differentiation and whether regulation of β-catenin is involved in modulation of cardiomyocyte differentiation by GSK-3α/β.

EXPERIMENTAL PROCEDURES

Transgenic Mice and MSC Culture—MSCs were isolated from bone marrow aspirates of 2–3-week-old C57BL/6 mice. Wild type mice, Tet-inducible GSK-3β transgenic mice (Tg-Tet-GSK-3β), Tg-Tet-GSK-3β mice cross-bred with CMV-tTA transgenic mice (Tg-Tet-GSK-3β-tTA) (16), and transgenic mice harboring the mouse Nkx2.5 (9.0 kb) promoter-LacZ (see below) were used. Tg-Tet-GSK-3β-tTA mice were fed doxycycline (Dox)-containing chow to suppress GSK-3β transgene expression. MSCs were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium/F-12 (Invitrogen) and mesenchymal basal medium (Stem Cell) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and 1% l-glutamine (Invitrogen). At passage three, cells were positive for CD105, CD29, and CD44, and negative for CD45. MSCs passed 3–5 times were used.

Plasmid Constructs and Adenoviral Vectors—Adenoviruses harboring GSK-3α, GSK-3β, dominant-negative GSK-3β (DN-GSK-3β), β-catenin, and LacZ have been described previously (16, 22, 23). To make GSK-3β with a nuclear localization signal (GSK-3β-NLS), cDNA encoding GSK-3β was subcloned into pEF/myc/nuc (Invitrogen). cDNA encoding Wnt11 was amplified by reverse transcription-polymerase chain reaction from mouse heart mRNA, subcloned into pCR2.1-TOPO (Invitrogen), and sequenced to confirm the correct sequence. cDNA encoding Wnt3a was purchased from Origene. Complementary hairpin sequences for GSK-3α, GSK-3β, β-catenin, c-Jun, and scramble (supplemental Table S1) were commercially synthesized and cloned into pSilencer 2.0—Adenovirus vector harboring tTA or rtTA transduced MSCs with or without 5-Aza were evaluated by immunostaining. Cells were co-stained with DAPI. The results are representative of at least 4 experiments.

FIGURE 1. Basal characteristics of mouse BM-derived MSCs. A, total RNA was prepared from MSCs at the 3rd and 10th passages. mRNA expression of Oct4 and Rex1, pluripotent stem cell markers, was evaluated by RT-PCR. B, protein expression of Oct3/4 (red) in MSCs at the 3rd passage was evaluated by immunostaining. Cells were co-stained with 4’,6-diamidino-2-phenylindole (DAPI). C and D, MSCs were treated with 5-Aza (5 μM) and cultured for 5 days. C, total RNA was prepared and mRNA expression of Nkx2.5, α-MHC, and GAPDH (internal control) evaluated by RT-PCR. D, protein expression of cTnI (green) in MSCs treated with or without 5-Aza was evaluated by immunostaining. Cells were co-stained with DAPI. The results are representative of at least 4 experiments.
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Dox (0.5 μg/ml, Clontech). Tet/tTA-MSCs isolated from Tg-Tet-GSK-3β-tTA mice were maintained in Dox containing medium and changed to normal culture medium for induction of GSK-3β transgene.

Western Blots—Total cell extracts were prepared, using cell lysis buffer containing 20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 0.5 μg/ml of leupeptin, and 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride. For immunoblot analyses, polyvinylidene difluoride membranes were incubated with 5% nonfat milk buffer containing primary antibody overnight, followed by incubation with anti-mouse IgG or anti-rabbit IgG (Cell Signaling Technology, 1:2500 dilution) for 3 h. The following antibodies were used as primary antibodies: GSK-3β (Cell Signaling Technology, 1:5000), GSK-3β (BD Biosciences, 1:5000), phospho-GSK-3β (Cell Signaling Technology, 1:5000), GSK-3α (BD Biosciences, 1:10000), Wnt3a (Santa Cruz Biotechnology, 1:2500), Wnt11 (R&D Systems, 1:2000), phospho-protein kinase C (Pan) (Cell Signaling Technology, 1:2500), sarcomeric α-actinin (Sigma, 1:10000), GATA4 (Santa Cruz Biotechnology, 1:5000), c-Jun (Cell Signaling Technology, 1:2500), troponin I (Santa Cruz Biotechnology, 1:5000), β-actin (Sigma, 1:10000), and GAPDH (Sigma, 1:5000).

Immunocytochemistry—MSCs were washed with phosphate-buffered saline, fixed with 4% paraformaldehyde for 10 min, permeabilized in 0.3% Triton X-100 for 10 min, and blocked in 3% bovine serum albumin for 1 h. The following antibodies were used as primary antibodies: Oct3/4 (Santa Cruz Biotechnology, 1:200 dilution), cardiac troponin I (Abcam, 1:200), sarcomeric α-actinin (Sigma, 1:5000), GSK-3β (BD Biosciences, 1:5000), GSK-3α (Abcam, 1:500), and c-Jun (Cell Signaling Technology, 1:500).

Reverse Transcriptase-PCR—Total RNA was extracted using TRIzol (Invitrogen) and 1 μg of RNA was used for cDNA synthesis (ThermoScript®, Ambion). The RT-PCR mixture (Promega) was incubated at 95 °C for 5 min followed by 95 °C for 30 s, 59 °C for 1 min, and 72 °C for 30 s for 34 cycles and then incubated at 72 °C for 7 min. PCR primers used in this study are shown in supplemental Table S1.

Methylation Specific PCR—Genomic DNA was extracted from MSCs using the Wizard Genomic DNA Purification Kit (Promega) and then treated with the CpGenome Fast DNA Modification Kit (Millipore). CpGenome-modified DNA (1 μg) was subjected to PCR with methylation- or non-methylation-specific primers (supplemental Table S1) (24).
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Transgenic Mice Harboring Nkx2.5-LacZ—cDNA containing mouse Nkx2.5 promoter-LacZ, which directs cardiac specific activation of luciferase (25), was kindly provided by Dr. K. Yutzey (The Children’s Hospital Research Foundation, Cincinnati, OH). Transgenic mice harboring the mouse Nkx2.5 (9.0 kb) promoter-luciferase were generated on FVB background.

Luciferase Assay—Plasmids harboring TOP flash (TCF-luciferase plasmid, Millipore) and FOP flash (mutant TCF-luciferase plasmid, Millipore) were transfected into MSCs using Lipofectamine reagent (Invitrogen), and the luciferase activity was measured with the Luciferase Assay System (Promega) after cell lysis with Passive Lysis Buffer (Promega).

FIGURE 3. GSK-3α, GSK-3β, and DN-GSK-3β overexpression in MSCs. A, protein expression of GSK-3α, GSK-3β, β-catenin, and β-actin was evaluated by immunoblot analyses in Ad-LacZ, Ad-GSK-3α, and Ad-GSK-3β-transduced MSCs. B, protein expression of β-catenin and β-actin was evaluated by immunoblot analyses in Ad-DN-GSK-3β-transduced MSCs. C, mRNA expression of Flk-1, Nkx2.5, atrial natriuretic factor, cTnC, α-MHC, GSK-3α, and GSK-3β was evaluated by RT-PCR. Expression of GAPDH was evaluated as an internal control. D, MSCs were prepared from transgenic mice harboring Nkx2.5-LacZ, MSCs were subjected to 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal) staining after GSK-3/β-, GSK-3α-, and GSK-3β-transduced MSCs. E, MSCs were evaluated by RT-PCR. Expression of GAPDH was evaluated as an internal control.

RESULTS

Murine Bone Marrow-derived MSCs Express Pluripotent Markers and 5-Aza Induces Expression of Cardiac Marker Genes in MSCs—MSCs at the third passage expressed Oct4 and Rex1 mRNA, pluripotent stem cell markers (Fig. 1A). Ninety-five percent of MSCs at the third passage were Oct3/4 positive (Fig. 1B). Although untreated bone marrow-derived mouse MSCs do not express cardiac marker genes, 5-Aza treatment (5 μM for 24 h), an established method of inducing bone marrow MSC differentiation into cardiomyocytes (20), induced mRNA expression of Nkx2.5, one of the earliest cardiac markers, and α-myosin heavy chain (α-MHC), a contractile protein (Fig. 1C). Although no cardiac troponin I (cTnI) positive cells were observed in control MSCs, 5-Aza induced a premature but clear striation pattern of cTnI in MSCs (Fig. 1D).

5-Aza Induces Cardiomyocyte Differentiation through an Increase in GSK-3β Protein and mRNA Expression—GSK-3β and β-catenin are important components of the canonical Wnt signaling pathway. To examine the effect of 5-Aza on the canonical Wnt signaling pathway, protein expression of GSK-3 isoforms and β-catenin was evaluated in 5-Aza-treated (5 μM for 24 h) and control MSCs. Expression of GSK-3α and GSK-3β was detectable but low. On the other hand, β-catenin was expressed abundantly in unstimulated MSCs. 5-Aza treatment increased expression of GSK-3α and GSK-3β in a time-dependent manner in MSCs, although induction of GSK-3α by 5-Aza was milder than that of GSK-3β (Fig. 2, A and B). Expression of GSK-3β at day 5 was significantly greater in 5-Aza-treated MSCs than in untreated MSCs (supplemental Fig. S1A). 5-Aza treatment did not induce up-regulation of GSK-3β in COS-7 cells, suggesting that the effect of 5-Aza is cell type-specific (supplemental Fig. S1B). Up-regulation of GSK-3α and GSK-3β by 5-Aza was also observed at the mRNA level (supplemental Fig. S1C). The promoter regions of GSK-3α and

Statistical Analyses—All values are expressed as mean ± S.E. Statistical analyses were performed using analysis of variance and Newman-Keuls multiple comparison test with a p < 0.05 considered significant.
GSK-3β contain CpG islands (supplemental Fig. S2). The CpG islands are methylated in untreated MSCs, but are demethylated after 5-Aza treatment (Fig. 2C). Although protein expression of β-catenin gradually increased in control MSCs, a progressive decrease in β-catenin was observed in 5-Aza-treated MSCs (Fig. 2, A and D), accompanied by decreases in TCF/LEF transcriptional activity as determined by TOP flash/FOP flash reporter gene assays (Fig. 2E).

GSK-3β Induces Cardiomyocyte Differentiation in MSCs—
To examine whether expression of GSK-3 isoforms mimics the effect of 5-Aza upon cardiomyocyte differentiation, GSK-3α or GSK-3β were overexpressed via adenovirus transduction in MSCs (Fig. 3A). Although GSK-3β overexpression reduced expression of β-catenin in MSCs, GSK-3α overexpression did not (Fig. 3A, see supplemental Fig. S6B). Adenovirus vectors harboring LacZ (Ad-LacZ) and DN-GSK-3β (Ad-DN-GSK-3β) were used as controls. As expected, Ad-DN-GSK-3β increased expression of β-catenin (Fig. 3B). Transduction of MSCs with adenovirus harboring GSK-3β (Ad-GSK-3β), but not Ad-LacZ or Ad-DN-GSK-3β, induced mRNA expression of mesoderm markers, including Flk-1 (26) (Fig. 3C and data not shown). Ad-GSK-3β-induced mRNA expression of cardiomyocyte markers, including Nkx2.5, cardiac troponin C (cTnC), α-MHC, and atrial natriuretic factor (Fig. 3C). Although Ad-GSK-3α also slightly induced mRNA expression of Flk-1, cTnC, and α-MHC, the extent of their up-regulation was less than that by Ad-GSK-3β. Transduction of Ad-LacZ or Ad-DN-GSK-3β did not significantly induce expression of the cardiomyocyte markers (Fig. 3C). To obtain genetic evidence of cardiomyocyte differentiation, we generated transgenic mice harboring a LacZ gene driven by the Nkx2.5 promoter (9.0 kb), a cardiac specific promoter (Tg-Nkx2.5-LacZ). Transduction of MSCs isolated from Tg-Nkx2.5-LacZ mice with Ad-GSK-3β increased the number of β-galactosidase positive cells, whereas transduction with Ad-DN-GSK-3β, Ad-GSK-3α, or Ad-LacZ did not (Fig. 3D and data not shown). Sarcomeric α-actinin protein expression was observed in MSCs transduced with Ad-GSK-3β, but not in MSCs transduced with Ad-DN-GSK-3β. On the other hand, only weak or negligible expression of sarcomeric α-actinin was observed in GSK-3α-overexpressing MSCs (Fig. 3E, see also Fig. 8D). Ad-GSK-3β exhibited stronger induction of α-actinin expression than 5-Aza treatment (supplemental Fig. S3). Taken together, these results suggest that GSK-3β induces cardiomyocyte differentiation in bone marrow-derived MSCs and that overexpression of GSK-3β induces cardiomyocyte differentiation more potently than that of GSK-3α.

Although transduction of MSCs with Ad-GSK-3α induced mRNA expression of both nestin, a neural marker, and Sox9, a chondrocyte marker, Ad-GSK-3β did not induce mRNA expression of either of them (supplemental Fig. S4). Transduction of MSCs with Ad-DN-GSK-3β induced mRNA expression of Sox9, but not nestin, suggesting that endogenous GSK-3β may inhibit chondrogenic differentiation in MSCs (supplemental Fig. S4).

To achieve up-regulation of GSK-3β by an alternative method, MSCs derived from Tet-GSK-3β transgenic mice were transduced with Ad-tTA (the Tet-Off system) or Ad-rTα (the Tet-On system), and then treated with or without Dox. GSK-3β expression was induced by withdrawing Dox in the Tet-Off system and by adding Dox in the Tet-On system (Fig. 4, A and B). The effect of Dox upon transgene expression was reversible (Fig. 4C), suggesting that GSK-3β expression can be regulated by Dox treatment in these MSCs. In both the Tet-On and Tet-Off systems, up-regulation of GSK-3β induced expression of Nkx2.5 and α-MHC in MSCs (Fig. 4D). Alternatively, Tet-inducible GSK-3β transgenic mice were crossed with transgenic mice harboring CMV-tTA and then MSCs were prepared from either Tet-GSK-3β/tTA (Tet) or Tet-GSK-3β-MHC and CMV-tTA mice. The GSK-3β transgene has a Myc tag. A, MSCs prepared from Tg-Tet-GSK-3β mice were transduced with adenovirus harboring tTA (Ad-tTA) and transgene expression was suppressed by Dox (0.5 μg/ml) treatment. Adenoviral vector LacZ (Ad-LacZ) was used as a control. B, MSCs prepared from Tg-Tet-GSK-3β mice were transduced with adenovirus harboring rTα and transgene expression was induced by Dox (0.5 μg/ml) treatment. Ad-LacZ was used as a control. C, GSK-3β expression was decreased by Dox and recovered after Dox was washed out in MSCs prepared from Tet-GSK-3β-MHC and CMV-tTA mice. D, the effect of inducible GSK-3β overexpression in the Tet-On (tTA) or Tet-Off (rTα) system upon mRNA expression of Nkx2.5 and α-MHC is shown. Ad-LacZ was used as a negative control. E and F, MSCs prepared from Tg-Tet-GSK-3β/tTA mice were cultured with or without Dox. mRNA expression of α-MHC and GAPDH was evaluated by real-time RT-PCR. Experiments were repeated at least 4 times.
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A

\[
\begin{array}{c|c|c|c|c}
 & Wnt3a & \beta\text{-actin} \\
\hline
Ad-LacZ & + & + & - & - \\
Ad-Wnt3a & - & - & + & + \\
\end{array}
\]

B

\[
\begin{array}{c|c|c|c|c}
 & Wnt11 & \beta\text{-actin} \\
\hline
Ad-LacZ & + & + & - & - \\
Ad-Wnt11 & - & - & + & + \\
\end{array}
\]

C

\[
\begin{array}{c|c|c|c|c}
 & \beta\text{-catenin} & \text{Phospho PKC (Pan)} & \text{GAPDH} \\
\hline
Ad-LacZ & + & - & - & - \\
Ad-Wnt3a & - & + & + & + \\
Ad-Wnt11 & - & - & + & + \\
Ad-GSK-3\beta & - & - & - & + \\
\end{array}
\]

D

\[
\begin{array}{c|c|c|c|c}
 & (a) Ad-GSK-3\alpha & (b) Ad-GSK-3\beta & (c) Ad-DN-GSK-3\beta \\
\hline
 & 100\mu m & 100\mu m & 100\mu m \\
\end{array}
\]

\[
\begin{array}{c|c|c|c|c}
 & (d) Ad-Wnt11 & (e) Ad-Wnt3a & (f) Ad-LacZ \\
\hline
 & 100\mu m & 100\mu m & 100\mu m \\
\end{array}
\]

E

\[
\begin{array}{c|c|c|c|c}
 & Flk-1 & Nkx2.5 & cTnC & \alpha\text{-MHC} \\
\hline
Ad-LacZ & + & - & - & - \\
Ad-Wnt11 & - & + & - & - \\
Ad-GSK-3\beta & - & - & + & + \\
\end{array}
\]

F

\[
\begin{array}{c|c|c|c|c}
 & Flk-1 & Nkx2.5 & cTnC & \alpha\text{-MHC} & \text{GAPDH} \\
\hline
Ad-LacZ & + & - & - & - & - \\
Ad-Wnt3a & - & + & - & - & - \\
Ad-GSK-3\beta & - & - & + & + & + \\
\end{array}
\]
of protein kinase C and Wnt3a caused a significant accumulation of β-catenin, suggesting that the non-canonical and canonical Wnt pathways were stimulated in MSCs in our experimental conditions (Fig. 5C). Up-regulation of GSK-3β induced the frequent appearance of tubular structures in MSCs (Figs. 5D and supplemental S5). Tubular structures are induced when MSCs are differentiated into the cardiomyocyte lineage (20, 27). Although up-regulation of GSK-3α and Wnt11 also induced some tubular structures, they were not as prominent as those induced by GSK-3β. Tubular structures were not observed in MSCs treated with Ad-LacZ, Ad-Wnt3a, or Ad-DN-GSK-3β (Figs. 5D and supplemental S5). Wnt11 induced mRNA expression of αMHC, but little or no expression of Flk-1, Nkx2.5, or cTnC, in MSCs (Fig. 5E), whereas Wnt3a did not induce mRNA expression of any of these marker genes (Fig. 5F). These results suggest that GSK-3β induces cardiomyocyte differentiation of MSCs more potently than the Wnt agonists.

Expression of GSK-3 Is Required for Cardiomyocyte Differentiation in MSCs—Because GSK-3 was up-regulated by 5-Aza and up-regulation of GSK-3β potently induced cardiomyocyte differentiation in MSCs, we next examined whether GSK-3 is required for induction of cardiomyocyte differentiation by 5-Aza in MSCs. MSCs were stimulated with 5-Aza in the presence or absence of known inhibitors of GSK-3. Treatment of MSCs with LiCl suppressed 5-Aza-induced down-regulation of β-catenin, suggesting that LiCl suppresses GSK-3 activity under 5-Aza treatment in MSCs (Fig. 6A). Although treatment of MSCs with LiCl induced expression of GATA4, LiCl alone did not induce expression of other cardiomyocyte marker genes. LiCl did, however, inhibit
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6-bromo-indirubin-3’-oxime (BIO) and SB216743, reversed 5-Aza-induced down-regulation of β-catenin (Fig. 6D). In addition, BIO and SB216743 inhibited 5-Aza-induced up-regulation of Flk-1, Nkx2.5, cTnC, and α-MHC mRNA (Fig. 6E) and sarcomeric α-actinin protein expression (supplemental Fig. S3). These results suggest that GSK-3 plays an essential role in mediating 5-Aza-induced cardiomyocyte differentiation in MSCs.

The Roles of the GSK-3 Isoforms in Mediating Cardiomyocyte Differentiation in MSCs—To evaluate the roles of endogenous GSK-3α and GSK-3β in mediating cardiomyocyte differentiation separately, we generated adenovirus vectors harboring shRNA-GSK-3α (Ad-sh-GSK-3α), shRNA-GSK-3β (Ad-sh-GSK-3β), and shRNA-scramble (Ad-sh-scramble) and confirmed that Ad-sh-GSK-3α and Ad-sh-GSK-3β selectively down-regulate GSK-3α and GSK-3β, respectively (Fig. 7A). Although Ad-sh-GSK-3β increased expression of β-catenin, Ad-sh-GSK-3α did not (Fig. 7A). Down-regulation of GSK-3β did not induce expression of Flk-1, a mesoderm marker, and the cardiomyocyte-specific genes, including αMHC, Nkx2.5, and cTnC (Fig. 7B). Unexpectedly, however, down-regulation of GSK-3α did induce mRNA expression of Flk-1 and cardiac specific genes (Fig. 7B). Down-regulation of GSK-3α, but not GSK-3β, also induced protein expression of α-actinin, cTnI, and GATA4, cardiomyocyte markers (Fig. 7C, see also Fig. 10C). Furthermore, although down-regulation of GSK-3β inhibited 5-Aza-induced up-regulation of mesoderm and cardiomyocyte markers, down-regulation of GSK-3α enhanced it (Fig. 7D). Essentially the same results were obtained with shRNA-GSK-3α targeting a distinct site (data not shown), suggesting that the effect of Ad-sh-GSK-3α was mediated by GSK-3α and not an off-target effect. These results suggest that endogenous GSK-3α and GSK-3β have opposite effects upon cardiomyocyte differentiation in MSCs. When GSK-3α was down-regulated in the presence of GSK-3β up-regulation, β-catenin remained down-regulated (Fig. 7E), whereas mesoderm and cardiomyocyte differentiation were enhanced (Fig. 7F), suggesting that up-regulation of GSK-3β and down-regulation of GSK-3α utilize distinct cellular mechanisms to induce cardiomyocyte differentiation in MSCs.

Differential Subcellular Localization of GSK-3 Isoforms May in Part Mediate Differential Effects of GSK-3α and GSK-3β upon Cardiomyocyte Differentiation in MSCs—Immunostaining with isoform-specific antibodies indicated that GSK-3α is localized primarily in the nucleus in MSCs. In contrast, GSK-3β is localized mainly in the cytosol but also partly in the nucleus (Fig. 8A). Transduction of MSCs with adenovirus harboring LacZ did not significantly change subcellular localization of endogenous GSK-3α or GSK-3β (Fig. 8B). Adenovirus-mediated overexpression of GSK-3α increased expression of GSK-3α in both the nucleus and cytoplasm and decreased expression of endogenous GSK-3β (Figs. 8B and supplemental S6A). Thus, overexpression of GSK-3α alters the subcellular distribution of GSK-3 isoforms. On the other hand, adenovirus-mediated overexpression of GSK-3β increased expression of GSK-3β in both the nucleus and cytoplasm, without signifi-

![Figure 7. The role of GSK-3α and GSK-3β in mediating cardiomyocyte differentiation of MSCs.](image-url)
but not GSK-3α, plays an essential role in regulating the cellular level of β-catenin in MSCs. To examine whether down-regulation of β-catenin is sufficient to induce expression of cardiomyocyte marker genes, β-catenin was down-regulated by adenovirus harboring shRNA-β-catenin (Ad-sh-β-catenin) (Fig. 9A). Down-regulation of β-catenin by Ad-sh-β-catenin led to the induction of mesoderm and cardiomyocyte markers, whereas shRNA-scramble had no effect (Fig. 9B). Up-regulation of β-catenin by adenovirus harboring β-catenin (Ad-β-catenin) (Fig. 9C) inhibited GSK-3β induced up-regulation of mesoderm and cardiomyocyte markers (Fig. 9D). These results suggest that endogenous GSK-3β, but not GSK-3α, plays an essential role in regulating the cellular level of β-catenin in MSCs. To examine whether down-regulation of β-catenin is sufficient to induce expression of cardiomyocyte marker genes, β-catenin was down-regulated by adenovirus harboring shRNA-β-catenin (Ad-sh-β-catenin) (Fig. 9A). Down-regulation of β-catenin by Ad-sh-β-catenin led to the induction of mesoderm and cardiomyocyte markers, whereas shRNA-scramble had no effect (Fig. 9B). Up-regulation of β-catenin by adenovirus harboring β-catenin (Ad-β-catenin) (Fig. 9C) inhibited GSK-3β induced up-regulation of mesoderm and cardiomyocyte markers (Fig. 9D). These results suggest that endogenous GSK-3β, but not GSK-3α, plays an essential role in regulating the cellular level of β-catenin in MSCs. To examine whether down-regulation of β-catenin is sufficient to induce expression of cardiomyocyte marker genes, β-catenin was down-regulated by adenovirus harboring shRNA-β-catenin (Ad-sh-β-catenin) (Fig. 9A). Down-regulation of β-catenin by Ad-sh-β-catenin led to the induction of mesoderm and cardiomyocyte markers, whereas shRNA-scramble had no effect (Fig. 9B). Up-regulation of β-catenin by adenovirus harboring β-catenin (Ad-β-catenin) (Fig. 9C) inhibited GSK-3β induced up-regulation of mesoderm and cardiomyocyte markers (Fig. 9D). These results suggest that endogenous GSK-3β, but not GSK-3α, plays an essential role in regulating the cellular level of β-catenin in MSCs. To examine whether down-regulation of β-catenin is sufficient to induce expression of cardiomyocyte marker genes, β-catenin was down-regulated by adenovirus harboring shRNA-β-catenin (Ad-sh-β-catenin) (Fig. 9A). Down-regulation of β-catenin by Ad-sh-β-catenin led to the induction of mesoderm and cardiomyocyte markers, whereas shRNA-scramble had no effect (Fig. 9B). Up-regulation of β-catenin by adenovirus harboring β-catenin (Ad-β-catenin) (Fig. 9C) inhibited GSK-3β induced up-regulation of mesoderm and cardiomyocyte markers (Fig. 9D).
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FIGURE 9. Down-regulation of β-catenin plays a critical role in mediating GSK-3β-induced cardiomyocyte differentiation in MSCs. A and B, MSCs were transduced with Ad-shRNA-scramble (Ad-sh-scramble) or Ad-shRNA-β-catenin (Ad-sh-β-catenin). A, protein expression of β-catenin and GAPDH (internal control) was evaluated by immunoblots. B, mRNA expression of Flik-1, Nkx2.5, α-MHC, cTnC, and GAPDH (internal control) was evaluated by RT-PCR. C, MSCs were transduced with Ad-LacZ or Ad-β-catenin and protein expression of β-catenin and GAPDH (internal control) was evaluated by immunoblots. D, MSCs were transduced with Ad-LacZ, Ad-β-catenin, Ad-GSK-3β, or Ad-β-catenin plus Ad-GSK-3β. mRNA expression of Flik-1, Nkx2.5, α-MHC, cTnC, and GAPDH (internal control) was evaluated by RT-PCR. E and F, MSCs were transduced with either Ad-sh-scramble or Ad-sh-β-catenin (E), or either Ad-LacZ or Ad-β-catenin (F). mRNA expression of Nestin, Sox9, and GAPDH (internal control) was evaluated by RT-PCR. In A–F, the results are representative of 3 experiments.

DISCUSSION

Expression of GSK-3 remains low when MSCs are uncommitted. However, GSK-3β is up-regulated when cardiomyocyte differentiation of MSCs is initiated by 5-Aza. Furthermore, up-regulation of GSK-3β is both necessary and sufficient for cardiomyocyte differentiation initiated by 5-Aza in MSCs. Unexpectedly, down-regulation, rather than up-regulation, of endogenous GSK-3α stimulated cardiomyocyte differentiation in MSCs. These results suggest that GSK-3β is an endogenous regulator of MSC differentiation and that GSK-3α and GSK-3β have opposite effects upon cardiomyocyte differentiation in MSCs.

5-Aza is a cytosine analogue and a demethylating agent that induces changes in chromatin structure, gene expression, cellular morphology, and survival in mammalian cells. Because phosphorylation of GSK-3β is not significantly affected by 5-Aza, 5-Aza must increase the total activity of GSK-3β primarily through up-regulation of GSK-3β mRNA. The promoter region of GSK-3β contains a prominent CpG island that is methylated in unstimulated MSCs, suggesting that 5-Aza induces up-regulation of GSK-3β through epigenetic modification of the promoter. Because GSK-3β stabilizes DNA methyltransferases, 5-Aza may initiate a positive feedback loop of GSK-3β promoter demethylation (28). At present, whether or not demethylation of the GSK-3β promoter is an endogenous mechanism for differentiation of adult stem cells into the cardiomyocyte lineage remains to be elucidated. In any event, GSK-3β may substitute for 5-Aza for induction of cardiomyocyte differentiation in MSCs, because clinical use of 5-Aza would be limited due to its nonspecific effects and obvious teratogenic actions.

GSK-3β is a central component of the Wnt pathway and negatively regulates β-catenin through phosphorylation-dependent proteolytic degradation (29). Although previous studies have shown that stimulation and inhibition of the Wnt signaling mechanism affect cardiomyocyte differentiation (8), up-regulation of GSK-3β induced cardiomyocyte markers more strongly than stimulation of either the canonical or non-canonical Wnt signaling pathways with Wnt3a and Wnt11, respectively. Because down-regulation of β-catenin alone also potently induces cardiomyocyte markers, modulating downstream components of the Wnt signaling pathway may induce cardiomyocyte differentiation more efficiently than stimulating the Wnt pathways at the receptor level.

It should be noted that GSK-3β not only regulates the Wnt pathway but also modulates a wide variety of signaling pathways, including other signaling cascades known to regulate stem cell differentiation, such as the Notch (30) and Hedgehog (31) pathways. Thus, up-regulation of GSK-3β may have a broader effect than selective stimulation of the Wnt pathway by the Wnt receptor ligand.

Increasing lines of evidence suggest that GSK-3α and GSK-3β have distinct cellular functions, despite the fact that they share 97% identity in their kinase domains and 36% identity overall. Our results suggest that GSK-3α and GSK-3β have distinct effects upon cardiomyocyte differentiation. Up-regulation of GSK-3β has a stronger effect upon cardiomyocyte differentiation in MSCs than up-regulation of GSK-3α. Although overexpression of GSK-3α slightly induces expression of cardiomyocyte markers, this effect may be mediated through promiscuous phosphorylation of GSK-3β substrates due to overexpression. In fact, adenovirus-mediated overexpression of GSK-3α substantially altered subcellular localization of GSK-3α in MSCs (see below). Importantly, although down-regulation of GSK-3β inhibited 5-Aza-induced cardiomyocyte differentiation, down-regulation of GSK-3α stimulated cardiomyocyte differentiation in MSCs. Previous studies have suggested that GSK-3α and GSK-3β could differentially affect cardiac development. Although GSK-3β knock-out mice exhibit cardiac defects consisting of malformation of the cardiac outflow tract and markedly thickened ventricular walls, contributing to their early mortality, GSK-3α knock-out mice show no significant cardiac defects (18). However, to our knowledge, the fact that GSK-3α and GSK-3β have opposite effects upon differentiation of stem cells has not been shown previously.

One possible explanation for the potential difference in their functions is that GSK-3α and GSK-3β exist in distinct subcel-
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For example, in adult mouse hearts, GSK-3α localized primarily in the nucleus, whereas GSK-3β exists primarily in the cytosol (17). GSK-3α localized in the nucleus phosphorylates and induces nuclear exit/proteolytic degradation of G1 cyclins, whereas endogenous GSK-3β localized in the cytosol does not induce nuclear exit of G1 cyclins in the mouse heart (17). Immunostaining of the GSK-3 isoforms suggest that GSK-3α is localized primarily in the nucleus, and GSK-3β primarily in the cytosol but also in the nucleus in MSCs. Forced expression of GSK-3β in the nucleus did not induce cardiomyocyte differentiation, whereas overexpression of GSK-3α induces cytosolic expression of GSK-3α and partially stimulates cardiomyocyte differentiation. We therefore speculate that endogenous GSK-3β more efficiently phosphorylates β-catenin in the cytosol, thereby inducing efficient proteolytic degradation, whereas endogenous GSK-3α primarily localized in the nucleus may regulate other targets, presumably transcription factors. Because GSK-3α and GSK-3β equally affect expression of β-catenin in some cell types, such as ES cells (19, 32), we speculate that subcellular localization of GSK-3α/β may be cell type- or developmental stage-dependent.

Importantly, up-regulation of GSK-3β and down-regulation of GSK-3α have additive effects upon cardiomyocyte differentiation in MSCs, consistent with the notion that they mediate cardiomyocyte differentiation through distinct cellular mechanisms. Our results suggest that down-regulation of β-catenin plays an important role in mediating the effect of GSK-3β upon MSC differentiation into the cardiomyocyte lineage. On the other hand, down-regulation of GSK-3α induces cardiomyocyte differentiation through up-regulation of c-Jun in MSCs. Because GSK-3 phosphorylates β-catenin and c-Jun (19, 33), thereby stimulating their degradation, it is likely that GSK-3β in the cytosol may phosphorylate β-catenin, whereas GSK-3α in the nucleus may phosphorylate c-Jun, thereby regulating cardiomyocyte differentiation in MSCs (supplemental Fig. S7). Interventions to selectively stimulate GSK-3β or inhibit GSK-3α may be considered independently or in combination with other methods to facilitate cardiomyocyte differentiation of MSCs for cell-based therapy in vivo.

We have successfully engineered MSCs that conditionally express GSK-3β through either withdrawal or application of Dox. Phasic modulation of the Wnt/β-catenin signaling mechanism effectively stimulates differentiation of progenitor cells into the cardiomyocyte lineage (10, 34). Activation of β-catenin

**FIGURE 10.** Up-regulation of c-Jun plays a critical role in mediating shRNA-GSK-3-α-induced cardiomyocyte differentiation in MSCs. A and B, MSCs were treated with Ad-shRNA-scramble (Ad-sh-scramble), Ad-shRNA-GSK-3-α (Ad-sh-GSK-3-α), or Ad-shRNA-GSK-3-β (Ad-sh-GSK-3-β). C–E, MSCs were transduced with Ad-sh-scramble, Ad-shRNA-c-Jun (Ad-sh-c-Jun), Ad-sh-GSK-3-α, or Ad-sh-c-Jun plus Ad-sh-GSK-3-α. A and C, protein expression of c-Jun and GAPDH (internal control) was evaluated by immunoblots. B, MSCs were subjected to staining with anti-c-Jun antibody, anti-sarcomeric α-actinin antibody, and 4′,6-diamidino-2-phenylindole (DAPI). D, mRNA expression of Flk-1, Nkx2.5, α-MHC, cTnC, and GAPDH (internal control) was evaluated by RT-PCR. E, mRNA expression of Nestin, Sox9, and GAPDH (internal control) was evaluated by RT-PCR. In A–E, the results are representative of 3 experiments.
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is required to maintain and expand cardiac progenitor cells (11, 35) but must be repressed to induce cardiomyocyte differentiation from cardiac progenitor cells (8). Thus, it would be interesting to test whether ex vivo engineered MSCs, in which the timing of expression of GSK-3β can be regulated by Dox treatment, enhance the efficacy of cell therapy in vivo.

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