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M-cadherin-inhibited phosphorylation of β -catenin augments differentiation of mouse myoblasts

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Abstract

β -catenin is essential for muscle development by regulating both cadherin-mediated cell-cell adhesion and canonical Wntless and Int1 (Wnt) signaling. The phosphorylation of β -catenin by Glycogen Synthase Kinase-3 β (GSK-3 β) at serine31/37/threonine41 regulates its stability and its role in canonical Wnt signaling. In this study, we investigated whether the N-terminal phosphorylation of β -catenin was regulated by M-cadherin, and if this regulation mediates M-cadherin's role in myogenic differentiation. These data show that the knockdown of M-cadherin expression by RNA interference (RNAi) in C2C12 myoblasts significantly increased the phosphorylation of β -catenin at Ser33/37/Thr41 and decreased the protein abundance of ser37/thr41-unphosphorylated active β -catenin. Furthermore, M-cadherin RNAi promoted TCF/LEF transcription activity but also blunted the initiation of myogenic progress by Wnt pathway activator lithium chloride (LiCl) or Wnt-3a treatment. Knockdown of β -catenin expression by RNAi decreased myogenic induction in myoblasts. Forced expression of a phosphorylation-resistant β -catenin plasmid (S33Y- β -catenin) failed to enhance myogenic differentiation, but it partially rescued C2C12 cells from M-cadherin RNAi-induced apoptosis. These data show for the first time that M-cadherin-mediated signaling attenuates β -catenin phosphorylation at Ser31/37/Thr41 by GSK-3 β and this regulation has a positive effect on myogenic differentiation induced by canonical Wnt signaling.

Keywords

M-cadherin; Myoblasts; Myotubes Wnt signaling; Myogenesis

INTRODUCTION

Muscle progenitor stem cells (i.e., satellite cells) are normally quiescent in adult muscles under basal physiological conditions. However, once they are activated in response to injury or exercise, they recapitulate development by entering the cell cycle, proliferate, and differentiate and finally fuse into myotubes (Zammit et al. 2006). It is clear that canonical Wntless and Int1 (Wnt) signaling plays an important regulatory role in many cellular events including proliferation, differentiation, and morphogenesis in many cell types including skeletal muscle (Zhang et al. 2012). β -catenin is a member of the armadillo family of proteins and it is a key transcription cofactor in the Wnt signaling pathway. β -catenin can

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modify the activity of the T cell factor/lymphoid enhancer factor (TCF/LEF) family of DNA binding proteins (Gottardi and Gumbiner 2001;Ishitani et al. 2003). It is also a central structural adaptor protein linking cadherins to the actin cytoskeleton in intercellular adherens junctions (Nelson and Nusse 2004). β -catenin-mediated canonical Wnt signaling is crucial to muscle development (Borello et al. 1999;Zhang et al. 2012). One of the key mechanisms by which the β -catenin/Wnt pathway regulates myogenesis, is to induce the expression of basic helix-loop-helix (bHLH) myogenic regulatory factors (MRFs) such as Myf5 and MyoD (Cossu et al. 1996;Tajbakhsh et al. 1998) and also inhibitor of differentiation 3 (Zhang et al. 2012). The β -catenin/Wnt pathway is also essential for the myogenic specification of muscle-derived CD45-positive stem cells after injury (Polesskaya et al. 2003) and differentiation of multipotent cells into myogenic cells (Rochat et al. 2004;Pan et al. 2005). Furthermore, the β -catenin/Wnt pathway is an important regulator of anabolic signaling in skeletal muscle (Armstrong et al. 2006;Armstrong and Esser 2005).

The paired phosphorylation of β -catenin at its NH₂ terminus by CK1 α and GSK-3 β plays a crucial role in regulating β -catenin stability and signaling. β -catenin that is phosphorylated at residues 33 and 37 is recognized by the β -TrCP E3-ligase complex, resulting in its ubiquitylation and rapid degradation by the 26S proteasome (Hart et al. 1999). This is an important mechanism to turn off Wnt signaling. An accumulated body of evidence suggests that N-terminus phosphorylation may directly modulate β -catenin-mediated signaling. Wnt signaling is mediated by N-terminal unphosphorylated β -catenin (Staal et al. 2002), which, readily accumulates in nuclei (Hendriksen et al. 2005;van et al. 2002). Thus, the GSK-3 β -dependent phosphorylation of β -catenin at N-Terminus may not only induce degradation as suggested by the classical view, but this may also directly impact β -catenin transcriptional activity. Thus, it is possible that the phosphorylation status alone will regulate β -catenin-mediated Wnt signaling without affecting its stability.

Without Wnt signaling, a sequential phosphorylation of β -catenin by serine/threonine kinases casein kinase I α (CKI α) at serine 45 and glycogen synthase kinase-3 β (GSK-3 β) at serine 33/37/threonine 41 results in ubiquitination and degradation of cytoplasmic β -catenin. Furthermore, degradation of β -catenin can occur by enhancing its polyubiquitination by FAF1 (Zhang et al. 2011). Activation of Wnt signaling leads to inactivation of GSK-3 β , resulting in accumulation of cytoplasmic β -catenin, which translocates to nucleus and binds to the TCF/LEF transcription factors to induce target gene expression. As a result, the key factors in β -catenin signaling include its stabilization and accumulation in the cytoplasm (Ikeda et al. 1998;Liu et al. 2002).

Several genes have been identified as having a role in myoblast differentiation. Among these, cadherins appear to play a key role in myogenesis (Liu et al. 2010), by inhibiting β -catenin/canonical Wnt signaling (Simcha et al. 2001;Stockinger et al. 2001;Kuphal and Behrens 2006;Sadot et al. 1998). Reports from several labs show that cadherins may also modulate β -catenin signaling via an adhesion-independent mechanism (Gottardi et al. 2001;Simcha et al. 2001;Stockinger et al. 2001). Recent data suggest that E-cadherin directly promotes β -catenin phosphorylation at the N-terminus by GSK-3 β in S2480 colon carcinoma cell lines (Maher et al. 2009). β -catenin phosphorylation has an inhibitory effect on Wnt signaling. This provides a potential mechanism to explain how cadherins regulate β -catenin signaling function at least in cancer cells, although it is not know if the signaling is similar in muscle cell lines.

M-cadherin is a member of the classical cadherin family of transmembrane glycoproteins that mediates calcium-dependent homophilic cell-cell adhesion. M-cadherin has been shown to be crucial in regulating myoblast alignment and fusion (Charrasse et al. 2006;Charrasse et al. 2007), and it is required for myogenic differentiation (Wrobel et al. 2007). We have

recently reported that M-cadherin-mediated signaling suppresses GSK-3 β activation and protects myoblasts against mitochondrial associated apoptosis during myogenic differentiation (Wang et al. 2011). However, the role for M-cadherin to regulate β -catenin phosphorylation at N-terminus by GSK-3 β in myoblasts and the potential impact that this regulation might confer to myogenic differentiation is unknown.

In the present study, we investigated the regulatory effect of M-cadherin signaling on β -catenin N-terminal phosphorylation by GSK-3 β and the impact of this regulation on myogenic differentiation. We utilized the C2C12 mouse myoblast line (Silberstein et al. 1986), which proliferates in normal culture conditions containing high serum, but differentiates spontaneously and rapidly fuse to form contractile myotubes under low serum conditions (Lavulo et al. 2008). This system provides a controlled model to study the role of M-cadherin during myoblast differentiation. In this study, we show that knockdown of M-cadherin expression promotes TCF/LEF transcription activity but decreases the availability of N-terminal unphosphorylated β -catenin (i.e., the active form of β -catenin) in confluent C2C12 myoblasts. This effect can be reversed by treatment with the Wnt pathway activator lithium chloride (LiCl). Furthermore, M-cadherin and β -catenin RNAi abolished both the myogenic and fibrotic induction by Wnt activation by LiCl. Forced expression of a phosphorylation-resistant mutated β -catenin induced a dramatic increase in TCF/LEF transcription activity but failed to induce myogenic differentiation by itself. However, it partially rescued apoptosis that was caused by M-cadherin RNAi. Together, these results suggest that M-cadherin signaling suppresses GSK-3 β -dependent β -catenin N-terminal phosphorylation and promotes myogenic differentiation.

MATERIALS AND METHODS

Culture Conditions

C2C12 myoblasts were purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic and antimycotic mixture solution (Invitrogen), in a humidified incubator under an atmosphere of 5% CO₂ at 37°C. The myoblasts were cultured in DMEM containing 2% horse serum and 1% antibiotic/antimycotic to induce differentiation. Recombinant Wnt-3a (working concentration: 50ng/ml) and recombinant Dickkopf-related protein (DKK-1, working concentration: 200ng/ml) were purchased from R&D Systems (Minneapolis, MN). Lithium chloride (LiCl; working concentration: 20mM) was purchased from Sigma-Aldrich (St. Louis, MO).

Inhibition of M-cadherin and β -catenin expression by RNA interference

*SMART*pool small interfering RNA (siRNA) targeted to M-cadherin and β -catenin mRNAs were purchased from Dharmacon/Thermo Scientific (Lafayette, CO). The M-cadherin-targeted siRNA consisted of duplexes targeting the following sequences: 5'-CGACACAGCUCUCAUCUAU-3'; 5'-GAAGGACGGCUGGUACAGA-3'; 5'-GAGCAAACGCUGAACGUCA-3'; 5'-UCGACGAGCACACGGGAGA-3'. The β -catenin-targeted siRNA consisted of duplexes targeting the following sequences: 5'-GAACGCAGCAGCAGUUUGU-3'; 5'-CAGCUGGCCUGGUUUGAUA-3'; 5'-GCAAGUAGCUGAUUUGAC-3'; 5'-GAUCUUAGCUUAUGGCAAU-3'. The mouse myoblasts were seeded in six-well plates 24 hours before transfection at a density of 1.7×10^5 per well. The transfection of siRNA was performed using either DharmaFECTTM-3 reagent (when transfected alone) or DharmaFECTTM-Duo reagent (when co-transfected with plasmids) (Thermo Scientific) according to the manufacturer's instructions. The final siRNA duplex concentration in each well was 100nM.

Plasmids and Transfection

The phosphorylation-resistant β -catenin plasmid (pcDNA3-S33Y- β -catenin), the β -catenin/Wnt reporter plasmid super 8x TOPFlash, and its mutated control plasmid, and the super 8x FOPFlash, were purchased from Addgene (Cambridge, MA). The plasmids were purified using an EndoFree plasmid Maxi Kit (Qiagen). The plasmids were transfected into the cells using either FuGENE 6 reagent (Roche) (when transfected alone) or DharmaFECT™-Duo (Thermo Scientific) (when co-transfected with other plasmids or with siRNA) according to the manufacturer's instructions.

Luciferase reporter assay

The cells were transfected with 2 μ g of super 8xTOPFlash or FOPFlash reporter plasmid and allowed to grow for 24–36 hours before further treatment. The myoblasts were harvested in lysis buffer and luciferase activity was measured in 20 μ l of each lysate using a Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Light units were measured using Berthold Autolumat Plus tube luminometer (Berthold Technology, Germany). The luciferase assays were normalized by the corresponding protein concentration of each sample. Each experiment was repeated for at least three times. TCF/LEF transcription activity was estimated from the ratio of TOPFlash to FOPFlash luciferase activity from triplicate experiments.

Cell surface biotinylation assay

A cell surface biotinylation assay was used to quantify the subcellular (membrane-bound vs. membrane-free) protein abundance of β -catenin at low and high cell densities according to published protocols (Le et al. 1999) with minor modifications. Briefly, the cells were washed three times with ice-cold PBS/Ca-Mg (138mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 9.6 mM Na₂HPO₄, 1mM MgCl₂, 0.1 mM CaCl₂) and incubated with EZ link Sulfo-NHS-SS-biotin (1mg/ml, Pierce) in PBS/Ca-Mg for 30 min at 4°C. The reaction was quenched by two washes with ice-cold 100 mM glycine in PBS/Ca-Mg for 10 min each and three times washes with ice-cold PBS/Ca-Mg. The cells were scraped from the dishes and lysed in ice-cold radioimmunoprecipitation assay buffer (RIPA) buffer containing protease inhibitor cocktail (Sigma, 1:100 dilution). The cell lysates were centrifuged at 15,000 rpm for 30 min at 4°C, and aliquots of supernatants were incubated with streptavidin beads (Sigma, 25 μ l of beads for 500 μ g/500 μ l of aliquot) for 1 hour at 4°C with rotation. The beads were washed three times with ice-cold RIPA buffer. The bound proteins were eluted with 30 μ l of Laemmli sample buffer (5X) for 5 min at 95–100°C. The whole cell lysates and the unbound fractions were separated by SDS-PAGE and processed for immunoblotting (Wang et al. 2011).

Antibodies and immunoblotting

Antibodies that were specific to phosphorylated β -catenin (serine31/37/threonine41) and cyclin D1 were purchased from Cell Signaling Technology (Danvers, MA). An antibody to M-cadherin was from Calbiochem (La Jolla, CA). The antibody to the serine37/threonine41-unphosphorylated active β -catenin was purchased from Millipore (Billerica, MA). The antibody to total β -catenin was purchased from Sigma (St. Louis, MO). Antibodies to Axin2, Troponin T, and GAPDH were obtained from Abcam (Cambridge, MA). All the secondary antibodies for immunoblotting including goat anti-rabbit or goat anti-mouse IgG conjugated with horseradish peroxidase (HRP), were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Immunoblotting of the cell lysates were conducted according to established protocols in our lab (Wang et al. 2011). Briefly, the cell lysates were diluted in 4X NuPAGE LDS sample buffer (Invitrogen) and heated at 70°C for 10 min. Equal amount of proteins were loaded in a 4–12% gradient polyacrylamide gel

(Invitrogen) and separated by electrophoresis. The proteins were transferred to a nitrocellulose membrane (Bio-Rad) and the membranes were blocked in 5% non-fat milk dissolved in Tris buffered saline with 0.05% Tween-20 (TBS-T) for 1 hour at room temperature and then probed with primary antibodies overnight at 4°C. This was followed by incubation with secondary antibodies for 1 hour at room temperature. The resulting signals were developed using Amersham enhanced chemiluminescence lighting (ECL) western blotting detection reagent kit (GE Health Care) and exposed to films. The protein signals were captured with a digital camera (Kodak 290). The densitometric analysis of the protein bands was obtained via KODAK 1D molecular imaging software.

Cell Imaging

The cells were grown on coverslips coated with 1% gelatin. Immunocytochemical staining was conducted on cells that were fixed with fresh 4% paraformaldehyde/PBS, permeabilized with 0.5% Triton X-100/PBS, and blocked with 1% BSA/PBS. The coverslips were incubated at 4°C overnight in antibodies against mouse anti- β -catenin (Sigma-Aldrich), rabbit anti- β -catenin (Cell Signaling), mouse anti-serine37/threonine41-unphosphorylated active β -catenin (Millipore), mouse anti-myosin heavy chain (MyHC) (Developmental Studies Hybridoma Bank), or rat anti-ER-TR7 (Santa Cruz Biotechnology) that were diluted with 1% BSA/PBS. Secondary antibody incubations occurred at 37°C for 1 hour with Alexa Fluor 546 anti-mouse, 488 goat anti-mouse, anti-rabbit, or anti-rat IgG (H+L) (Molecular Probes). The nuclei were counter-stained with 4,6-diamidino-2-phenylindole (DAPI) and the cells were mounted in VECTASHIELD mounting medium (Vector). Digital images were obtained with a Zeiss LSM510 confocal laser scanning microscope using AIM software (Carl Zeiss MicroImaging). The exposure time was 8 seconds and the pinhole size for capturing the image was set as 1.

Myogenic Differentiation Index and Fusion Index

After 48-hours in differentiation media, the cells were stained for myosin heavy chains (MyHC) and nuclei as described above. MyHC is expressed in differentiated myotubes but not in undifferentiated myoblasts. The number of DAPI-positive nuclei was counted from 10 non-overlapping areas of each coverslip. The differentiation index was determined by identifying myotubes with three or more nuclei and calculating the ratio of the number of nuclei in MyHC-positive cells divided by the total number of nuclei in the same field. The fusion index was calculated as the ratio of the number of nuclei located in myotubes with three or more nuclei, divided by the total number of nuclei in the same field (Hall et al. 2011; Wang et al. 2011).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

A TUNEL assay (In Situ Cell Death Detection Kit; Roche) was used to identify the extent of apoptotic nuclei in adherent myoblasts as reported previously (Siu et al. 2005; Hao et al. 2011). The nuclei of all cells were counter-stained with DAPI. The number of TUNEL- and DAPI-positive nuclei was counted in ten images that were chosen randomly from non-overlapping areas of each group. The data were presented as the apoptotic index, which was determined by dividing total number of TUNEL-positive nuclei by the total number of DAPI-positive nuclei.

Statistical analyses

The results are given as means $\pm$ standard error of mean (SE). Statistical analyses were performed using the SPSS 13.0 software package. A one-way analysis of variance (ANOVA) was used to compare differences in all measured variables. $P < 0.05$ was considered statistical significant.

RESULTS

GSK-3 β -dependent phosphorylation of β -catenin at the N-terminus is reduced, yet TCF/LEF transcription activity is down-regulated in C2C12 myoblasts at high cell density

β -catenin signaling and Wnt activity are regulated by cell confluence in various types of cells (Ishibe et al. 2006; Steel et al. 2005). The expression level and pattern of cadherin proteins vary as the cell density changes. Cadherin is central partner that binds to β -catenin and in turn, regulates both the subcellular distribution and transcription activity of β -catenin. Thus, to examine the phosphorylation status of β -catenin at the N-terminus by GSK-3 β , mouse C2C12 mouse myoblasts were seeded at $2.0 \times 10^3/\text{cm}^2$ to obtain a low cell density (~20–30% confluent) or $2.1 \times 10^4/\text{cm}^2$ to reach a high cell density (~100% confluent) within 48 hours. Immunoblot analyses showed that the protein abundance of phosphorylated β -catenin at residues serine33/37/threonine41 of the N-terminus decreased, and those of “active” serine37/threonine41-unphosphorylated β -catenin and total β -catenin increased when cells were confluent (Figure 1a). In addition, the protein abundance of two target proteins for canonical Wnt signaling, Axin2 and cyclin D1 were decreased. We next examined the subcellular expression pattern of β -catenin at different cell densities. β -catenin was located in both the nuclei and the cell cytoplasm especially in perinuclear regions in cells at a low density (Figure 1b). In contrast, β -catenin was detected in the cytoplasm and also more prominently in adjacent cell-cell contacting regions when the cells were confluent (Figure 1b'). The protein abundance of membrane-bound as compared to membrane-free β -catenin was then evaluated via a cell surface biotinylation assay. As shown in Figure 1c, the ratio of membrane-bound/membrane-free β -catenin was significantly higher in cells that were grown at a high cell density as compared with cells grown at a low density. This finding is consistent with the data showing that a large portion of β -catenin translocates to cell-cell adhesive membrane regions in confluent cells (Figure 1b').

To examine the difference in canonical Wnt signal activity in cells at different densities, C2C12 cells were transfected with the TOPFlash reporter vector and its mutant control FOPFlash vector, in either low or high cell densities as described above. TCF/LEF transcription activity, as determined from the ratio of luciferase activity in TOPFlash vector-transfected cells normalized to the signals from the FOPFlash vector-transfected cells, was significantly less in cells that were grown at high density, as compared with cells that were grown at a low density (Figure 1d, 1e). However, in spite of the decreased TCF/LEF transcription activity, myogenic differentiation, as shown by immunofluorescent staining of myosin heavy chain (MyHC)-positive myotubes in differentiation medium for 48 hours, was comparatively less in cells grown at a low density (Figure 1f), but clearly enhanced in confluent C2C12 myoblasts (Figure 1f'). These data suggest that phosphorylation of β -catenin at its N-terminus by GSK-3 β is suppressed, and TCF/LEF transcription is also down-regulated when C2C12 myoblasts become confluent. Our results show that the myogenic potential is much higher in confluent myoblasts as compared with cells with a low density, but the TCF/LEF transcription activities change in the opposite way. Thus, the high myogenic outcome of confluent myoblasts is independent of TCF/LEF transcription activity.

M-cadherin RNAi increases phosphorylation of β -catenin N-terminus at Ser31/37/Thr41 but enhances TCF/LEF transcription activity

We have reported previously that M-cadherin-mediated signaling suppresses GSK- β activation in confluent myoblasts (Wang et al. 2011). To further investigate if M-cadherin-mediated signaling modulates GSK-3 β -dependent N-terminal phosphorylation of β -catenin, and the impact of this potential modulation on TCF/LEF transcription activity in confluent C2C12 myoblasts, we inhibited M-cadherin expression in confluent C2C12 myoblasts via M-cadherin RNA interference (RNAi) and subsequently treated the cells with lithium

chloride (LiCl), an established GSK-3 β inhibitor and Wnt activator. LiCl inhibits GSK-3 β activity by competing with ATP in the ATP-binding site of the kinase via directly competing with magnesium and by increasing the inhibitory phosphorylation of the Serine 9 residue of GSK-3 β (Klein and Melton 1996). The knockdown efficacy of M-cadherin expression was verified by immunoblotting (Figure 2a). The protein abundance of phospho- and total β -catenin as well as the unphosphorylated active β -catenin (ABC) were analyzed by immunoblotting. The protein abundances of both total β -catenin and unphosphorylated active β -catenin were significantly increased when GSK-3 β activity was inhibited by LiCl, (Figure 2b) whereas, knockdown of M-cadherin by RNAi, prevented this effect (Figure 2c', c''). In addition, M-cadherin RNAi alone significantly increased the phosphorylation of β -catenin at its N-terminus and inhibition of GSK-3 β activity effectively reversed this induction (Figure 2c). Compared with the control cells that were transfected with scrambled siRNA, knocking down M-cadherin by RNAi did not alter the protein abundance of total β -catenin (Figure 2c''), but it decreased the abundance of unphosphorylated active β -catenin. This effect could be rescued by inhibiting GSK-3 β activity via LiCl treatment (Figure 2c'). These results suggest that M-cadherin-mediated signaling attenuates phosphorylation of β -catenin at its N-terminus and maintains unphosphorylated active β -catenin in a GSK-3 β -dependent manner.

We further investigated the subcellular expression pattern of the unphosphorylated active β -catenin in confluent myoblasts and the effect of M-cadherin RNAi and/or inhibition of GSK-3 β activity on β -catenin. β -catenin was localized primarily to cell-cell contact regions and in the cell's cytoplasm without inhibition of GSK-3 β activity (Figure 3a,b). Staining patterns with the anti-ABC antibody demonstrated that unphosphorylated active β -catenin mainly resides in the cytoplasmic space (Figure 3a', 3a'', 3b', 3b''), and not at cell-cell contacting membrane regions. In addition, inhibition of GSK-3 β activity via LiCl treatment, translocates unphosphorylated active β -catenin into the cell nuclei (Figure 3a', 3b'). These results suggest that the unphosphorylated active form of β -catenin is most responsive to inhibition of GSK-3 β activity-induced Wnt activation. However, M-cadherin RNAi did not alter the expression pattern of ABC (Figure 3c', 3c'', 3d', 3d''), and total β -catenin (Figure 3c, 3c'', 3d, 3d''), with or without inhibition of GSK-3 β activity via LiCl treatment.

We then examined the change in TCF/LEF transcription activity in response to the knock down of M-cadherin mRNA using reporter (luciferase) assays. As shown in Figure 4a, in spite of the statistically significant decrease in the protein abundance of ABC, TCF/LEF transcription activity was increased in cells transfected with M-cadherin-targeted siRNA compared with that in control groups. To further verify M-cadherin's role in regulating TCF/LEF transcription activity, we co-transfected the cells with M-cadherin-targeted siRNA and TOPFlash/FOPFlash-reporter plasmids and then treated the cells with either LiCl or recombinant Wnt-3a for 12 hours (Figure 4b). The luciferase activity was measured in cells at the end of treatments. The reporter (luciferase) assays (Figure 4a, 4b) were normalized by the corresponding protein concentration of each sample. Each experiment was repeated for at least three times. Both inhibition of GSK-3 β activity by LiCl and treating the cells with Wnt-3a induced a significant increase in TCF/LEF transcription activity and both inductions were significantly enhanced in cells transfected with M-cadherin-targeted siRNA (Figure 4b).

Reducing M-cadherin expression blunts myogenic and fibrotic induction, which occurs by inhibition of GSK-3 β activity. To further examine the effect of knocking down M-cadherin gene expression on the phenotype of myoblasts induced by inhibition of GSK-3 β activity or increasing Wnt activity, C2C12 myoblasts were transfected with either an M-cadherin-targeted or a scrambled non-targeted siRNA then treated with LiCl (an inhibitor of GSK-3 β activity) or recombinant Wnt-3a for 12 hours. The cells were treated for 48 hours to

induce myotubes, and then harvested for immunoblotting analysis. The protein abundance of Troponin T, an established marker for terminal myogenic differentiation (Breitbart and Nadal-Ginard 1987; Yao et al. 1992), was significantly increased in cells after inhibition of GSK-3 β activity by LiCl to increase Wnt signaling, or by Wnt-3a-treatment compared with vehicle control cells (Figure 5a, 5b). However, M-cadherin RNAi significantly attenuated the induction of Troponin T expression in both vehicle and LiCl/Wnt-3a-treated cells. Further morphological examination via immunofluorescent staining of MyHC-positive myotubes and nuclei (Figure 6a) showed that inhibition of GSK-3 β activity by LiCl treatment (which increases Wnt signaling), induced a significant increase in both myogenic differentiation (Figure 6b) and fusion (Figure 6b') as determined from the differentiation and fusion indexes. The myogenic differentiation and fusion inductions were both significantly blunted by M-cadherin RNAi (Figure 6b–6').

Since dysregulated increases in Wnt signaling could potentially skew the myogenic progenitor cells into fibrotic lineages (Brack et al. 2007; Brack et al. 2008), we examined the expression of a fibroblast marker, ER-TR7 (Brack et al. 2007; Van et al. 1986) in cells after the various treatments (Figure 7a–a''). Inhibition of GSK-3 β activity by LiCl induced a substantial increase in the expression of ER-TR7 in C2C12 myoblasts (Figure 7a') while M-cadherin RNAi effectively abrogated this induction (Figure 7a''). These results suggest that M-cadherin plays a positive role in mediating myogenic and fibrotic induction by Wnt signal activation caused by inhibition of GSK-3 β activity by LiCl.

Inhibiting β -catenin expression and canonical Wnt signaling reverses myogenic and fibrotic induction by inhibiting GSK-3 β activity

To confirm the role of β -catenin/Wnt signaling in mediating myoblast phenotype induced by inhibition of GSK-3 β activity, we treated the cells either with β -catenin RNAi to inhibit β -catenin expression, or with DKK-1 (Dickkopf related protein-1), an established antagonist of canonical Wnt signaling. This was followed by treating the cells with LiCl for 12 hours to inhibit GSK-3 β activity and differentiation medium for 48 hours to induce myogenic differentiation. The efficacy of β -catenin knockdown by RNAi was verified by immunoblotting of β -catenin protein abundance (Figure 8a). The cell morphology of differentiated cells was examined by immunofluorescent staining of MyHC-positive myotubes and nuclei (Figure 8b–b''). The differentiation (Figure 8c) and fusion (Figure 8c') indices were determined and compared among cells after the appropriate treatments. While myotube formation increases with LiCl-induced inhibition of GSK-3 β activity, this was largely prevented in β -catenin RNAi treated cells as seen by a significant reduction in both differentiation and fusion of myoblasts as compared to cells treated with LiCl (Figure 8c–c'). Both DKK-1 (Figure 8b''–c'), and β -catenin RNAi (Figure 8b–c'), attenuated the increased myogenic induction by LiCl treatment; however, β -catenin RNAi had the greatest effect (Figure 8b'–c'). Similarly, both β -catenin RNAi and DKK-1 treatment significantly blocked the expression of the fibrotic marker, ER-TR7, which was induced by LiCl treatment (Figure 9a–a'') but a greater effect was seen by inhibiting β -catenin expression (Figure 9b). These results show that the myogenic and fibrotic induction by inhibition of GSK-3 β activity is mediated by β -catenin/Wnt signaling.

Forced expression of phosphorylation-resistant β -catenin partially rescued the blunting effect of reducing M-cadherin expression on the myogenic phenotype induced by inhibition of GSK-3 β activity

To further clarify the importance of phosphorylation of the N-terminus of β -catenin in regulating myogenesis, we transfected a phosphorylation-resistant mutated β -catenin (S33Y- β -catenin) plasmid into C2C12 myoblasts and induced myogenic differentiation. The expression efficacy of the β -catenin plasmid was verified by immunoblotting the protein

abundance of phospho-, unphosphorylated, and total β -catenin (Figure 10a). Forced expression of S33Y- β -catenin in C2C12 myoblasts induced a significant increase in TCF/LEF transcription activity (Figure 10b) but failed to increase the protein abundance of Troponin T as determined by immunoblotting after transfection with the S33Y- β -catenin plasmid (Figure 10c), β -catenin or M-Cadherin (data not shown). This observation indicates that the expression of the phosphorylation-resistant mutant form of β -catenin increased TCF/LEF transcription activity yet had no effect on myogenic differentiation of C2C12 cells. TCF/LEF activity was also increased over control levels, with inhibition of M-cadherin RNA and it was further increased when M-cadherin knockdown was combined with the S33Y- β -catenin plasmid. In contrast, LiCl-induced inhibition of GSK-3 β activity in cells that were co-transfected with both S33Y- β -catenin and M-cadherin siRNA, yielded an increased protein abundance of Troponin T compared with cells that were transfected with M-cadherin siRNA alone (Figure 10d–d'). This suggests that the phosphorylation-resistant form of β -catenin attenuates the blunting effect of M-cadherin RNAi on myogenic induction by LiCl treatment.

Forced expression of phosphorylation-resistant (mutated) β -catenin partially attenuated apoptosis exacerbated by M-cadherin RNAi during myogenic differentiation

We have shown previously that M-cadherin RNAi exacerbated apoptosis in myoblasts during myogenic differentiation (Wang et al. 2011). We examined this further in an attempt to determine if the phosphorylation status of β -catenin N-terminus plays a role in regulating the apoptotic phenotype caused by M-cadherin RNAi. M-cadherin-targeted siRNA and S33Y- β -catenin plasmids were either transfected alone or co-transfected into C2C12 myoblasts followed by culturing the cells in differentiation medium for 48 hours to induce myotubes formation. At the end of the treatments, the cells were fixed and stained with an anti-MyHC antibody to evaluate differentiated myotubes. *In situ* apoptotic DNA fragmentation was examined after staining the cells with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and the nuclei were counter-stained with 4', 6-diamidino-2-phenylindole (DAPI) (Figure 11a–b'''). The indices for differentiation and apoptosis were calculated and compared among cells with different treatments. Forced expression of S33Y- β -catenin alone did not alter the myogenic (Figure 11c) or the apoptotic (Figure 11d) outcomes in C2C12 cells at the end of 48-hour myogenic differentiation. However, co-transfection of S33Y- β -catenin with M-cadherin siRNA partially rescued the impaired myogenic differentiation (Figure 11b''', c), and attenuated the exacerbated apoptosis caused by M-cadherin RNAi alone (Figure 11d). These results suggest that the alteration of the phosphorylation status of β -catenin at its N-terminus may mediate the apoptotic phenotype induced by disruption of M-cadherin-mediated signaling.

DISCUSSION

β -catenin/Wnt signaling is important for initiation and progression of skeletal muscle development and regeneration. Furthermore, β -catenin is required to maintain muscle cell proliferation and it acts as a molecular switch to regulate myogenic differentiation (Tanaka et al. 2011). This is the first study to show that M-cadherin-mediated signaling regulates the β -catenin N-terminal phosphorylation at Ser31/37/Thr41, and therefore, β -catenin/Wnt signaling and this promotes myogenic differentiation in a TCF/LEF transcription-independent manner. In this study we report that reduced M-cadherin gene expression increased GSK-3 β -dependent phosphorylation of β -catenin at the N-terminus Ser31/37/Thr41, decreased the protein abundance of unphosphorylated signaling-active β -catenin, and blunted myogenic induction caused by elevated Wnt signaling via inhibition of GSK-3 β activity or Wnt-3a treatment. Forced expression of phosphorylation-resistant β -catenin (to increase active β -catenin) failed to enhance myogenic differentiation but it partially rescued

the myogenic and apoptotic phenotype caused by M-cadherin RNAi. Interestingly, these effects are independent of changes in TCF/LEF transcription activity. The results from the experiments in this study are summarized in Table 1.

Cadherin regulation of β -catenin transcription

It is clear that cadherin proteins modulate β -catenin transcription activity in a variety of cells including Chinese hamster ovary and SW480 colon carcinoma cell lines (Sadot et al. 1998). In addition, transient expression of exogenous E-cadherin in has been reported to arrest cell growth via inhibiting β -catenin transcriptional activity in epithelial and fibroblastoid cells (Stockinger et al. 2001). Cadherins also play an important role in myogenesis (Liu et al. 2010), by inhibiting β -catenin/canonical Wnt signaling (Kuphal and Behrens 2006).

Translocation of β -catenin in myogenesis

Translocation of β -catenin appears to play an important role in determining its activity in various cell lines (Kuphal and Behrens 2006;Gottardi et al. 2001). We have demonstrated in this study that the unphosphorylated active β -catenin (ABC) accumulates in cytoplasm regions and not at cell-cell contacts in confluent myoblasts. In response to inhibition of GSK-3 β activity to enhance Wnt activity (by LiCl treatment), ABC but not total β -catenin translocates into nuclei, indicating that ABC is responsive to Wnt stimulation. Similar to our observation in myoblasts, several other labs have demonstrated that β -catenin translocates to cell-cell junctions and this increase occurs during the initiation of myogenic differentiation (Goichberg et al. 2001). As RhoA activity is required for β -catenin recruitment to intercellular adhesions sites (Charrasse et al. 2002), it is important to note that N-cadherin-dependent cell-cell contact activates muscle-specific promoters and RhoA in C2C12 cells. Our results suggest that M-cadherin-mediated signaling helps in maintaining a cytosolic pool of unphosphorylated signaling-active β -catenin, which plays a critical role in myogenic induction by Wnt stimulation.

M-cadherin regulates the phosphorylation status on the N-terminus Ser31/37/Thr41 of β -catenin

β -catenin is important in myoblast differentiation by downregulating myostatin, and inducing hypertrophy (Bernardi et al. 2011), and loss of β -catenin prevents muscle fiber hypertrophy (Armstrong et al. 2006). In the current study, we show for the first time that inhibition of M-cadherin expression by RNAi increases phosphorylation of β -catenin on the N-terminus at Ser31/37/Thr41, decreases the protein abundance of unphosphorylated active β -catenin, and impairs the myogenic induction that is caused by either inhibition of GSK-3 β activity or Wnt-3a treatment. Together these results indicate that M-cadherin-mediated signaling attenuates GSK-3 β -dependent phosphorylation of the β -catenin N-terminus at Ser31/37/Thr41 and helps in maintaining a cytosolic pool of unphosphorylated signaling-active β -catenin, which plays a critical role in myogenic induction by Wnt stimulation. In this study, we treated the cells with Wnt3a, but we did not test if other Wnt family members would result in different results. This is possible because it has been reported that Wnt3a overexpression decreased differentiation but Wnt 4 did not alter C2C12 differentiation (Tanaka et al. 2011). However, evaluation of M-Cadherin on the various Wnt signaling proteins was beyond the scope of this study.

M-cadherin-mediated signaling attenuates phosphorylation of β -catenin N-terminus at Ser31/37/Thr41 via suppressing GSK-3 β activity

We have shown previously that M-cadherin suppresses GSK-3 β activity in confluent myoblasts (Wang et al. 2011). In the present study we further demonstrate that while a large portion of β -catenin is detected at cell-cell contact membrane regions, in confluent

myoblasts, unphosphorylated active β -catenin (i.e., ABC catenin) is located mainly in cytoplasmic regions of the cell rather than at cell-cell contacts. This observation suggests that the phosphorylation regulatory effect by M-cadherin is not simply a result of direct binding to unphosphorylated active β -catenin, but rather in an adhesion-independent manner. Furthermore, decreasing M-cadherin expression, increases phosphorylation of β -catenin at the N-terminus Ser31/37/Thr41 position and decreases the protein abundance of unphosphorylated active β -catenin. Inhibition of GSK-3 β by LiCl treatment significantly reverses this effect. These results indicate that M-cadherin-mediated signaling attenuates phosphorylation of β -catenin N-terminus at Ser31/37/Thr41 through suppressing GSK-3 β activity. The exact mechanism by which M-cadherin-mediated signaling attenuates phosphorylation of the β -catenin N-terminus at Ser31/37/Thr41 still remains to be elucidated.

TCF/LEF transcription activity is less dependent on the phosphorylation status of β -catenin N-terminus at Ser31/37/Thr41, than mechanical sequestration of β -catenin by M-cadherin

Our results show that the myogenic potential is much higher in confluent myoblasts as compared with cells with a low density, but the TCF/LEF transcription activities change in the opposite way (Figure 1a–f'). Although knockdown of M-cadherin by RNAi promotes TCF/LEF transcription activity at both steady state and LiCl/Wnt-3a-treatment conditions (Figure 1e, 10b), it blunts the myogenic differentiation under those conditions. While inhibiting the gene expression of M-cadherin and the forced expression of the phosphorylation-resistant form of β -catenin induced a substantial increase in TCF/LEF transcription activity, it failed to induce a similar increase in myogenic differentiation by itself. These results show that there is a disconnection between the level of TCF/LEF transcription activity and the outcome of myogenic differentiation in myoblasts. This observation suggests that β -catenin may mediate Wnt signaling and promote myogenic differentiation independent of its role in enhancing TCF/LEF transcription activity. These findings are however, consistent with reports from treated LIM2537 cells, a poorly-differentiated colon cancer cell line, which when they are treated with the potent differentiating agent sodium butyrate, reduces GSK-3 β activity by 34%, stabilizes the level of cytoplasmic β -catenin but there is no increase in β -catenin/TCF target genes c-myc and cyclin D1 (Vincan et al. 2000).

Previous work has shown that β -catenin interacts with I-mfa (an inhibitor of the MyoD family of muscle regulatory transcription factors (MRFs)) and this interaction relieved the transcription activity suppression and cytosolic sequestration of MRFs by I-mfa (Pan et al. 2005). This observation suggests that a β -catenin-dependent but TCF/LEF-independent regulatory mechanism impacts MRFs during myogenesis. Similarly, Kim and colleagues (Kim et al. 2008) reported that TCF/LEF activity is dispensable for β -catenin's role in promoting myogenic differentiation. Instead, β -catenin directly interacts with MyoD and enhances its transcriptional activity that is necessary for myogenic differentiation (Kim et al. 2008). Interestingly, the results in our present study show that TCF/LEF transcription activity is increased in response to M-cadherin RNAi in spite of the decreased availability of unphosphorylated active β -catenin, suggesting that TCF/LEF transcription activity might be more responsive to the relief of mechanical sequestration of β -catenin by M-cadherin knockdown and less dependent on the change in phosphorylation status of β -catenin N-terminus at Ser31/37/Thr41, at least in C2C12 myoblasts.

Fibrotic induction caused by Wnt stimulation is reversed by M-cadherin and β -catenin

Although skeletal muscle progenitor cells are committed to be myogenic lineages and do not spontaneously adopt non-myogenic fates (Starkey et al. 2011), hyper-activation of Wnt

signaling may induce the premature termination of the expansion of myogenic progenitor cells and push the myogenic progenitor cells towards fibroblast lineages, resulting in the increased fibrosis in muscle tissues (Brack et al. 2007; Brack et al. 2008). Consistent with this finding, we demonstrated that inhibiting GSK-3 β activity to enhance Wnt signaling in C2C12 myoblasts prior to differentiation, gave rise to the expression of a fibroblast marker, ER-TR7. The fibrotic induction caused by Wnt stimulation via inhibiting GSK-3 β activity (i.e., LiCl treatment) could be effectively reversed by inhibition of the expression of either M-cadherin or β -catenin expression.

Phosphorylation of β -catenin at the N-terminus Ser31/37/Thr41 position mediates apoptosis that occurs from reduced M-cadherin expression during myogenic differentiation

We have shown previously that knockdown of M-cadherin expression via RNAi in both C2C12 myoblasts and primary muscle progenitor cells exacerbates apoptosis during myogenic differentiation. This increase in apoptotic signaling was partially rescued by inhibition of GSK-3 β activity (Wang et al. 2011). In the current study, we extend these observations by showing that M-cadherin RNAi increased phosphorylation of β -catenin N-terminus at Ser31/37/Thr41 and forced expression of the phosphorylation-resistant mutated β -catenin partially rescued the apoptosis induced by M-cadherin RNAi. These observations suggest that alteration in the phosphorylation levels of β -catenin N-terminus at Ser31/37/Thr41 and β -catenin/Wnt signaling might be at least partially responsible for mediating M-cadherin RNAi-induced apoptosis during myogenic differentiation. Our findings here are consistent with reports that show that β -catenin/Wnt signaling plays an important role in promoting survival of either skeletal or cardiac myoblasts under various conditions (Almeida et al. 2005; Du et al. 2009; Hahn et al. 2006; Li et al. 2011).

Conclusion

β -catenin is a key player in signal transduction pathways during muscle development. Novel data in this study show that M-cadherin-mediated signaling attenuates β -catenin phosphorylation at Ser31/37/Thr41 by GSK-3 β and this regulation has a positive effect on myogenic differentiation induced by canonical Wnt signaling. Unphosphorylated β -catenin is the active form of this protein, and M-cadherin reduces the degree to which β -catenin becomes phosphorylated at the N-terminus Ser31/37/Thr41 position. Thus, M-cadherin helps to maintain a high cytosolic pool of the N-terminal unphosphorylated (at Ser31/37/Thr41) signaling-active β -catenin, which in turn, plays a key role in mediating canonical Wnt signaling to promote myogenic differentiation in a TCF/LEF-independent manner (Figure 12). M-cadherin mediated reduction in phosphorylation of β -catenin along with various Wnt ligands (Tanaka et al. 2011) may provide important mechanisms that regulate translocation of β -catenin to control differentiation of muscle cells.

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Figure 1. Phosphorylation and subcellular distribution of β-catenin, TCF/LEF transcription activity, and myogenic differentiation in C2C12 myoblasts at different cell densities

a C2C12 myoblasts were plated at either a low density ($2.0 \times 10^3/\text{cm}^2$), or a high density ($2.1 \times 10^4/\text{cm}^2$). 48 hours after plating, the cells were harvested in lysis buffer for immunoblotting analysis of the protein abundance of phosphorylated (Ser33/37/Thr41), Ser37/Thr41-unphosphorylated active β-catenin (ABC) and total β-catenin. Protein abundance of Axin2 and cyclin D1 were also examined. GAPDH was used as a loading control.

b Representative confocal images of β-catenin staining in C2C12 myoblasts at low density. **b'** Representative confocal images of β-catenin staining in C2C12 myoblasts at high density as described in Figure 1a. The objective magnification is 63×. The length of scale bar is 10 μm.

c Representative immunoblots of protein abundance of β-catenin in subcellular fractions (membrane-bound versus membrane-free) of cells at low or high density as determined by cell surface biotinylation assay. **c'** Representative immunoblots of protein abundance of β-catenin in total cell lysates.

d Comparison of ratios of β-catenin in membrane-bound fraction to that in membrane-free fraction in cells of different densities. Each data point (mean ± SEM) is the mean of three independent experiments. *, $P < 0.05$ vs. the low density group.

e C2C12 myoblasts transfected with either a TOPFlash or a FOPFlash plasmid. The cells were then plated at low or high cell density. 48 hours later the cells were harvested for luciferase activity measurement to determine the TCF/LEF transcription activity. Each data point (mean ± SEM) is the mean of three independent experiments. *, $P < 0.05$ vs. the low density group.

f Representative images of MyHC-positive myotubes in cells at low density or high density (**f'**) after culture in differentiation medium for 48 hours. MyHC: red; DAPI: blue. Images were acquired at 20×. The scale bar is 100 μm.

Figure 2. Effect of reducing M-cadherin expression and inhibiting GSK-3 β activity on β -catenin phosphorylation and subcellular distribution, and TCF/LEF transcription activity

a Representative immunoblot of protein abundance of M-cadherin in C2C12 myoblasts with different treatments (M-, M-cadherin siRNA transfection; SiCON, Non-targeted scrambled siRNA transfection; NC, Non-treated control cells with same culture condition). GAPDH was probed as a loading control.

b Representative immunoblots of protein abundance of phospho-, unphosphorylated active β -catenin (ABC) and total β -catenin in C2C12 myoblasts in either M-cadherin siRNA-transfected (M-) or non-targeted scrambled siRNA-transfected (SiCON) cells treated with LiCl (20mM) or PBS as a vehicle control for 12 hours. Non-treated cells with same culture condition were used as a normal control (NC). GAPDH was probed as a loading control.

c Densitometric analyses of immunoblots of phospho- β -catenin normalized to GAPDH. *, $P < 0.05$ vs. the SiCON plus Vehicle group. †, $P < 0.05$ vs. M- plus vehicle group.

c' Densitometric analyses of immunoblots of ABC normalized to GAPDH. *, $P < 0.05$ vs. the SiCON plus Vehicle group. †, $P < 0.05$ vs. M- plus vehicle group.

c'' Densitometric analyses of immunoblots of total β -catenin (β -CAT) normalized to GAPDH. *, $P < 0.05$ vs. the SiCON plus Vehicle group. †, $P < 0.05$ vs. SiCON plus LiCl group.

Figure 3. Effect of reducing M-cadherin expression and inhibiting GSK-3 β activity on β -catenin subcellular distribution

Representative confocal images of immunofluorescent staining of total β -catenin (**a,b,c,d**), ABC (**a', b', c', d'**), DAPI (**a'', b'', c'', d''**) and the merged image showing β -catenin, ABC and DAPI staining (**a''', b''', c''', d'''**). The conditions included: non-targeted scrambled siRNA-transfected (SiCON) cells (**a–a'''**; **b–b'''**), M-cadherin-RNAi-treated (M-) (**c–c'''**; **d–d'''**) cells, that were treated with PBS as a control (**b–b'''**; **c–c'''**) or LiCl to inhibit GSK-3 β activity (**a–a'''**; **d–d'''**), for 12 hours. ABC, green; β -catenin, red; DAPI, blue. Images were acquired at 63 $\times$. The length of scale bar is 10 μ m.

Figure 4. Effect of reducing M-cadherin expression and inhibiting GSK-3 β activity on β -catenin phosphorylation and subcellular distribution, and TCF/LEF transcription activity

a TCF/LEF transcription activity in M-cadherin-RNAi (M-), non-targeted scrambled siRNA-transfected (SiCON) or normal control (NC) C2C12 myoblasts as determined by TOPFLASH/FOPFLASH luciferase activity. *, $P < 0.05$ vs. the control groups.

b TCF/LEF transcription activity in M-cadherin-RNAi (M-), non-targeted scrambled siRNA-transfected (SiCON) or normal control (NC) C2C12 myoblasts that were treated with LiCl (20mM) or recombinant Wnt-3a (50ng/ml) for 12 hours as determined by TOPFLASH/FOPFLASH luciferase activity. *, $P < 0.05$ vs. the normal control (NC) group. **, $P < 0.01$ vs. NC group. †, $P < 0.05$ vs. the SiCON plus LiCl treatment group. #, $P < 0.05$ vs. the SiCON plus Wnt-3a treatment group.

Figure 5. Effect of reducing M-cadherin expression and inhibiting GSK-3 β activity on myogenic differentiation in C2C12 myoblasts

a Representative immunoblots of protein abundance of Troponin T, an established marker for terminal myogenic differentiation, in M-cadherin-RNAi-treated (M-) or non-targeted scrambled siRNA-transfected (SiCON) cells that were treated with LiCl (20mM), recombinant Wnt-3a (50ng/ml), or DMSO as a vehicle control for 12 hours, followed by culture in differentiation medium for 48 hours. GAPDH was probed as a loading control.

b Densitometric analysis of immunoblots of Troponin T normalized to GAPDH. *, $P < 0.05$ vs. the SiCON plus Vehicle group. #, $P < 0.05$ vs. M- plus Vehicle group. †, $P < 0.05$ vs. the correspondent vehicle/LiCl/Wnt-3a-treatment in SiCON groups.

Figure 6. Differentiation and fusion of myoblasts after inhibition of M-cadherin expression or GSK-3 β activity

Representative confocal images of MyHC-positive C2C12 myotubes after treatments with PBS (a), LiCl (a'), or LiCl plus M-cadherin RNAi (LiCl plus M- (a'')), followed by culture in differentiation medium for 48 hours. MyHC: red; DAPI: blue. Images were acquired at 20 $\times$. The length of scale bar is 100 μ m.

The differentiation index (b) and fusion index (b') was determined, and compared among cells with above treatments. b–b'; *, $P < 0.05$ vs. the PBS-treated group. †, $P < 0.05$ vs. the LiCl-treated group.

Figure 7. Effect of reducing M-cadherin expression and inhibiting GSK-3 β activity on the fibrotic potential of differentiated C2C12 myoblasts

Representative confocal images of immunofluorescent staining with a fibrotic marker, ER-TR7, in C2C12 myoblasts after treatments after treatments with PBS (**a**), LiCl(**a'**), or LiCl plus M-cadherin RNAi (LiCl plus M-)(**a''**). **a–a''** ER-TR7: green; DAPI: blue. Images were acquired at 20 $\times$. The length of scale bar is 100 μ m.

b Quantitative analysis of mean fluorescence intensity of ER-TR7 staining in cells with PBS, LiCl, or LiCl plus M-cadherin RNAi (LiCl plus M-). *, $P < 0.05$ vs. the PBS-treated group. †, $P < 0.05$ vs. the LiCl-treated group.

Figure 8. The effect of reducing β -catenin gene expression and inhibiting canonical Wnt signaling on myogenic induction via inhibition of GSK-3 β activity

a A representative immunoblot of protein abundance of β -catenin in C2C12 myoblasts under non-treated control cells (NC), after transfection with a non-targeted scrambled siRNA (SiCON), or after knocking down β -catenin gene expression via transfection with RNAi (β -cat RNAi, β -catenin-targeted siRNA transfection). GAPDH was used as a loading control.

Representative confocal images of MyHC-positive C2C12 myotubes to indicate differentiation in C2C12 cells after transfection with a non-targeted scrambled siRNA (SiCON) (**b**), after knocking down β -catenin gene expression via transfection with RNAi (β -cat RNAi, β -catenin-targeted siRNA transfection) (**b'**), or DKK-1(200ng/ml) (**b''**), to inhibit canonical Wnt signaling. All treatments were followed by the addition of LiCl to inhibit GSK-3 β activity. The treatment conditions were for 12 hours, followed by incubation in differentiation medium for 48 hours. MyHC: red; DAPI: blue. Images were acquired at 20 $\times$. The length of scale bar is 100 μ m.

The differentiation index (**c**), and the fusion index (**c'**), were quantified for the C2C12 cells after the treatments described (**a–b''**). *, $P < 0.05$ vs. the LiCl plus SiCON group.

Figure 9. The effect of reducing β -catenin gene expression and inhibiting canonical Wnt signaling on fibrotic induction via inhibition of GSK-3 β activity

Representative confocal images of immunofluorescent staining of the fibrotic marker, ER-TR7 in C2C12 myoblasts after transfection with a non-targeted scrambled siRNA (SiCON) (a), after knocking down β -catenin gene (β -CAT) expression via transfection with RNAi (β -cat RNAi, β -catenin-targeted siRNA transfection) (a'), or DKK-1(200ng/ml), to inhibit canonical Wnt signaling a''. All treatments were followed by the addition of LiCl to inhibit GSK-3 β activity. ER-TR7: green; DAPI: blue. Images were acquired at 20 $\times$. The length of scale bar is 100 μ m.

b Quantitative analysis of mean fluorescence intensity of ER-TR7 staining in cells with the treatments described in Figure 8. *, $P < 0.05$ vs. the LiCl plus SiCON group.

Figure 10. The effect of forced expression of phosphorylation-resistant mutated S33Y-β-catenin on TCF/LEF transcription activity and myogenic differentiation

a Representative immunoblots of protein abundance of phospho-, unphosphorylated and total β-catenin in C2C12 myoblasts with different treatments (S33Y-β-cat, S33Y-β-catenin plasmid transfection; EV, empty vector transfection; NC, Non-treated control cells with same culture condition). GAPDH was probed as a loading control.

b TCF/LEF transcription activity in untreated normal control (NC), empty vector-transfected (EV), M-cadherin siRNA transfection (M-), S33Y-β-catenin plasmid-transfected (S33Y-β-CAT), or M-cadherin siRNA plus S33Y-β-catenin plasmid-transfection in C2C12 myoblasts was determined by measuring TOPFlash/FOPFlash luciferase activity. *, $P < 0.01$ vs. the control groups; †, $P < 0.05$ vs. M- plus S33Y-β-catenin group.

c Representative immunoblots of protein abundance of Troponin T in S33Y-β-catenin-transfected (S33Y-β-CAT), empty vector-transfected (EV) or normal control (NC) C2C12 myoblasts after culture in differentiation medium for 48 hours. GAPDH was probed as a loading control.

d Representative immunoblots of protein abundance of Troponin T in the following four groups of cells: normal control (NC), LiCl-treated (LiCl), LiCl plus M-cadherin siRNA-transfected (LiCl/M-), LiCl plus M-cadherin-siRNA and S33Y-β-catenin plasmid-co-transfected (LiCl/M-/S33Y-B-CAT), and differentiation for 48 hours.

d' Densitometric analyses of immunoblots of Troponin T normalized to GAPDH. *, $P < 0.05$ vs. NC group. #, $P < 0.05$ vs. LiCl group. †, $P < 0.05$ vs. LiCl plus M- group.

Figure 11. Effect of forced expression of phosphorylation-resistant mutated S33Y-β-catenin on apoptosis and myogenic differentiation in response to M-cadherin RNAi

C2C12 cells were grown on coverslips, co-transfected with M-cadherin-targeted (M-) or non-targeted scrambled siRNA (SiCON) with S33Y-β-catenin plasmid (S33Y-β-CAT) or an empty vector (EV) for 36 hours. The cells were then cultured in differentiation medium for 48 hours.

Representative confocal images of immunofluorescent staining for MyHC-positive C2C12 myotubes stained with DAPI and in situ DNA fragmentation was labeled by TUNEL staining following treatments: SiCON (a, a''), or M- (a', a''') after treatment with EV (a, a'), or with S33Y-β-CAT (a'', a'''). MyHC: red; DAPI: blue, TUNEL: green. The scale bar = 100 μm.

Representative confocal images of immunofluorescent staining for DAPI stained nuclei and in situ DNA fragmentation was labeled by TUNEL staining in C2C12 myotubes that were treated with: SiCON (b, b''), or M- (b', b''') after treatment with EV (b, b'), or with S33Y-β-CAT (b'', b'''). TUNEL: green; DAPI: blue. Images were acquired at 20×. The scale bar = 100 μm.

c The differentiation index of C2C12 cells that were exposed to the above treatments. *, $P < 0.05$ vs. SiCON/EV group. †, $P < 0.05$ vs. M-/EV group.

d The fusion index of C2C12 cells exposed to the treatments described above. *, $P < 0.05$ vs. SiCON/EV group. †, $P < 0.05$ vs. M-/EV group.

Figure 12. Schematic summary of the regulatory role of M-cadherin's control of β -catenin phosphorylation in myogenic differentiation.

Cadherin is an established inhibitor for β -catenin/canonical Wnt signaling. In myoblasts, it inhibits β -catenin activity by mechanical sequestration, attenuates the phosphorylation of β -catenin N-terminus at Ser31/37/Thr41 to increase the active ABC form of β -catenin, and promotes β -catenin activity in a TCF/LEF-independent manner.

Table 1

Summary of Major Biological Outcomes

Treatment	Desired Outcome	Biological Outcome			
		Unphosphorylated β -catenin (active/ABC)	Differentiation	Fibrosis	TCF/LEF
M-cadherin siRNA	↓ M-cadherin	↓	↓	↓	↓
LiCl	↓ GSK-3 β activity	↓	↓	↓	↓
M-cadherin siRNA + LiCl	↓ M-cadherin + ↓ GSK-3 β activity	↓	↓	↓	↓
S33Y- β	↓ ABC	↓	↓		↓
M-Cadherin siRNA + S33Y- β	↓ M-Cadherin + ↓ ABC	↓	↓		↓
β -catenin siRNA + LiCl	↓ β -catenin + ↓ GSK-3 β activity		↓	↓	

The major findings are summarized by arrows to indicate an increase, (↓) decrease (↑) or no change (→) in biological outcome measures.