β-Catenin regulates myogenesis by relieving I-mfa-mediated suppression of myogenic regulatory factors in P19 cells

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Wnt/β-catenin signaling plays a critical role in embryonic myogenesis. Here we show that, in P19 embryonic carcinoma stem cells, Wnt/β-catenin signaling initiates the myogenic process depending on β-catenin-mediated relief of I-mfa (inhibitor of MyoD Family a) suppression of myogenic regulatory factors (MRFs). We found that β-catenin interacted with I-mfa and that the interaction was enhanced by Wnt3a. In addition, we found that the interaction between β-catenin and I-mfa was able to attenuate the interaction of I-mfa with MRFs, relieve I-mfa-mediated suppression of the transcriptional activity and cytosolic sequestration of MRFs, and initiate myogenesis in a P19 myogenic model system that expresses exogenous myogenin. This work reveals a mechanism for the regulation of MRFs during myogenesis by elucidating a β-catenin-mediated, but lymphoid enhancing factor-1/T cell factor independent, mechanism in regulation of myogenic fate specification and differentiation of P19 mouse stem cells.

Wnt

Myogenic regulatory factors (MRFs), which belong to the family of basic helix-loop-helix (bHLH) transcription factors, are the “master genes” that control the events leading to skeletal muscle development. These MRFs, including Myogenin, MyoD, Myf5, and MRF4, form heterodimers with the E-type bHLH transcription factors to regulate gene transcription (1–5). In addition, transcription suppressors including I-mfa (inhibitor of MyoD Family a) also negatively regulate transcriptional activity of MRFs. I-mfa binds to and inhibits bHLH MRFs via its C-terminal Cys-rich domain and prevents MRF from retention in the nucleus (6). I-mfa is broadly expressed in both embryonic and extraembryonic tissues, including the presomatic mesoderm, dermomyotome, and sclerotome of mouse embryos (6, 7). This expression pattern of I-mfa led to a hypothesis that I-mfa may inhibit the activity of MRFs that are expressed at low levels in the presomitic mesoderm until the inhibition is relieved. In addition to MRFs, I-mfa inhibits the activity of another bHLH transcription factor Mesh2 (7). Recently, a human I-mfa domain-containing protein, HIC, was identified for its ability to regulate Tat- and Tax-mediated expression of viral promoters (8). HIC or XIC, the Xenopus homolog of HIC, also contains a Cys-rich C-terminal domain that shares a high degree of homology with that of I-mfa. Both XIC and I-mfa have been shown to bind to and inhibit the activity of Xenopus Wnt/β-catenin signaling target transcriptional factor XTCf3 in additional to MRFs (9).

The Wnt family of secretory glycoproteins is one of the major families of developmentally important signaling molecules and plays important roles in embryogenesis including generation of cell polarity, specification of cell fate, and regulation of proliferation and differentiation. Studies using Drosophila, Xenopus, and mammalian cells have established a canonical Wnt (Wnt/β-catenin) signaling pathway. Wnt proteins bind to cell surface receptors Frizzled (Fz) and low-density lipoprotein-receptor related protein-5/6 and prevent glycoprotein synthase kinase 3-dependent phosphorylation of β-catenin, thus leading to the stabilization and accumulation of soluble β-catenin. Stabilized β-catenin interacts with transcription regulators, including lymphoid enhancing factor-1 (LEF1) and T cell factors (TCFs), to regulate gene transcription (10–13).

A myriad of evidence demonstrates that Wnt/β-catenin signaling plays important roles in myogenic fate determination and differentiation (14–16). Canonical Wnt molecules were found to be one class of signaling molecules that regulate the specification of skeletal myoblasts in the paraxial mesoderm during embryo development. Studies suggest that canonical Wnts, including Wnt1, Wnt3a, and Wnt7a, can induce location-specific expression of MRFs in embryos (17–19). The role of Wnt/β-catenin signaling in specification of skeletal muscles does not appear to be limited to embryonic development, and it was also shown that Wnt proteins can induce myogenesis in CD45· stem cells during muscle regeneration (20).

Previous studies that investigated the effect of ectopic expression of MRFs on the developmental potential of P19 embryonic carcinoma stem cells found that expression of MRFs alone in these cells did not result in their differentiation into skeletal muscle without aggregation (16, 21). P19 cells are pluripotent stem cells that can be induced to differentiation simulating the biochemical and developmental processes that occur in early embryogenesis. Aggregation of P19 cells led to mesoderm induction evidenced by the expression of a mesoderm marker Brachyury T (22). Treatment with DMSO will further the differentiation into cardiac and skeletal muscles as well as other mesodermal and endodermal cell types (23). The failure of differentiation of P19 cells expressing MRFs into skeletal muscle, which also occurred in other stem cells (24), together with the observation of expression of Myf5 in the presomitic mesoderm whose further development requires further stimulation of signaling molecules (6), suggests that negative regulation of MRFs may exist, and the suppression needs to be relieved in order for the cells to differentiate. In this report, we describe a mechanism by which I-mfa-mediated suppression of MRFs can be relieved. We found that β-catenin, whose levels in cells are accentuated by canonical Wnt signaling, binds I-mfa and relieves its inhibitory effects on the transcriptional activity of MRFs, and demonstrated that this mechanism plays an important role in myogenesis.

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Abbreviations: MRF, myogenic regulatory factor; LEF1, lymphoid enhancing factor-1; TCF, T cell factor; HA, hemagglutinin; siRNA, small interfering RNA.

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**Materials and Methods**

**Plasmid Constructs.** The full-length cDNA or mutants of mouse I-mfa, β-catenin, MyoD, Myogenin, and Myf5 were generated by PCR or RT-PCR and verified by DNA sequencing, hemagglutinin (HA)/Myc-epitope tags were introduced to the C termini of these molecules, and GG epitope tags were introduced to the N termini. pCMV plasmid is the vector for all mammalian expression constructs.

All mutants of mouse I-mfa and β-catenin are depicted in Fig. 1. I-mfa siRNA was expressed by using a pSuper-derived vector (25) that contains a neomycin resistant gene expression unit. The I-mfa target siRNA sequences are GTTGCAGACGCATCCATCT and GTTGCAGACGCATCCATCT. The total levels of I-mfa, MyoD, Myogenin, and Myf5 were established. All of the clones showed the similar results, but we present the results from only one.

**Yeast Two-Hybrid Screening.** The two-hybrid system and the mouse embryonic E10.5 cDNA library were purchased from Invitrogen/Life Technologies. Two-hybrid screening was carried out as suggested by the manufacturer (Invitrogen). The transfection was stopped after 3 h. The MRF reporter gene system consists of the MRF-luciferase reporter gene (27) and GFP expression construct. Generally, for reporter gene assays in NIH 3T3 or P19 cells, total amount of expression plasmid is 500 ng per well (24-well plate), including 100 ng of MRF reporter and 50 ng of GFP and other cDNA (500 ng per well). Small interfering RNA (siRNA) was transfected at 20 μM per well. For immunoprecipitation, HEK cells in six-well plates were transfected with 1,000 ng per well cDNA, with each plasmid at 100 ng per well. LacZ was used to make up the total.

**Transfection, Reporter Gene Assay, and Immunoprecipitation.** Transfection was carried out by using Lipofectamine Plus as suggested by the manufacturer (Invitrogen). The transfection was stopped after 3 h. The MRF reporter gene system consists of the MRF-luciferase reporter gene (27) and GFP expression construct. Generally, for reporter gene assays in NIH 3T3 or P19 cells, total amount of expression plasmid is 500 ng per well (24-well plate), including 100 ng of MRF reporter and 50 ng of GFP and other cDNA (500 ng per well). Small interfering RNA (siRNA) was transfected at 20 μM per well. For immunoprecipitation, HEK cells in six-well plates were transfected with 1,000 ng per well cDNA, with each plasmid at 100 ng per well. LacZ was used to make up the total.

**Cell Fraction Preparation.** Cells in six-well plates were washed once with ice-cold PBS and scraped into hypotonic buffer containing 10 mM Hepes (pH 7.9), 1.5 mM MgCl2, 1 mM EDTA, 10 mM KCl, 0.5 mM DTT, and a mixture of protease inhibitors. After incubating on ice for 10 min, cells were homogenized in a homogenizer and centrifuged at 15,000 × g. The supernatant was collected as the cytosolic fraction. The pellets were dissolved in a buffer containing 20 mM Hepes (pH 7.9), 1.5 mM MgCl2, 1 mM EDTA, 420 mM NaCl, 25% glycerol, 0.5 mM DTT, and a mixture of protease inhibitors. After 30-min incubation on ice, the sample was centrifuged at 15,000 × g. The supernatant was collected as the nuclear fraction.

**Immunofluorescence Staining and Microscopy.** P19 cells on coverslips were washed once with ice-cold PBS and then fixed for 30 min in PBS containing 4% paraformaldehyde at 4°C. Fixed cells were incubated in CMA (chloroform/methanol/acetic, 1:2:1, vol/vol) and then methanol for 30 min at -20°C. After rehydrating with PBS...
at room temperature, coverslips were stained with anti-MHC antibody (1:100) followed by FITC-conjugated anti-mouse IgG (Jackson ImmunoResearch). Nuclei were stained with DAPI.

Results
I-mfa Interacts with β-Catenin. In our yeast two-hybrid screening experiment using the first ten arm-repeats of β-catenin as bait, we identified five I-mfa clones in 36 positive clones out of ~2 million yeast transformants. Among these five I-mfa clones, four contain the full-length reading frame, and one lacks the N-terminal two amino acids. The interaction between β-catenin and I-mfa was subsequently confirmed by coimmunoprecipitation of endogenous β-catenin and I-mfa in HEK 293T cells (data not shown) and of endogenous β-catenin and I-mfa in the P19 cells (Fig. 1A). In addition, the interaction between β-catenin and I-mfa was enhanced by a canonical Wnt ligand, Wnt3a (Fig. 1A). Given that Wnt3a elevated the level of free β-catenin without overtly changing the total β-catenin level, the above result suggests that canonical Wnts can regulate the β-catenin–I-mfa interaction by regulating the levels of free β-catenin. To further confirm that the interaction between I-mfa and β-catenin is direct, we carried out an in vitro pull-down assay using recombinant I-mfa and β-catenin partially purified from Escherichia coli. When recombinant HA-tagged I-mfa was pulled down by an anti-HA antibody, β-catenin was readily detected in the complex (Fig. 1B). This result, together with the fact that the interaction between I-mfa and β-catenin initially revealed by a yeast two-hybrid screen, indicates that this is, most likely, a direct interaction.

We next identified β-catenin sequences that are responsible for the interaction. First, we determined whether a β-catenin dominant negative mutant whose C-terminal 98 amino acids are replaced with a transcription suppressor domain from Xenopus Engrailed-2 (the resultant β-catenin mutant is designated β-catenin-NN; Fig. 1C; ref. 15) could interact with I-mfa. As shown in Fig. 1D, β-catenin-NN could still coimmunoprecipitated with I-mfa, suggesting that the C-terminal 98 amino acids of β-catenin that are required for β-catenin transcription activation may not be involved in the interaction with I-mfa. We used deletion mutagenesis to identify residues 308–352 of β-catenin, which are required for the interaction with I-mfa (Fig. 1C). Subsequently, we found that mutation of residues R342 or K345 led to a reduction in the interaction (Fig. 1C) and that mutation of both residues (the resultant β-catenin mutant is designated β-catenin-MM) almost completely abolished the interaction (Fig. 1C and D).

To delineate the regions of I-mfa that are involved in interactions with β-catenin, two I-mfa mutants were generated; I-mfa-N contains the N-terminal 161 amino acids, and I-mfa-C contains the C-terminal 122 amino acids (Fig. 1E). As shown in Fig. 1F, only the full-length I-mfa was able to interact with β-catenin, but both full-length I-mfa and I-mfa-C can bind to Myogenin and other MRFs (data not shown), which is consistent with a published result (6).

β-Catenin Attenuates the Interactions of I-mfa with MRFs. Because I-mfa has been shown to interact with MRFs, we investigated whether β-catenin and MRFs compete for the binding of I-mfa. Expression of β-catenin led to a reduction in the interaction of I-mfa with Myf5 (Fig. 2A, compare lane 3 with 4) or myogenin (data not shown). As anticipated, β-catenin expression had no effect on the interactions between Myf5 and I-mfa-C (Fig. 2A, lanes 7 and 8), because I-mfa-C does not interact with β-catenin (Fig. 1E). Together with the result that expression of β-catenin-MM failed to disrupt the interaction between I-mfa and Myf5 (Fig. 2A, compare lanes 3 and 5), we conclude that the interaction between β-catenin and I-mfa is also required for the attenuation of the interaction of I-mfa with MRFs by β-catenin.

β-Catenin Relieves I-mfa-Mediated Transcriptional Suppression of MRFs. The competition between β-catenin and MRFs for binding I-mfa also suggests that β-catenin may be able to regulate transcriptional activities of MRFs by relieving I-mfa-mediated suppression. We examined the effect of β-catenin on myogenin-mediated transcriptional activity by using an MRF-specific reporter gene assay (27) in NIH 3T3 cells. In these cells, myogenin-induced transcriptional activity was readily repressed by coexpression of I-mfa or I-mfa-C (Fig. 2B, bars 5). The results that coexpression of
Fig. 3. Canonical Wnt and β-catenin stimulate monolayer P19 cell differentiation into skeletal muscle by relieving I-mfa-mediated suppression of myogenin. (A–C) Wnt1, β-catenin, or I-mfa siRNA induces myogenesis independently of LEF1 transcriptional activity. P19 cells stably expressing Myogenin-HA (P19[Myogenin]) or a control plasmid (P19[Ctr]) were transiently transfected as indicated. After 3-day culture as monolayer, cells were fixed and stained with an anti-MHC antibody and DAPI (A) or harvested and separated into cytosolic or whole cell fractions for Western blot analysis (B). Quantification of Western blot analysis is shown in C. The effects of I-mfa siRNA on endogenous I-mfa protein and β-tubulin (an internal control) are shown. (D) Effect of endogenous β-catenin on the interaction of I-mfa with Myogenin. P19[Myogenin] cells were treated with or without Wnt3a for 3 h. Cells were harvested for cell fractionation and immunoprecipitation with an anti-HA or anti-c-Myc (as a control) antibody.
w was prevention of MRF from retention in the nucleus (6). Thus, we examined whether β-catenin can reverse this effect of I-mfa on MRFs. Consistent with the previous observations, I-mfa or I-mfa-C expression in P19 cells reduced the levels of coexpressed myogenin in the nuclei without changing the total levels of myogenin (Fig. 2C, lanes 2 and 3). Coexpression of β-catenin was able to reverse the reduction in nuclear myogenin levels caused by I-mfa (Fig. 2C, lanes 2 and 5), but not I-mfa-C (Fig. 2C, lanes 3 and 6). These results further confirm the ability of β-catenin to reverse I-mfa-mediated suppression of MRFs in a manner dependent on the interaction between β-catenin and I-mfa.

β-Catenin Relieves I-mfa-Mediated Suppression of MRFs in Myogenesis. To investigate whether β-catenin-mediated relief of I-mfa’s suppression of MRFs is of biological significance, we examined the role of I-mfa-β-catenin interaction in the regulation of myogenesis. P19 embryonic cells can be induced to differentiate into skeletal muscle in a number of models. One of those is the monolayer P19 differentiation model in which canonical Wnt proteins can stimulate the skeletal muscle formation, provided that exogenous myogenin is supplied (16) (Fig. 3 A, B, and C). Skeletal muscle differentiation was evaluated by the detection of the expression of a muscle marker MHC using immunostaining (Fig. 3A) or Western analysis (Fig. 3B and C). However, we found that this canonical Wnt-stimulated myogenesis was independent of LEF1-mediated gene transcription, because ΔC-LEF1, a potent dominant negative mutant that can block β-catenin-LEF1 pathway (28), had no effect on Wnt1-induced myogenesis in this model (Fig. 3, A4g, B lane 6, and C bar 6). Together with the fact that Wnt1-induced myogenesis in this model could be still blocked by a dominant negative Dvl mutant Dvl-DEP (29, 30) (Fig. 3Ah, B lane 7, and C bar 7), the canonical Wnt is likely to regulate myogenesis via a mechanism that is downstream of Dvl, but upstream of LEF1.

Knowing that β-catenin can bind to I-mfa that is expressed endogenously in P19 cells, we hypothesized that Wnt may stimulate myogenesis in this model by attenuating I-mfa-mediated suppression of myogenin via β-catenin. This hypothesis is consistent with the observations that the levels of free β-catenin appeared to be correlated with the extents of myogenin (Fig. 3B) and that I-mfa-C, a I-mfa mutant that cannot bind to β-catenin, is more potent than the wild-type I-mfa in blocking Wnt-induced myogenesis (Fig. 5, which is published as supporting information on the PNAS web site). In addition, the hypothesis is supported by the results that expression of β-catenin or, importantly, β-catenin-N, the mutant that cannot activate LEF1 activity, was able to stimulate myogenesis (Fig. 3 A j and k and C) and that these β-catenin-mediated effects could be blocked by exogenous expression of I-mfa (Fig. 3A j and C bar 10). To confirm the involvement of endogenous I-mfa, we examined the effect of siRNA-mediated suppression of I-mfa expression on myogenesis in the P19 cells stably expressing myogenin. We found that expression of I-mfa-specific siRNA induced myogenesis, and that this induction could not be blocked by ΔC-LEF1, although it could be blocked by excessive expression of I-mfa (Fig. 3A n-p and C). To verify the siRNA specificity, we used a second siRNA that targets a different sequence on I-mfa mRNA and found that this siRNA had the similar ability to induce myogenesis in the P19[myogenin] cells (Fig. 6, which is published as supporting information on the PNAS web site). Finally, we investigated whether canonical Wnt signaling is able to regulate the I-mfa-MRF interaction via endogenous β-catenin. Myogenin from P19 cells treated with or without Wnt3a was immunoprecipitated, and the levels of I-mfa were determined. Wnt3a treatment elevated the free β-catenin level and decreased the level of I-mfa associated with myogenin (Fig. 3D), indicating that Wnt signaling is capable of regulating I-mfa-myogenin interaction via endogenous β-catenin.

Stimulation of Canonical Wnt to Myogenesis in P19[myogenin] Cells Depends on I-mfa and β-Catenin Interaction. Although the aforementioned results showing that a reduction in the I-mfa level by siRNA or an increase in the level of an β-catenin mutant that cannot activate LEF1 both led to myogenesis in P19 cells stably expressing myogenin provides strong correlative evidence for the requirement of the interaction between β-catenin and I-mfa for canonical Wnt signaling-regulated myogenesis, we sought for more direct evidence. We wanted to substitute endogenous β-catenin in P19 cells with β-catenin-MM, the β-catenin mutant that cannot interact with I-mfa, but retains the same ability to activate LEF1 (Fig. 7, which is published as supporting information on the PNAS
If our hypothesis that the interaction between β-catenin and I-mfa is required for canonical Wnt-induced myogenesis is true, Wnt should not induce myogenesis in these P19 cells expressing β-catenin-MM*. To carry out the substitution, we diminished the expression of endogenous β-catenin by using a β-catenin-specific siRNA (Fig. 4A–31) and expressed a β-catenin-MM form (β-catenin-MM*) from a cDNA in which the siRNA target sequence was mutated via introduction of silent mutations, so that expression of β-catenin-MM* is not affected by β-catenin siRNA. As a control, we also expressed a wild-type β-catenin (β-catenin*) with the siRNA target sequence being silently mutated. As shown in Fig. 4A, although β-catenin siRNA significantly suppressed the expression of normal β-catenin, it was unable to affect the expression of either β-catenin* or β-catenin-MM*. When myogenesis was examined, expression of β-catenin siRNA abrogated Wnt1-induced myogenesis as expected (Fig. 4B bars 1–4 and C a and b). Whereas coexpression of β-catenin* rescued siRNA-mediated inhibitory effect on Wnt1-induced myogenesis, coexpression of β-catenin-MM* showed little rescuing activity (Fig. 4B bar 5–8 and C c–f). Given that the mutations in β-catenin-MM do not affect its activation LEF-1 or its interaction with LEF-1, Axin, or antigen-presenting cells (data not shown), but does affect I-mfa (Fig. 1D), these results lead us to conclude that the interaction between β-catenin and I-mfa is essential for canonical Wnt-induced myogenesis in this P19 differentiation model.

Discussion

I-mfa, whose expression is initiated after mesoderm formation, is widely distributed in the somite, notochord, and neural tube. It is a potent transcriptional suppressor for myogenic “master regulator” MRFs. Although I-mfa-mediated suppression may be regulated by differential expression of I-mfa, we show here that the suppression can also be acutely regulated by Wnt/β-catenin signaling during myogenic differentiation and that this Wnt-activated β-catenin-mediated relief of I-mfa’s suppression of MRFs has an important role in myogenesis. Thus, canonical Wnts regulate gene transcription not only in the LEF-1/TCF-dependent, but also independent, mechanisms.

In early embryogenesis, skeletal muscle is derived from the somite, which forms in the paraxial mesoderm. Strong evidence suggests that Wnts can lead somatic myogenic cells to become the muscle lineage by regulation of the expression and activity of MRFs (14, 32–35). Although LEF1/TCF may directly regulate MRF expression, I-mfa-mediated inhibition of MRFs activity has to be presumably relieved in cells expressing I-mfa. I-mfa is expressed in the presomitic mesoderm and dermomyotome; thus, it is reasonable to believe that I-mfa-mediated suppression of MRF activity is to be relieved by β-catenin, whose levels are elevated by canonical Wnts, such as Wnt1 and -3a. In addition, canonical Wnts are also required for further development of myogenic processes.

For instances, expression of MRFs in many stem cells, including P19 cells (16, 21), and in the presomitic mesoderm (6) do not lead immediate differentiation into skeletal muscles. Our results show that the I-mfa-mediated suppression of MRFs has to be relieved through the interaction between β-catenin and I-mfa before cells expressing MRF can proceed in the myogenic differentiation, providing a possible explanation for the requirement of Wnt proteins in these cases.

Mice lacking I-mfa exhibit genetic background-dependent phenotypes (7). I-mfa-deficiency results embryonic lethality in the C57BL background, probably because of severe placental defects. Because the disruption of the Wnt2 gene, a canonical Wnt gene, led to placental defects associated with a lack of the giant cells (36), which are the opposite of those associated with I-mfa-deficiency, some of I-mfa-deficiency-associated defects in the placenta may be the result of the increase in Wnt activity. In the 129/Sv background, I-mfa deficiency is associated with delayed neural tube closure, attenuated expression of Pax-1 and scleraxis, and skeletal patterning defects indicative of suppressed chondrogenesis. The latter phenotype is in agreement with the observation that overexpression of canonical Wnt inhibits Pax-1 expression and suppresses chondrogenesis (17). Thus, some of the phenotypes exhibited by mice lacking I-mfa may be the result of the increases in Wnt signaling activity, which is consistent with our findings that I-mfa can suppress Wnt signaling. The existence of I-mfa homologs (such as HIC, which contains I-mfa C-terminal domain, and also binds to β-catenin and MRFs; data not shown) and other MRF suppressors undoubtedly adds layers of complexity and difficulty for unambiguous interpretation of the phenotypes or the lack of thereof for these mice.

In summary, we describe a mechanism by which I-mfa-mediated transcriptional suppression of MRFs can be relieved by β-catenin and Wnt/β-catenin signaling. This finding expands the list of transcription factors that can be acutely regulated by elevated β-catenin levels that occur upon the activation of Wnt/β-catenin signaling. Because I-mfa has also been shown to inhibit Wnt target transcription factor LEF-1/TCFs, the similar mechanism by which β-catenin relieve I-mfa’s suppression may be envisioned. More work is needed to characterize the effect of β-catenin on regulation of I-mfa’s suppression of LEF-1/TCFs.

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