β-catenin relieves I-mfa-mediated suppression of LEF-1 in mammalian cells

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Summary

We have previously shown that β-catenin interacts with a transcription suppressor I-mfa and, through this interaction, canonical Wnt signaling could relieve I-mfa-mediated suppression of myogenic regulatory factors (MRFs). In this study, we found that, based on this interaction, I-mfa-mediated suppression of the Wnt transcription factor T-cell factor/lymphoid enhancing factor-1 (TCF/LEF-1) can also be relieved. Our work showed that knocking down endogenous I-mfa expression mimics canonical Wnt treatment by inducing myogenesis and increasing Wnt reporter gene activity, endogenous Wnt target gene expression and expression of MRFs in P19 cells. More importantly, these I-mfa small interfering RNA (siRNA)-induced effects could be blocked by a dominant-negative mutant of LEF-1, confirming the involvement of the TCF/LEF-1 pathway. In addition, we found that β-catenin could compete with I-mfa for binding to LEF-1 and relieve the inhibitory effects of I-mfa in overexpression systems. Furthermore, canonical Wnt was able to reduce the levels of endogenous I-mfa associated with LEF-1, while increasing that of I-mfa associated with β-catenin. All of the evidence supports a conclusion that I-mfa can suppress myogenesis by inhibiting TCF/LEF-1 and that canonical Wnt signaling may relieve the suppression through elevating β-catenin levels, which in turn relieve I-mfa-mediated suppression.

Key words: Wnt, β-catenin, I-mfa, LEF-1, Myogenesis, P19 carcinoma stem cells

Introduction

Wnt signaling plays important roles in embryogenesis, including generation of cell polarity, specification of cell fate and regulation of proliferation and differentiation. Its misregulation is also known to be involved in tumorigenesis. The β-catenin-mediated signaling mechanism is the best characterized of the Wnt pathways and highly conserved among *Drosophila*, *Xenopus* and mammalian systems. In this pathway, Wnt proteins bind to cell surface receptors Frizzled (Fz) and low-density-lipoprotein-receptor (LDLR)-related protein-5 (LRP5) and LRP6 and suppress the degradation of β-catenin through disrupting function of the degradation complex composed of glycogen-synthase kinase 3β (GSK3β), adenomatous polyposis coli (APC), axin and casein kinases, etc. Free β-catenin translocates to the nucleus where it functions as a coactivator of the transcription factors T-cell factor/lymphoid enhancing factor-1 (TCF/LEF-1) to activate transcription of Wnt target genes (Dale, 1998; Gumbiner, 1998; Nusse, 2005; van Noort and Clevers, 2002; Wodarz and Nusse, 1998).

The Wnt/β-catenin signaling pathway has been reported to play key roles in myogenic fate determination and differentiation (Ridgeway et al., 2000; Cossu and Borello, 1999; Petropoulos and Skerjanc, 2002). Studies showed that canonical Wnts, including Wnt1, Wnt3a and Wnt7a, could induce location-specific expression of myogenic regulatory factors (MRFs), including Myf5 and MyoD, in embryos (Fan et al., 1997; Capdevila et al., 1998; Tajbakhsh et al., 1998). Besides, P19 cells are pluripotent embryonic carcinoma cells that differentiate into several cell types and it has been reported that Wnt signaling is necessary and sufficient for skeletal myogenesis in P19 cells (Petropoulos and Skerjanc, 2002). In addition, the Wnt/β-catenin signaling was also shown to induce myogenesis in $CD45^+$ stem cells during muscle regeneration (Polesskaya et al., 2003). I-mfa was identified as an inhibitor of basic helix-loop-helix (bHLH) transcription factors that include MRFs by preventing their nuclear localization and DNA binding (Chen et al., 1996). The inhibitory effects of I-mfa are dependent upon its conserved cysteine- rich C-terminal domain. In addition to MRFs, I-mfa was reported to inhibit the activity of another bHLH transcription factor, Mash2 (Kraut et al., 1998). A related human I-mfa domain-containing protein (HIC) has also been identified as a regulator of Tat- and Tax-mediated expression of viral promoters (Thebault et al., 2000). More recently, it was reported that I-mfa could also regulate Wnt signaling (Snider et al., 2001; Kusano and Raab-Traub, 2002). I-mfa and XIC (the *Xenopus* homolog of I-mfa) were shown to bind to the C-terminal part of XTcf3, a transcription factor of the *Xenopus* Wnt/β-catenin signaling pathway, and thereby inhibit its transcriptional activity (Snider et al., 2001).

In a previous study we identified the interaction between I-mfa and β-catenin and demonstrated that this interaction is required for canonical Wnts to relieve I-mfa-mediated

suppression of MRFs during myogenesis in a P19 model system that stably expressed myogenin (Pan et al., 2005). In this study we found that I-mfa, which is expressed endogenously in P19 cells, also suppressed Wnt target transcription factors TCF/LEF-1 and myogenesis in an aggregation model in the absence of exogenous myogenin. We further demonstrated that β-catenin is capable of relieving the suppression by I-mfa, thus expanding our understanding of the mechanisms by which β-catenin regulates gene transcription.

Results

In our previous study we showed that canonical Wnt signaling can release the inhibitory effect of I-mfa on MRFs via β-catenin–I-mfa interaction to induce myogenesis of P19 cells that stably expressed an exogenous MRF myogenin in a monolayer myogenesis model (Pan et al., 2005). To determine the role of I-mfa in a P19 myogenic model independent of exogenous expression of myogenin, we established a stable P19 cell line that expressed siRNA specific to I-mfa (Fig. 1A) and tested these cells for myogenic differentiation in an aggregation culture model. This aggregation culture model does not require exogenous myogenin, but efficient induction of myogenic differentiation requires inducers (Ridgeway et al., 2000; Petropoulos and Skerjanc, 2002). Dimethyl sulfoxide (DMSO) is a non-specific differentiation inducer, which induces the differentiation into not only skeletal muscle, but also cardiac muscles (Edwards et al., 1983). Canonical Wnt proteins have been shown to skew P19 differentiation toward skeletal muscle (Ridgeway et al., 2000). These observations were confirmed in our studies (Fig. 1B); both DMSO and Wnt3a, a canonical Wnt protein, stimulated skeletal myogenic differentiation, which was monitored by the expression of a specific marker myosin heavy chain (MHC) using western-blot analysis (Fig. 1B) and immunostaining (Fig. 1C). Consistent with our expectation that I-mfa functions as a key transcription suppressor for MRFs, the cell lines that stably expressed I-mfa siRNA underwent myogenic differentiation even in the absence of DMSO or canonical Wnt proteins (Fig. 1B, lane 3 and Fig. 1Cc). To examine whether Wnt target transcription factor TCF/LEF-1 have a role in the myogenic induction by I-mfa siRNA, we also established cell lines that stably express a dominant-negative LEF-1 mutant, ΔC-LEF-1, in the presence or absence of I-mfa siRNA (Fig. 1A, lanes 3,4). ΔC-LEF-1 has previously been shown to be a strong canonical Wnt signaling inhibitor (Pan et al., 2005; Pandur et al., 2002), and it does not contain an I-mfa binding domain and cannot bind to I-mfa (Tajbakhsh et al., 1998; Pan et al., 2005). We found that cells coexpressing ΔC-LEF-1 and I-mfa siRNA failed to undergo myogenic differentiation (Fig. 1B, lanes 7,8 and Fig. 1Cf), suggesting that canonical Wnt signaling might have an important role in this myogenic model.

In the monolayer model that expresses exogenous myogenin, expression of ΔC-LEF-1 had no effect on myogenic differentiation, and canonical Wnt signaling regulates myogenic differentiation by relieving the transcriptional suppression of MRFs by I-mfa through the direct interaction between β-catenin and I-mfa (Pan et al., 2005). The inhibitory effect of ΔC-LEF-1 in the aggregation culture model suggests that canonical Wnt signaling might have additional roles in this model system. Previously, canonical Wnt signaling was reported to induce the expression of MRFs both in embryos

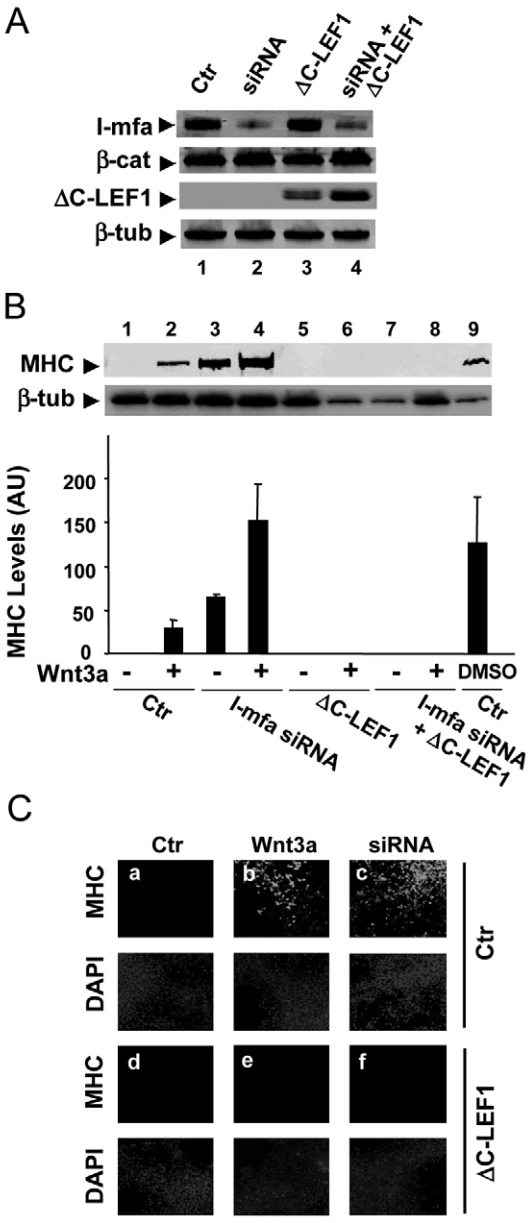

Fig. 1. Stably expressing I-mfa siRNA in P19 cells initiate myogenesis. (A) Protein expression levels in different kinds of stable cell lines. The levels of endogenous I-mfa, β-catenin and β-tubulin and exogenous ΔC-LEF-1-HA were determined by western-blot analysis. β-tubulin was measured as an internal control. (B,C) P19 cell lines that stably express a control plasmid (Ctr), I-mfa siRNA (siRNA) or ΔC-LEF-1, or coexpress ΔC-LEF-1 and I-mfa siRNA were induced to differentiate after aggregation in the presence or absence of Wnt3a. At Day 9, cells were collected. MHC protein levels were analyzed via western blotting and quantified through the use of an Odyssey II quantification system as previously described (Pan et al., 2005) (B). Cells were also fixed and stained with DAPI (blue) and an anti-MHC antibody (green) (C). We established several individual clones for every kind of stable cell line. All the clones showed similar results, and we present only the results from one of them.

(Fan et al., 1997; Capdevila et al., 1998; Tajbakhsh et al., 1998) and in the P19 cells (Ridgeway et al., 2000; Snider et al., 2001). These observations might provide an explanation to reconcile

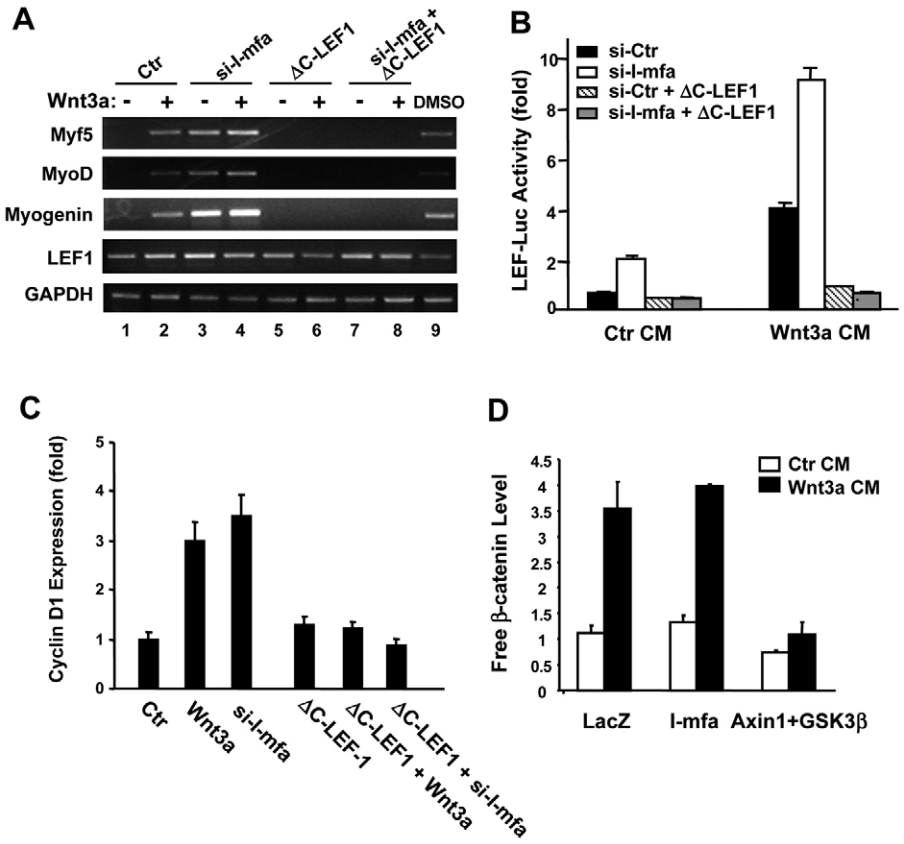

Fig. 2. I-mfa inhibits endogenous Wnt/β-catenin signaling in P19 cells. (A) P19 cells, as shown in Fig. 1B,C, were collected for RNA isolation and semi-quantitative RT-PCR analysis as described in the Materials and Methods. (B) I-mfa suppresses LEF-1 reporter activity in P19 cells. Cells were first transfected with control siRNA (si-Ctr) or I-mfa siRNA (si-I-mfa) and two days later with the LEF-1 reporter system. Twenty-four hours later, cells were treated with control-conditioned medium (Ctr CM) or Wnt3a-conditioned medium (Wnt3a CM) for an additional 8 hours before the activity was determined. (C) I-mfa siRNA elevate the expression of the Wnt target gene. Individual clones for different kinds of P19 stable cell line were cultured as a monolayer, and treated with Ctr CM or Wnt3a CM for 6 hours before RNA isolation. Semi-quantitative RT-PCR was performed for cyclin D1 and GAPDH detection. Relative mRNA levels of cyclin D1 were quantified by Labwork 2.0 UVP software and normalized with the amount of GAPDH. (D) I-mfa does not affect Wnt-induced free β-catenin accumulation. P19 cells were transfected with I-mfa, GSK3β and Axin1 expression plasmids or LacZ as indicated in the figure. Eighteen hours post-transfection, cells were treated with Ctr CM or Wnt3a CM for a further 6 hours and then collected for cell-fraction preparation and β-catenin ELISA assay.

the discrepancy in the requirement of TCF/LEF-1-dependent signaling between the two myogenic model systems: canonical Wnts act through LEF-1/TCF to induce expression of MRFs in the aggregation model, whereas in the monolayer model one of the MRFs, myogenin, was provided and thus the LEF-1/TCF pathway was not needed. To test this hypothesis, we examined the expression of MRFs including Myf5, MyoD and myogenin during myogenic differentiation in the aggregation model. Both canonical Wnt and DMSO treatments induced the expression of these MRFs (Fig. 2A, lanes 2,9). Importantly, the induction could be blocked by ΔC-LEF-1 (Fig. 2A, lane 6). Consistent with the ability of I-mfa siRNA to induce myogenesis in this model, it also induced the expression of MRFs (Fig. 2A, lane 3). The fact that ΔC-LEF-1 blocked I-mfa-induced expression of MRFs but not the expression of LEF-1 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fig. 2A, lanes 7,8) suggests that I-mfa siRNA-induced MRF expression might require the LEF-1/TCF pathway in the aggregation model. The recent finding that XIC and I-mfa inhibit XTcf3 transcriptional activity in frog embryos (Snider et al., 2001) provides a possible explanation for the inhibition of I-mfa siRNA-induced myogenesis and MRF expression by ΔC-LEF-1; I-mfa might inhibit LEF-1/TCF-mediated transcription. To test this idea, we examined the effect of I-mfa siRNA on the activation of a LEF-1 reporter gene in P19 cells. P19 cells not only express endogenous I-mfa (Fig. 1A) (Pan et al., 2005) but also endogenously express at least two canonical Wnts, Wnt3 and Wnt8a (Smolich and Papkoff, 1994). Transfection of I-mfa siRNA led to marked increases in LEF-1 reporter gene activity, and this activation could be blocked by (ΔC-LEF-1 (Fig. 2B), suggesting that I-mfa might suppress endogenous Wnt signaling in P19 cells. The presence of exogenous canonical Wnt ligand could further stimulate the reporter gene activity, which was also blocked by ΔC-LEF-1 (Fig. 2B). The observation that I-mfa siRNA- and Wnt3a-induced expression of cyclin D1, a well-characterized Wnt target gene (Shtutman et al., 1999), was inhibited by ΔC-LEF-1 (Fig. 2C) further supports the idea that I-mfa might suppress TCF/LEF-1 activity in P19 cells. Since expression of I-mfa (Fig. 2D) or I-mfa siRNA (data not shown) did not affect the free β-catenin level even in the presence of Wnt3a ligand, in contrast to GSK3β and Axin1 expression (Fig. 2D), we conclude that I-mfa most likely inhibits Wnt–β-catenin signaling downstream of β-catenin. This conclusion is consistent with the finding that I-mfa binds to TCF/LEF-1 and inhibits the transcriptional activity (Snider et al., 2001).

The aforementioned evidence clearly demonstrates that endogenous I-mfa can inhibit myogenesis, expression of MRFs and Wnt target and reporter gene expression in P19 cells. Nevertheless, Wnt3a appears to be able to circumvent I-mfa-mediated inhibition to induce myogenesis, MRF expression and Wnt target and reporter gene expression in these cells. Knowing that β-catenin binds to I-mfa and relieves I-mfa-mediated suppression of MRFs, we postulate that canonical Wnts might be able to relieve I-mfa-mediated suppression of TCF/LEF-1 through β-catenin. To test this hypothesis, we examined the effect of β-catenin on the interaction between I-mfa and LEF-1. Since the interaction between LEF-1 and β-catenin might complicate result interpretation, we used a LEF-1 mutant, ΔN-LEF-1, which lacks the N-terminal 33 amino

acids and is unable to interact with β-catenin (Tetsu and McCormick, 1999). The deletion does not, however, affect its interaction with I-mfa (Fig. 3A, lane 3). As shown in Fig. 3A, ΔN-LEF-1 and β-catenin appeared to compete for binding to I-mfa. In other words, the presence of ΔN-LEF-1 reduced the level of β-catenin in the immunocomplexes pulled down through I-mfa (Fig. 3A; compare lanes 1 and 4 of the top panel) and the presence of β-catenin decreased the level of ΔN-LEF-1 precipitated with I-mfa (Fig. 3A, compare lanes 3 and 4 of the second panel). However, β-catenin expression did not affect the interaction between ΔN-LEF-1 and I-mfa-C, which contains the 122 C-terminal residues of I-mfa (Fig. 3A, lanes 7,8). I-mfa-C binds to LEF-1, but not β-catenin (Pan et al., 2005), thus suggesting that the interaction between β-catenin and I-mfa is required for the competition between β-catenin and LEF-1 for binding to I-mfa. This conclusion is further supported by the finding that β-catenin-MM, which has mutated residues R342 and K345, and a low affinity for I-mfa (Pan et al., 2005), had little effect on the interaction between ΔN-LEF-1 and I-mfa (Fig. 3A, compare lanes 3 and 5). In addition, we also used wild-type LEF-1 and a mutant form of β-catenin-H470A (von Kries et al., 2000), which can still bind to I-mfa but not to LEF1/TCFs, in such a competition assay, and found the similar competitive effect as that using ΔN-LEF-1 and wild-type β-catenin (data not shown).

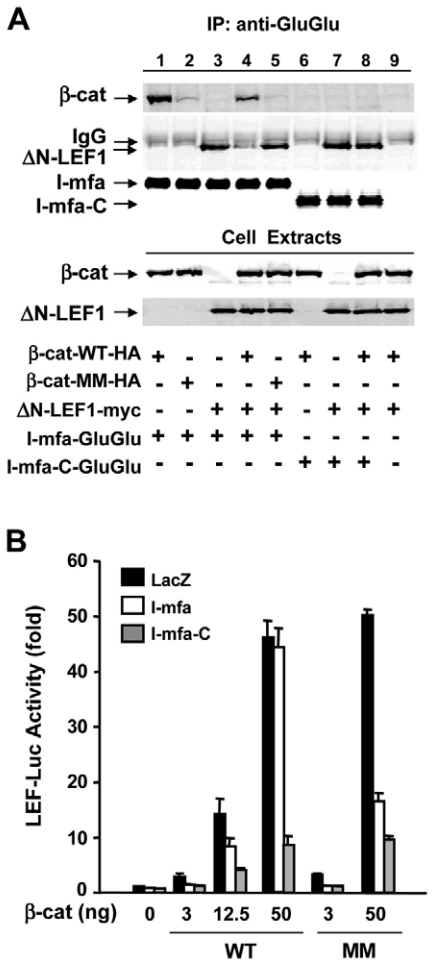

Fig. 3. β-catenin relieves I-mfa-mediated suppression of LEF-1. (A) β-catenin competes with LEF-1 for binding to I-mfa. HEK 293T cells were transfected with plasmids as indicated. Immunoprecipitation was performed with an anti-GluGlu antibody and analyzed with an anti-HA, anti-GluGlu or anti-Myc antibody. (B) β-catenin relieves I-mfa inhibition of the LEF-1 reporter gene in mammalian cells. For NIH3T3 cells, transfection was performed with the LEF-1 reporter gene system and expression plasmid as indicated in the figures. Luciferase activity was determined 24 hours after transfection.

The competition between β-catenin and LEF-1 for I-mfa binding suggests that β-catenin, the cellular levels of which are drastically regulated by canonical Wnt signaling, might regulate transcriptional activities of LEF-1 through relieving I-mfa-mediated suppression in addition to its direct activation of LEF-1. To determine the effects of increased expression of β-catenin on I-mfa-mediated suppression of transcriptional activity of LEF-1, we performed the LEF-1 reporter gene assay in NIH3T3 fibroblast cells in which there was no endogenous I-mfa or canonical Wnt proteins (data not shown). In NIH3T3 cells expression of β-catenin led to increases in the reporter gene activity in a dose-dependent manner (Fig. 3B). Coexpression of wild-type I-mfa was able to suppress most of β-catenin-mediated activation of LEF-1 at low doses of β-catenin transfection; almost 100% inhibition by I-mfa when 3 ng of β-catenin expression plasmid were transfected, and approximately 50% when 12.5 ng were transfected (Fig. 3B). However, the ability of I-mfa to inhibit β-catenin dissipated as more β-catenin was transfected (Fig. 3B). Transfection of 50 ng β-catenin completely abolished I-mfa-mediated inhibition (Fig. 3B), suggesting that β-catenin relieves the suppression when there is enough of it. However, β-catenin failed to relieve the suppression imposed by I-mfa-C (Fig. 3B), the I-mfa mutant that does not bind to β-catenin, but still binds to and inhibits LEF-1 (Fig. 3A) (Snider et al., 2001) regardless of the amount of β-catenin being transfected. Importantly, overexpression of β-catenin-MM, the β-catenin mutant, could not reverse the inhibition imposed by I-mfa even though β-catenin-MM and β-catenin showed the same transcriptional activity in the absence of I-mfa (Fig. 3B). All these data clearly demonstrate that β-catenin is capable of relieving I-mfa-mediated transcriptional suppression of LEF-1 and that the relief requires the interaction between I-mfa and β-catenin.

To confirm that β-catenin is capable of disrupting the I-mfa and LEF-1 interaction in P19 cells, we examined whether canonical Wnt-induced accumulation of free β-catenin can attenuate the I-mfa–LEF-1 interaction. P19 cells were treated with or without Wnt3a, and an endogenous LEF-1-associated protein complex was immunoprecipitated with a LEF-1-specific antibody. As shown in Fig. 4, Wnt3a treatment increased the level of free β-catenin while reducing the level of I-mfa coimmunoprecipitated with LEF-1, and at the same time more β-catenin proteins were associated with LEF-1. These results once again strongly suggest that Wnt signaling is able to attenuate the I-mfa–LEF-1 interaction and thus I-mfa-mediated suppression of LEF-1 activity through β-catenin in vivo.

Discussion

Expression of I-mfa is initiated after mesoderm formation and I-mfa is widely distributed in the somite cells, notochord and neural tube (Chen et al., 1996). I-mfa is a potent

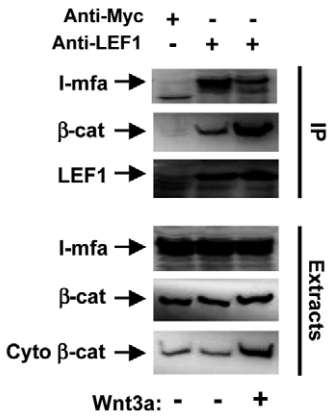

Fig. 4. Effect of endogenous β-catenin on LEF-1–I-mfa interactions. P19 cells were treated with or without Wnt3a for 3 hours. Cells were harvested for cell fractionation and immunoprecipitation with an anti-LEF-1 or anti-Myc antibody (control antibody). Endogenous LEF-1, β-catenin and I-mfa were detected with their respective antibodies.

transcriptional suppressor for the canonical Wnt signaling target TCF/LEF-1 and myogenic 'master regulators' MRFs (Buckingham, 2001; Pownall et al., 2002; Rudnicki and Jaenisch, 1995; Weintraub et al., 1991; Yun and Wold, 1996). In addition, I-mfa might suppress other bHLH transcription factors (Kraut et al., 1998). In our previous study we showed that β-catenin interacted with I-mfa and that canonical Wnt signaling could relieve I-mfa-mediated suppression of MRFs through the β-catenin and I-mfa interaction. In this study we found that I-mfa suppressed Wnt target transcription factors TCF/LEF-1 and that β-catenin could relieve this suppression as well.

P19 cells were derived from a tumor of an early mouse embryo and are pluripotent in nature. They can be differentiated into various cell types depending on the environments. Aggregation and DMSO treatment induces the expression of the mesoderm marker Brachyury (T). In addition, several developmentally important factors, including Wnt proteins and bone morphogenetic proteins BMP2/4, are expressed during the aggregation. All of this is reminiscent of what occurs in the embryo. In early embryogenesis, skeletal muscle is derived from the somite, which forms in the paraxial mesoderm. Strong evidence suggests that development signals, including Wnts, hedgehogs and BMPs produced from the surface ectoderm, axial structures including the dorsal neural tube and notochord, and the lateral mesoderm commit somatic mesodermal cells to become the muscle lineage by regulation of the expression and activity of MRFs (Cossu and Borello, 1999; Christ and Ordahl, 1995; Currie and Ingham, 1998; Molkentin and Olson, 1996). The expression of Myf5 in the dermomyotome appears to be controlled by canonical Wnts because the Myf5 expression could be initiated by co-culture of cells expressing Wnt1 (Capdevila et al., 1998). The lack of dermomyotome in Wnt1 and Wnt3a double-knockout mouse embryos further confirms the importance of canonical Wnts in myogenic lineage specification (Ikeya and Takada, 1998). Whereas TCF/LEF-1 might directly regulate expression of MRFs, I-mfa-mediated inhibition of TCF/LEF-1 activity is 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 the activity of TCF/LEF-1 is relieved by β-catenin, whose levels are elevated by canonical Wnts such as Wnt1 and 3a.

Mice lacking I-mfa exhibit genetic background-dependent phenotypes (Kraut et al., 1998). I-mfa-deficiency results in embryonic lethality in the C57Bl background, which is probably because of severe placental defects. Since the disruption of the Wnt2 gene, a canonical Wnt gene, led to placental defects associated with a lack of the giant cells (Monkley et al., 1996), which are the opposite of those associated with I-mfa-deficiency, some of the I-mfa- deficiency-associated defects in the placenta might be the result of the increase in Wnt activity. In the 129/Sv background, I-mfa deficiency is associated with delayed neutral 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 (Capdevila et al., 1998). Thus, some of the phenotypes exhibited by mice lacking I-mfa might be the result of the increases in Wnt signaling activity, which is consistent with our findings that I-mfa can suppress Wnt signaling. Since I-mfa suppresses not only TCF/LEF-1 but also MRFs and probably other transcription factors, which may all be relived by canonical Wnt signaling, the complex phenotypes exhibited by I-mfa-deficient mice could be a result of altered regulation of LEF-1/TCFs, MRFs and/or other factors. The existence of I-mfa homologs and other

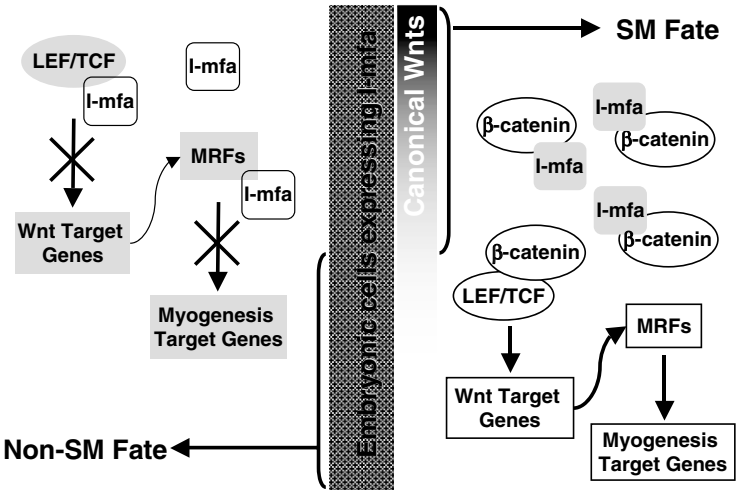

Fig. 5. A model for the regulation of MRFs and TCF/LEF-1 by β-catenin and I-mfa in myogenesis. In the absence of a canonical Wnt signal, I-mfa suppresses transcriptional activities of MRF and TCF/LEF-1 by binding to them. In the presence of canonical Wnt proteins, canonical Wnt signaling is activated, and the levels of β-catenin increase in the cytoplasm and nucleus are elevated. β-catenin then binds to I-mfa and relieves I-mfa suppression of TCF/LEF-1 and MRF, which is required for myogenic specification and development.

MRF and LEF-1/TCF suppressors undoubtedly adds additional layers of complexity and difficulty for unambiguous interpretation of the phenotypes or the lack of them for these mice.

In summary, the present work, together with our previous work, reveals multiple roles of β-catenin-mediated signaling in regulation of myogenesis, as depicted in Fig. 5. When I-mfa is present, β-catenin not only functions as a transcription activator for LEF-1/TCFs but it also has to counteract the inhibitory effect of I-mfa, relieving suppression of I-mfa on both canonical Wnt signaling downstream target-transcription factors and myogenic factors. The dual mechanisms by which β-catenin regulates LEF-1/TCF-dependent transcription might provide wider Wnt signaling dynamics that might be demanded in the diverse developmental or physiological processes regulated by Wnt proteins. These findings evidently expand the list of transcription factors that can be acutely regulated by elevated β-catenin levels that occur upon the activation of canonical Wnt signaling, thus furthering our understanding of Wnt signaling that is involved not only in the regulation of developmental processes and tissue regeneration, but also in many pathological processes including cancer.

Materials and Methods

Cell cultures

Mouse NIH3T3 fibroblast cells and human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% fetal bovine serum (FBS). P19 cells were cultured in DMEM-F12 (Sigma) containing 10% FBS (HyClone). Wnt3a-containing conditioned medium was prepared as previously described (Mao et al., 2001).

To establish stable P19 cell lines, cells were transfected with pPERL–I-mfa–siRNA and selected with G418 (800 ng/ml). At least two clones for each stable cell line were established. To obtain the stable cell lines expressing or coexpressing ΔC-LEF-1, cells were transfected with pcDNA3-Hygro-ΔC-LEF1-HA that contains a hygromycin-resistant gene. Cells were then selected with G418 (200 ng/ml) and hygromycin (200 ng/ml). At least four clones for each stable cell line were established. All the clones showed similar results, but we only present the results from one of the clones.

For the aggregation model (Ridgeway et al., 2000; Petropoulos and Skerjanc, 2002), differentiation was initiated by plating 5×10^5 cells (P19[Ctr], P19[I-mfa siRNA], P19[ΔC-LEF-1] or P19[I-mfa siRNA + ΔC-LEF-1]) into 60 mm Petri dishes (Fisher) in α-MEM (Invitrogen) plus 10% FBS (HyClone), with or without control medium or Wnt3a conditioned medium or 1% DMSO. After two days, aggregates were transferred into new Petri dishes with fresh medium and with or without Wnt3a conditioned medium, and then cultured for an additional 48 hours. Finally, aggregates were transferred into normal tissue culture dishes for a further nine days. Media were changed every two days.

Plasmid constructs

The LEF-1 reporter genes were kindly provided by Rudolf Grosschedl (UCSF). The mutants of mouse I-mfa, β-catenin and LEF-1 were generated by PCR or reverse transcriptase-PCR and verified by DNA sequencing. HA/Myc-epitope tags were introduced to the C-termini of these clones, whereas GluGlu epitope tags were introduced to the N-termini of the clones. I-mfa siRNA was expressed using a pSuper-derived vector (Brummelkamp et al., 2002), designated pPERL, which contains a neomycin-resistant gene-expression unit. The sequence of I-mfa target siRNA, the structure of ΔC-LEF1 [1-256 amino acids of human LEF1, lacking high mobility group (HMG) box and C-terminal sequence] and β-catenin-MM (R342A, K345A) were previously described (Pan et al., 2005).

Transfection and reporter gene assay

Transfection was performed in 24-well plates using Lipofectamine Plus as suggested by the manufacturer (Life Technologies, Rockville, MD). The transfection was stopped after 3 hours. For reporter gene assays, cell extracts were collected 24 hours later. Cell lysates were measured for fluorescence intensity emitted by green fluorescent proteins (GFP) in a FL600 fluorometer (Bio-Tek), and then the luciferase substrate (Boehringer Mannheim Luciferase Assay Kit) was added to determine the luciferase activities using a MicroLumate Plus (Perkin Elmer) luminometer. Luminescence intensity is normalized against fluorescence intensity.

Immunoprecipitation assays and western blotting

For immunoprecipitation, cells using six-well plates were lysed with the lysis buffer containing 1% Nonidet P-40, 137 mM sodium chloride, 20 mM Tris, pH 7.4, 1 mM dithiothreitol, 10% glycerol, 10 mM sodium fluoride, 1 mM pyrophosphoric acid, 2 mM sodium vanadate and Complete™ protease inhibitors (Roche Biochemical, used as the instructions suggest). The cell lysates were incubated with 1 μl of anti-tag antibody and 25 μl of protein A/G sepharose beads for 3 hours in 4°C. The immunocomplexes were pelleted and washed three times with cold lysis buffer in the absence of protease and phosphatase inhibitors.

For western-blot analysis, proteins were analyzed by SDS-PAGE and by western blotting using nitrocellulose transfer membrane (Schleicher and Schuell BioScience). Results were visualized and quantified by an Odyssey Infrared Imaging System 9120 (LI-COR). Mouse anti-GluGlu, anti-Myc and anti-HA monoclonal antibodies were purchased from Bibco. Mouse anti-β-catenin monoclonal antibody was purchased from BD Bioscience (San Jose, CA). Mouse anti-β-tubulin monoclonal antibody was purchased from Sigma. MF20 and anti-MHC were purchased from DSHB (Iowa City, IA). Anti-GFP monoclonal antibody was purchased from Roche. Anti-I-mfa chicken antibody was as previously used (Ma et al., 2003).

Total RNA extraction and RT-PCR detection

Total RNA was harvested and prepared with TRIzol™ reagent (Invitrogen). Four micrograms of total RNA were used with 40 μl of Superscript II™ RT (Invitrogen) for first-strand cDNA synthesis. PCR reactions were analyzed at the linage range with specific primers.

Cell-fraction preparation and β-catenin ELISA

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 $MgCl_2$, 1 mM EDTA, 10 mM KCl, 0.5 mM dithiothreitol and a cocktail of protease inhibitors. After incubating on ice for 10 minutes, cells were homogenized in a homogenizer and centrifuged at 15,000 ***g***. The supernatant was collected as the cytosol fraction and then put into a β-catenin ELISA assay kit (Assay Designs, Ann Arbor, MI). ELISA data is normalized with protein level quantification (Pierce, prod. no. 23228).

Immunofluorescence staining and microscopy

P19 cells on coverslips were washed once with ice-cold PBS and then fixed for 30 minutes in PBS containing 4% paraformaldehyde at 4°C. Fixed cells were incubated in chloroform-methanol-acetone (CMA, 1:2:1, v/v) and then methanol for 30 minutes, respectively, at –20°C. After rehydration with PBS at room temperature, coverslips were incubated with 1% bovine serum albumin for 1 hour and then with anti-MHC antibody (1:100) for a further hour. FITC-conjugated anti-mouse immunoglobulin G (Jackson Immunoresearch Laboratories) was used as the second antibody. Nuclei were stained with DAPI for 1 minute. Cells were examined using a fluorescence microscope (Leica TCS SP2).

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