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14. ABSTRACT

Multi-potent stem cell populations found in adult tissues have been of great interest because they serve as reservoirs for tissue renewal after trauma, disease and aging. One important type of adult stem cell derived from bone marrow is the mesenchymal stem cell (MSC), which contributes to the regeneration of mesenchymal tissues such as bone, cartilage, muscle, tendon and adipose. However, lack of knowledge at the molecular level on the regulatory mechanisms underlying the self-renewal and differentiation of MSCs has limited the potential use of MSCs in practical applications such as tissue engineering and gene therapy. In this report I describe a novel form of crosstalk between the TGF-β and Wnt signaling pathways and its functional role in regulating the proliferation and osteogenic differentiation of human MSCs. We show that TGF-β induces rapid nuclear translocation of β-catenin in MSCs in a Wnt signaling-independent fashion. TGF-β does not affect the stability of β-catenin, but requires the activity of the TGF-β type I receptor and the presence of Smad3. Functionally, this pathway is required for the stimulation of MSC proliferation and the inhibition of MSC osteogenic differentiation by TGF-β likely through the combined actions of β-catenin and Smad3 to regulate downstream target genes. These results provide evidence for a novel mode of cooperation between the TGF-β and Wnt signaling pathways in this specific cellular context, and suggest a potentially important role for this distinct signaling pathway in the control of self-renewal and differentiation of mesenchymal stem cells.

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Abstract

Multi-potent stem cell populations found in adult tissues have been of great interest because they serve as reservoirs for tissue renewal after trauma, disease and aging. One important type of adult stem cell derived from bone marrow is the mesenchymal stem cell (MSC), which contributes to the regeneration of mesenchymal tissues such as bone, cartilage, muscle, tendon and adipose (Pittenger et al. 1999; Caplan 2005). However, lack of knowledge at the molecular level on the regulatory mechanisms underlying the self-renewal and differentiation of MSCs has limited the potential use of MSCs in practical applications such as tissue engineering and gene therapy (Caplan 2000b; Caplan and Bruder 2001).

In this report I describe a novel form of crosstalk between the TGF-β and Wnt signaling pathways and its functional role in regulating the proliferation and osteogenic differentiation of human MSCs. We show that TGF-β induces rapid nuclear translocation of β-catenin in MSCs in a Wnt signaling-independent fashion. TGF-β does not affect the stability of β-catenin, but requires the activity of the TGF-β type I receptor and the presence of Smad3. Functionally, this pathway is required for the stimulation of MSC proliferation and the inhibition of MSC osteogenic differentiation by TGF-β likely through the combined actions of β-catenin and Smad3 to regulate downstream target genes. These results provide evidence for a novel mode of cooperation between the TGF-β and Wnt signaling pathways in this specific cellular context, and suggest a potentially important role for this distinct signaling pathway in the control of self-renewal and differentiation of mesenchymal stem cells.

Introduction

TGF-β and Wnt signaling pathways function in a wide range of biological processes, and play a crucial role in mesenchyme-derived tissue generation such as bone formation and limb development. TGF-β signaling is primarily mediated by type I and type II receptors. In response to TGF-β, both Smad2 and Smad3 can be phosphorylated on serine residues in the C-terminus by the TGF-β type I receptor kinase. These two Smads can then separately form heteromers with Smad4, translocate to the nucleus and bind to DNA in a sequence specific fashion and act as transcription factors. Consistent with their role as transcription regulators, Smad proteins have been found to interact with a large numbers of other transcription factors, as well as coactivators p300/CBP and transcriptional repressors, including HDAC (Massague and Chen 2000; Shi and Massague 2003). Wnt signaling is initiated by the binding of Wnt to its cell surface receptor Frizzled and co-receptor LRP5 or LRP6 to activate a signaling cascade. Activation of the Wnt pathway leads to stabilization and nuclear accumulation of β-catenin, the primary effector of Wnt signaling. After moving into the nucleus, β-catenin can modulate gene transcription through association with members of the TCF transcription factor family (Logan and Nusse 2004).

β-catenin stabilization and translocation is crucial for Wnt signaling. In the absence of Wnt signal, the cytoplasmic pool of β-catenin is constantly degraded through a process controlled by a degradation complex consisting of adenomatous polyposis coli (APC), Axin, glycogen synthase kinase-3β (GSK-3β), and casein kinase I (CKI) (Korinek et al. 1997; Kishida et al. 1998; Kishida et al. 1999; Hinoi et al. 2000). In this complex, APC initiates the assembly of the complex, Axin functions as a scaffold, CKI and GSK-3β facilitate phosphorylation on the N-terminus of β-catenin (Li et al. 1999; Yanagawa et al. 2000; Liu et al. 2002; Yanagawa et al. 2002). Phosphorylation directs β-catenin to ubiquitination and degradation by the 26S proteasome complex (Liu et al. 1999).Wnt signaling inhibits activity of this complex, resulting in the accumulation of β-catenin protein for nuclear translocation. The mechanism of β-catenin nuclear accumulation remains to be elucidated. No classical nuclear localization signal (NLS) and nuclear export signal (NES) have been identified on β-catenin. A possible mechanism arises from the considerable structural similarity shared by β-catenin and importin β, which is in extensive helical confirmation. Like importin β, β-catenin has self-translocation ability through nuclear pore complex (NPC) (Yokoya et al. 1999), probably mediated by its ARM repeats. Interestingly, these features are shared by Smad2 and Smad3, which can directly bind to nucleoporin and translocate in and out of nucleus without adaptor proteins (Xu, 2002).

Recently, in addition to Wnt, certain other growth factors such as androgen receptors (Mulholland et al. 2002; Pawlowski et al. 2002), hepatocyte growth factors and mullerian inhibiting substance (MIS) have also been shown to regulate the nuclear translocation of β-catenin. However, the molecular mechanism of the regulation, as well as the biological significance, is not clear. In another report, TGF-β is shown to regulate β-catenin, likely through induction of Wnt protein expression during the process of chondrogenesis (Zhou et al. 2004). Furthermore, β-catenin has been shown to form complexes with Smads to induce transcription of specific genes during Xenopus development (Labbe et al. 2000; Nishita et al. 2000), suggesting cross-talk of the TGF-β

and Wnt pathways is important in certain biological processes. In this report, we present studies on the cross-talk of TGF-β and Wnt in the context of MSCs. Specifically, we find that TGF-β can induce β-catenin nuclear translocation in MSCs and this pathway is mediated by TGF-β signaling members including the type I receptor and Smad3. β-catenin nuclear translocation in response to TGF-β is a possible mechanism for TGF-β induced proliferation in MSCs and other mesenchymal derived cells.

Materials and methods

Human mesenchymal stem cells (MSCs)

Three separate batches of MSCs and growth media were purchased from Cambrex Bio Science (PT-2501, PT-2500). The cells were cultured according to the protocols provided by the manufacture.

Osteogenic differentiation and alkaline phosphatase staining

MSCs were cultured in osteogenic supplementary (OS) media for osteogenic differentiation. OS media: 100 nM dexamethasone, 0.25 mM ascorbic acid, 10 mM β-glycerophosphate. Alkaline phosphatase staining kit was purchased from Sigma (86C-1KT) and staining was performed according to the manual provided by the kit.

Antibodies and immunoprecipitation

Immunoprecipitation and Western blot were carried out as described(Shen et al. 1998). Antibodies to β-catenin (Transduction lab), GSK-3β (Santa Cruz), Smad3 I-20 (Santa Cruz), Smad3 (Zymed), phspho-Smad2 (Cell signaling), p53 (Santa Cruz), Lamin (Santa Cruz), β-tubulin (Santa Cruz), LEF1 (Oncogene), HA Y-11 (Santa Cruz), Myc 9E10 (Roche) were used to detect proteins and for immunoprecipitation. HRP-conjugated antibodies to mouse or rabbit IgG were purchased from Zymed

Wnt3A conditioned media

Wnt3A conditioned and control media were generated from LWnt-3A cells and L cells respectively (ATCC, CRL-2647 and CRL-2648). Nearly confluent cells were split 1:10 into 10 ml culture media (10% DMEM) in 10 cm^2 petri dishes and grown for 4 days (almost confluent). Media was then collected sterile filtered, fresh media was added, and cells were cultured for another 3 days. Media was collected, filtered and combined with preciously collected media, and separated in to 10 ml aliquots to be stored at -80 ^{0}C.

Antibodies and Reagents

Protesome inhibitors lactocystin, MG132 and LLNL were purchased form Calbiochem, SD208 and chycloheximide. Chycloheximide, and SD208. Antibodies are: GSK-3β (Santa Cruz), Smad3 I-20 (Santa Cruz), Smad3 (Zymed), phospho-Smad2 (Cell signaling), Lamin (Santa Cruz), β-tubulin (Santa Cruz), γ-tubulin (Sigma), p53 (Santa Cruz).

Adenovirus generation

Adenoviral constructs of DVL-ΔPDZ were generated according to the protocol developed by Tong-Chuan He in the Vogelstein group (He et al. 1998b). LEF1ΔC or DVL-ΔPDZ were first cloned into the shuttle vector, pAdtrack-CMV. This was the linearized by digesting with PmeI, and subsequently cotransformed into E. coli. BJ5183 cells with adenoviral backbone plasmid pADEasy-1. Recombinants were selected for kanamycin resistance, and recombination was confirmed by PacI digestion, which released a 3 kb or 4.5 kb small fragment and a 30kb large fragment. Recombinant DNA was retransformed into a general plasmid propagation strain and then purified by CsCl gradient ultracentrifugation. Finally, the linearized recombinant DNA was transfected into an

adenovirus packaging cell line, 293. Viruses were typically generated after 10 days. 293 cells containing virus particles were collected and broken to release virus. Multiple rounds of infection are needed to generate high titer viral stock.

Whole cell lysis and western-blot

Cells cultured in 10cm dish as desired were harvested by trypsin digestion (for MSCs) or scraping (most other cell types). After collection, cells were lyased in an appropriate amount of universal lysis buffer (50mM Tris pH 7.5, 150 mM NaCl, 50 mM NaF, 0.5% NP-40) plus other additives (1 mM PMSF, 1 mM DTT, 0.5 mM Na_3VO_4, and 1X protease inhibitors). Cell lysates were seperated by SDS-PAGE gel and used for western blot analysis.

Cytoplasmic and nuclear fractionation

MSCs were collected by trypsin digestion and washed with PBS one time before addition of cytoplasmic lysis buffer (10 mM Hepes pH 8.0, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 300 mM sucrose, 0.1% NP-40, 10 mM NaF, 20 mM β-glycerophosphate, 10 mM Na_3VO_4, 1X protease inhibitors, 0.5 mM PMSF). Cells were lysed for 10 minutes on ice and then quick spun for 15 seconds to collect cytosolic lysate. Pellets were washed two times with cytoplasmic lysis buffer and then lysed with nuclear lysis buffer (50 mM Hepes pH 7.9, 250 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% NP-40, 0.1% glycerol, 10 mM NaF, 10 mM Na_3VO_4, 1 mM DTT, 1X protease inhibitors, 0.5 mM PMSF) for 30 minutes on ice. The lysates were spun for 20 minutes at 14,000 rpm at 4^0C to collect nuclear lysates. Lysates were seperated in SDS-PAGE for Western blot analysis.

Immunofluoresence staining

MSCs were grown on cover-slips at 500 cells/well in 6-well-plate. Cells were fixed in 3% paraformaldehyde/2% sucrose/ PBS for 10-15 minutes, and washed with PBS three times. Cells were then permeablized with 0.2% Triton-X 100 in PBS for 10 minutes and rinsed three times with PBS. The cover slips were blocked in a drop of PBTN (PBS, 0.1% Triton and 3% normal goat serum) for 20 minutes in a humidified chamber followed by an incubation with a drop of primary antibody diluted in PBTN (generally 1:100) at 4^0C in a wet chamber over night. Cover-slips were then washed three times in PBT (PBS, 0.1% Triton) and incubated in a drop of secondary antibody diluted in PBTN (generally 1:200) at room temperature for 20 minutes. Cover-slips were then washed three times in PBT and 1X Hoechest was included in the last wash to stain the nuclei. After rinsing in PBS, cover-slips were mounted in 90% glycerol/PBS with 1 mg/ml of anti-bleach (p-phenylenediamine, sigma P-1519) and were observed by confocal microscope.

Immunoprecipitation

The MSCs were plated at 10000-20000 cells/plate in a 10 cm plate and then treated with 50 pM TGF-β for 1 and 2 hours the next day. Cells were collected by trypsin digestion and lysed in universal lysis buffer (50mM Tris pH 7.5, 150 mM NaCl, 50 mM NaF, 0.5% NP-40) plus other additives (1 mM PMSF, 1 mM DTT, 0.5 mM Na_3VO_4, and 1X protease inhibitors), and whole cell lysates were collected and used for immunoprecipitation. The desired amount of lysate was load into 1.5 ml tube with 1 μg

of the desired antibodies. Samples were incubated and rotated for 2 hours at 4^0C. 10 µl protein A/protein G sepharose bead mix (1:1) was added into the reaction and rotated for another hour at 4^0C. One antibody/beads/lysis buffer and one beads/lysate reaction were included as controls. The mixture was washed three times with lysis buffer and each time the supernatant was removed gently. After the third wash, 20 µl lysis buffer and 10 µl of 4X western-blot loading buffer were added and the samples were seperated in SDS-PAGE gel and analyzed by western-blot.

Construction of Smad3 RNAi

Smad3 RNAi was generated from shRNA cloned into the pSuper-retro vector (oligoengine). The primers used as shRNA are:

forward,5'GATCCCCGGCCATCACCACGCAGAACTTCAAGAGAGTTCTGCGTGG TGATGGCCTTTTTGGAAA 3'

reverse,5'AGCTTTTCCAAAAAGGCCATCACCACGCAGAACTCTCTTGAAGTTCT GCGTGGTGATGGCCGGG 3'

The annealed primers were cloned into BglII/HindIII sites of pSuper-retro and recombinants were identified by non-BglII digestion and sequencing. Then the correct constructs were co-transfected with retrovirus help vectors into 293T cells to generate retrovirus as described in chapter 2. MSCs infected with retrovirus containing Smad3 shRNA were then selected by 1 µg/ml puromycin. Cells that survived the selection were lysed and analyzed by western-blot for Smad3 protein level.

Research Results

1. TGF-β induces proliferation and inhibits osteogenic differentiation of MSCs

TGF-β potently inhibits the growth of most cell types, including epithelial, endothelial, lymphoid and hematopoietic cells, whereas it stimulates the proliferation of certain mesenchyme-derived cells, such as osteoblasts and MSCs (Borton et al. 2001). It is not clear, however, if this cell context-dependent and unique pro-proliferation activity of TGF-β is extended to bone marrow-derived, plural-potent mesenchymal stem cells, although a few reports suggest it is very likely (Cassiede et al. 1996).

To address this important question, we tested the effect of TGF-β on MSC proliferation using the ^{3}H-thymidine incorporation assay. As shown in Fig. 1A, we found that the proliferation of those primary human MSCs was stimulated by TGF-β. This discovery provides us with a model system of primary cells to study the molecular mechanism of TGF-β-induced proliferation. For further confirmation, we stained MSCs with a mouse monoclonal antibody against Ki-67, a nuclear cell proliferation-associated antigen. This procedure has been widely used to detect cycling cells as a measure of cell proliferation (Kubbutat et al. 1994). As shown in Fig. 1B, more cells stained positively with Ki-67 after treatment with TGF-β, suggesting that TGF-β stimulates the proliferation of MSCs. Fig. 1C is the quantification of results from counting 10 viewing areas under the microscope. This result is consistent with the data of ^{3}H-thymidine incorporation assay shown in Fig. 1A.

To test if TGF-β can also affect the differentiation program of MSCs, we performed the osteogenic assay to measure the production of ALP by culturing the MSCs in the OS media in the presence or absence of 50 nM TGF-β. As shown in Fig. 1D, culturing the MSCs in the OS media without TGF-β led to a significant enhancement in the staining of ALP, as expected for the induced differentiation of those MSCs toward an osteogenic cell fate. In contrast, the presence of TGF-β in the OS media resulted in a much lower level of ALP staining, strongly suggesting that TGF-β inhibits the osteogenic-inductive effect of the OS media and maintains MSCs in an undifferentiated state.

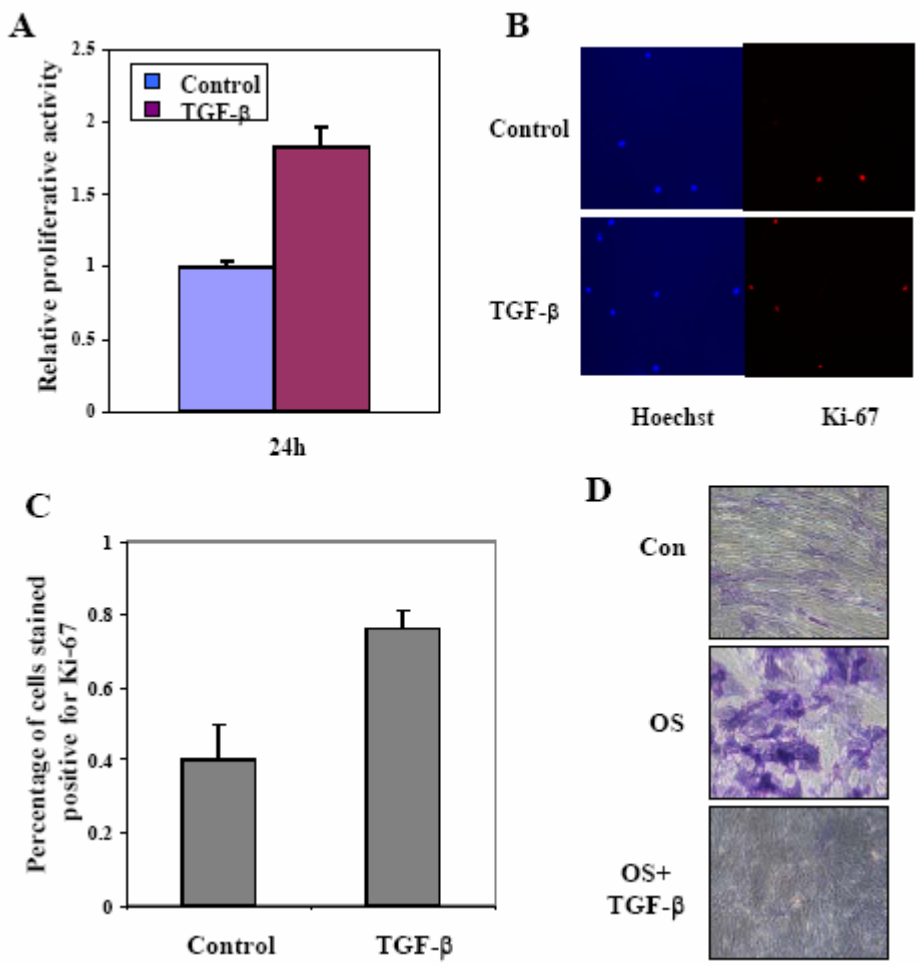

Figure 1. TGF-β induces proliferation and inhibits osteogenic differentiation of human MSCs (A) Proliferation of human MSCs was examined by 3H-thymidine incorporation after the cells were untreated or treated with TGF-β for 24 hours and relative proliferation activities are shown. Error bars were calculated from three duplicate experiments. (B) MSCs were treated with TGF-β for 36 hours before immuno-fluorescence staining with a mouse monoclonal antibody against Ki-67 (BD PharMingen). Hoechst staining was used to detect nuclei. (C) Quantification of Ki-67 positive nuclei counted from 10 viewing areas under the microscope. (D) Alkaline phosphatase activity was probed as a readout for osteogenic differentiation after MSCs were cultured in osteogenic supplementary media in the absence or presence of TGF-β for 10 days.

2. Wnt induces proliferation and inhibits osteogenic differentiation of MSCs

Since MSCs located in the same growth environment (bone marrow) as hematopoitic stem cells (HSCs), it is likely that these two types of plural-potent cells may be subject to regulation by similar groups of cytokines. Because Wnt signaling pathway has been reported to be involved in the stimulation of proliferation and inhibition of differentiation of HSCs, we reasoned that Wnt could have a similar effect on the activities of MSCs. Using RT-PCR, we examined the expression pattern of Wnt family members by the MSCs and found that these cells do indeed express at least four different Wnt genes.

Since it is difficult to obtain biologically active recombinant Wnt protein for in vitro assays, we tested our hypothesis through the use of mutant forms of two Wnt pathway components, Frizzled, the receptor for Wnt, and β-catenin, the effector for Wnt signaling. Fz8CRD is a truncated form of Frizzled 8 that contains only the Wnt ligand-association domain, the CRD domain, and has no transmembrane domain (Fig. 2-1A). In this form, Fz8CRD functions as a competitive inhibitor of Wnt signaling by sequestering Wnt proteins and consequently blocking the activation of the pathway (Hsieh et al. 1999). The β-catenin mutant, contains amino acid substitutions at the four phosphorylation sites at its N-terminus and thus can not be phosphorylated by the kinase GSK-3β (Barth et al. 1999) (Fig. 2-2A). As a result, this mutant form of β-catenin is stabilized and remains constitutively active (Fig. 2-2B).

To test if Wnt activity is required for the maintenance of MSC proliferation, we incubated the cells with conditioned media containing Fz8CRD. The presence of Fz8CRD in the media was confirmed by western-blot (Fig. 2-1B). As shown in Fig. 2-2C, the blockage in autocrine Wnt signaling resulted in a significant reduction in cell proliferation of MSCs. Consistent with the anti-proliferative effect of blocking Wnt signaling, MSCs treated with Fz8CRD displayed a more differentiated phenotype, as indicated by the increased staining of ALP, a marker for differentiation into osteoblasts (Fig. 2-1D). As an alternative approach to test the same hypothesis, we introduced the mutant β-catenin into the MSCs by retroviral infection. Subsequently, the proliferation and differentiation characteristics of the MSCs stably expressing the mutant β-catenin were compared to those of the vector control-infected cells. As shown in Fig. 2-2C and 2-2D, the presence of a higher level of β-catenin in the MSCs led to a higher proliferation rate and a reduced ability to differentiate into cells of osteogenic lineage. Taken together, these data strongly suggest that the Wnt signaling pathway exerts the same pro-proliferation and anti-differentiation effects on MSCs as on HSCs (Reya et al. 2003).

These results indicate that the activities of MSCs are likely regulated by the Wnt family of proteins, possibly through an autocrine mechanism. In the following chapters, we will test if the presence of this autocrine system for Wnt signaling contributes to the unique response and signaling mechanism of TGF-β in MSCs.

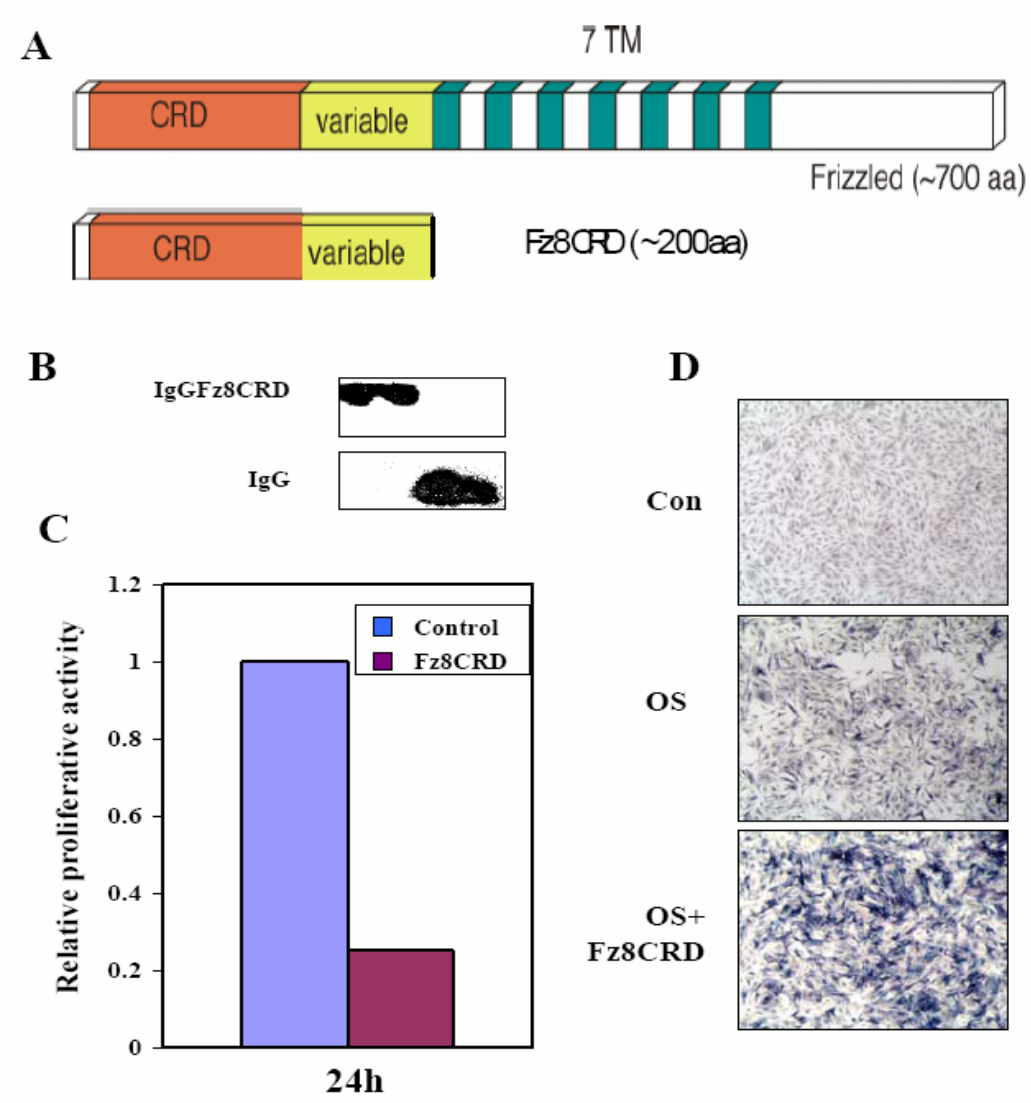

Figure 2-1. The effects of Fz8CRD on proliferation and osteogenic differentiation of human MSCs. (A) Schematic structure of Frizzled and Fz8CRD. (B) Expression of Fz8CRD. pRK5- IgG-Fz8CRD or pRK5-IgG were transiently transfected into 293T cells and Fz8CRD conditioned and control media were collected three days later. Protein expression was detected by anti-IgG antibody (C) ^{3}H thymidine incorporation was performed after cells cultured in Fz8CRD conditioned media for 24 hours and fold induction is shown. (D) Cells were cultured in OS media or OS plus Fz8CRD media for 10 days. Osteogenic differentiation was determined by alkline phosphatase (ALP) staining.

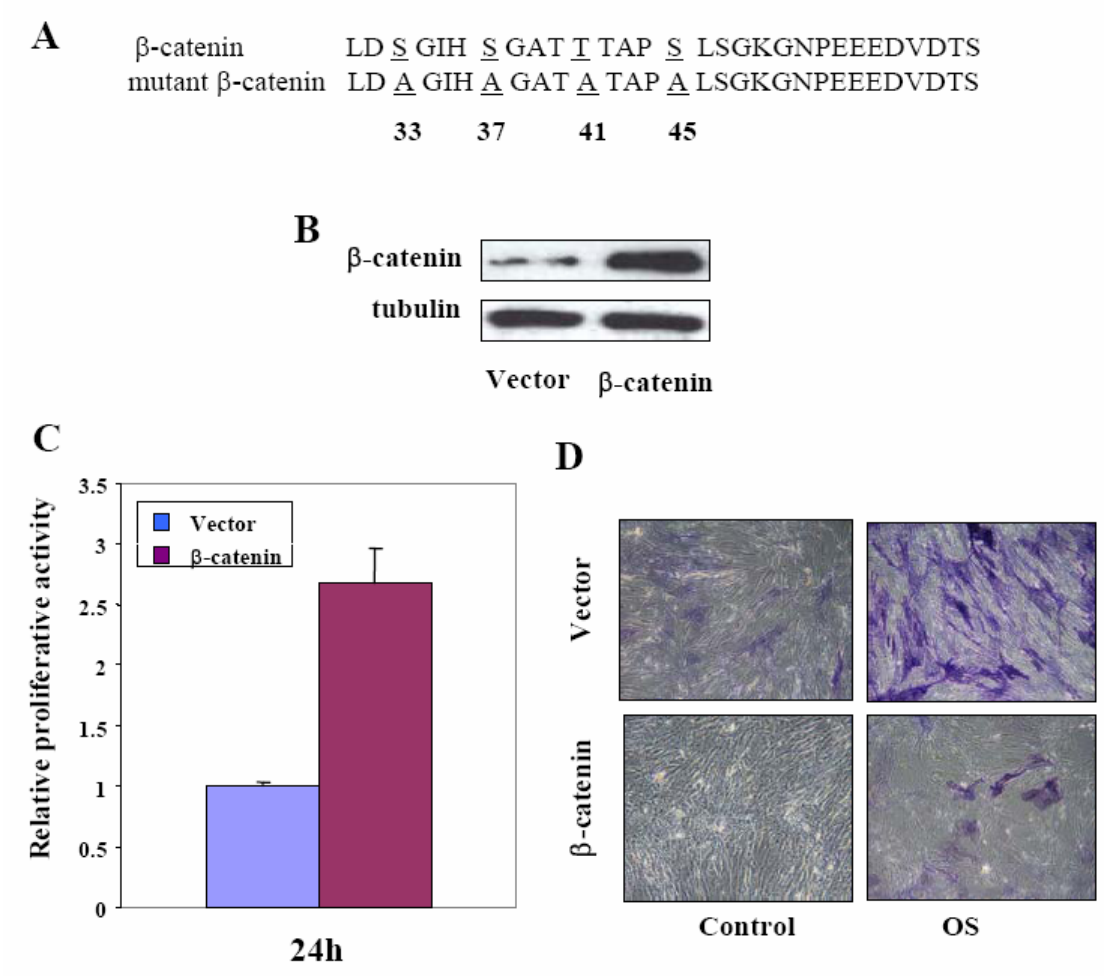

Figure 2-2. Constant active β-catenin induces proliferation and inhibits osteogenic differentiation of human MSCs (A) Mutant sites of β-catenin. The four serine/threoines were replaced by alanine. (B) MSCs were infected with MSCV-mutant β-catenin or vector control retroviruses. Ectopic expression of the mutant β-catenin is shown. (C) Proliferation of MSCs infected with the mutant β-catenin or vector control retroviruses was examined by 3H-thymidine incorporation. Relative proliferation activities are shown and error bars were calculated as standard deviation from three duplicate experiments. (D) Alkaline phosphatase activity was probed for osteogenic differentiation after MSCs were infected with the mutant β-catenin or vector control retroviruses and cultured in osteogenic supplementary media for 10 days.

3. TGF-β induces nuclear translocation of β-catenin in Mesenchymal stem cells

Based on our observations that TGF-β, just as Wnt does, stimulates the proliferation and inhibits the differentiation of MSCs, we hypothesized that a common signaling pathway may be utilized by the two unrelated stimuli. In fact, the fundamental question of the molecular mechanism by which TGF-β stimulate rather than inhibit the proliferation of certain mesenchyme-derived cells has for a long time remained a major challenge in the TGF-β field. Using the MSC model system, we tested if components of the Wnt signaling pathway, such as β-catenin, could serve as mediators of the TGF-β pro-proliferation signal in MSCs.

To do this, we stimulated primary human MSCs that were derived from adult human bone marrow with either Wnt3A or TGF-β. In our initial experiments, we took the approach of biochemical fractionation to separate the nuclear and cytoplasmic fractions of cells treated with TGF-β or Wnt-containing conditioned media. As shown in Fig. 3-1A, a significant amount of β-catenin appeared in the nucleus of MSCs after 2 hrs incubation with Wnt3A-conditioned medium as determined by nuclear/cytoplasmic fractionation. To our surprise, we found that TGF-β was also capable of inducing the nuclear translocation of β-catenin in a manner similar to Wnt3A treatment, since an increasing amount of β-catenin was detected in the nuclear fraction 1 to 2 hr after the cells were treated with TGF-β (Fig. 3A). To verify this highly intriguing result, we used immunofluorescence imaging to directly visualize the localization of β-catenin. As shown in Fig. 3B, the nuclear staining of endogenous β-catenin was significantly increased in MSCs 1 hr after treatment with TGF-β, confirming the data from the fractionation experiments. When MSCs were plated at a low cell density to maintain their undifferentiated state, strong staining of β-catenin in the nucleus was detected in more than 90% of the cells following treatment with TGF-β.

Importantly, β-catenin accumulation in the nucleus was rapid in response to TGF-β treatment, suggesting that the TGF-β-induced β-catenin nuclear translocation in MSCs is likely to be mechanistically distinct from that of the slow accumulation of β-catenin in the nucleus in response to TGF-β as previously reported in the context of chondrogenesis of mesenchymal stem cells (Tuli et al. 2003; Zhou et al. 2004). It is hypothesized that during chondrogenesis, TGF-β upregulates Wnt protein expression, which subsequently leads to stabilization and translocation of β-catenin into the nucleus. Our follow-up studies suggest that TGF-β-induced β-catenin nuclear translocation in MSCs occurs through a completely different mechanism.

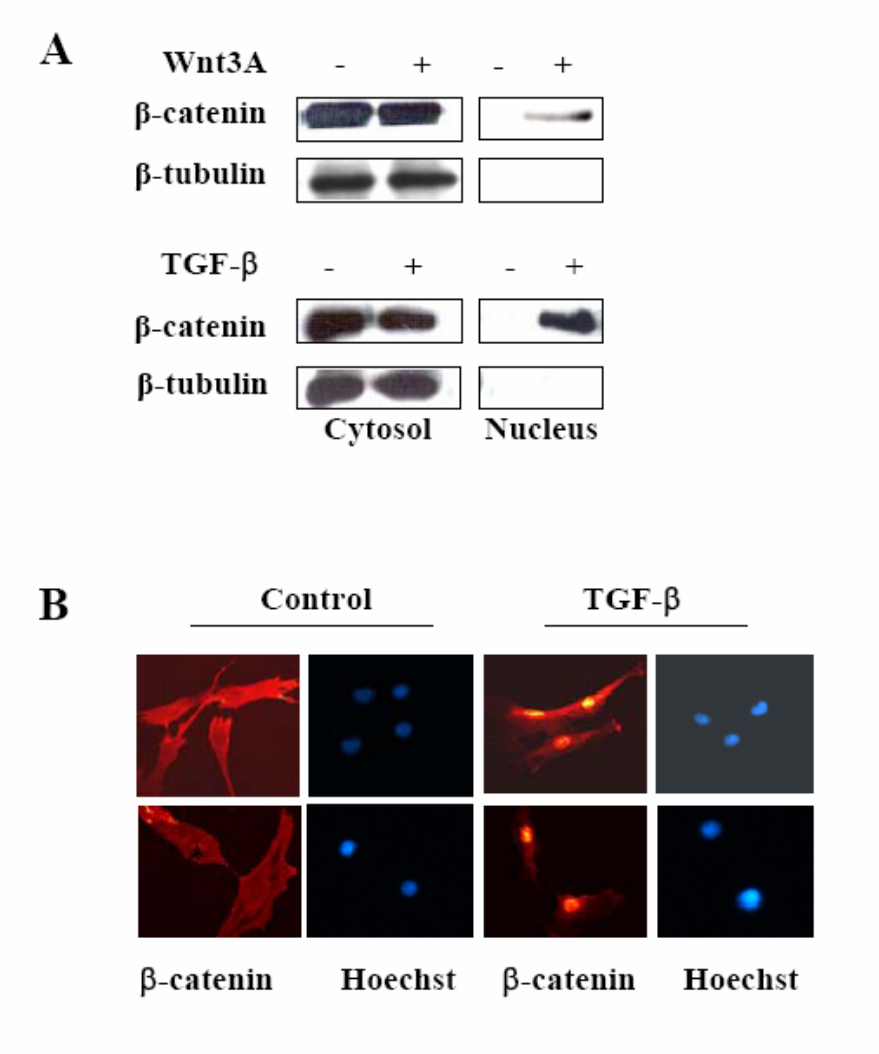

Figure 3. TGF-β induces nuclear translocation of β-catenin in human MSCs (A) Cytosolic and nuclear fractions of protein lysates were isolated from human MSCs treated or untreated with TGF-β for 2 hours or Wnt3A for 6 hours. Western blot was probed with an anti-β-catenin monoclonal antibody. (B) β-catenin localization was detected by immunofluorescence with the anti-β-catenin antibody after cells were treated or untreated with TGF-β for 1 hour.

4. TGF-β induces β-catenin nuclear translocation without affecting the steady-state protein level of β-catenin

Wnt-induced nuclear accumulation of β-catenin has been established in multiple cellular systems as the consequence of β-catenin stabilization (Orford et al. 1997). To determine if TGF-β induces β-catenin nuclear translocation via a similar mechanism, we measured the steady-state protein levels of β-catenin in MSCs in the presence or absence of TGF-β. Interestingly, no change in the levels of β-catenin was observed after the MSCs were treated with TGF-β for 24 hrs (Fig. 4A), suggesting that β-catenin nuclear translocation in response to TGF-β is not mediated by a significant change in the stability of β-catenin in MSCs. As a control, Wnt3A treatment still induced an increase in β-catenin protein levels in this cellular context (Fig. 4A). To explore this question further, we tested whether the stability of β-catenin was regulated by the ubiquitin/proteasome-mediated protein degradation pathway in MSCs. As shown in Fig. 4B, the steady-state levels of β-catenin in MSCs were not affected by the presence of two different proteasome inhibitors, lactacystin and MG132, as indicated by the lack of higher molecular weight (HMW) β-catenin species that likely represent polyubiquitinated β-catenin. In contrast, the HMW β-catenin was readily detected in the presence of these inhibitors in C57MG cells (Fig. 4B). Importantly, treatment of MSCs with the same proteasome inhibitors dramatically increased the level of p53 protein, suggesting that the proteasome-mediated protein degradation pathway is intact in these cells (Fig. 4C and 4D). In addition, following treatment of MSCs with TGF-β, we could not detect any β-catenin that was phosphorylated at several regulatory sites known to be involved in the modulation of its stability by using the corresponding phospho-specific antibodies (Liu & He, 2002) (Fig. 4E). Taken together, these results suggest that β-catenin nuclear translocation in response to TGF-β is not mediated by a significant change in the stability of β-catenin, but rather by a novel mechanism that may be specific to MSCs.

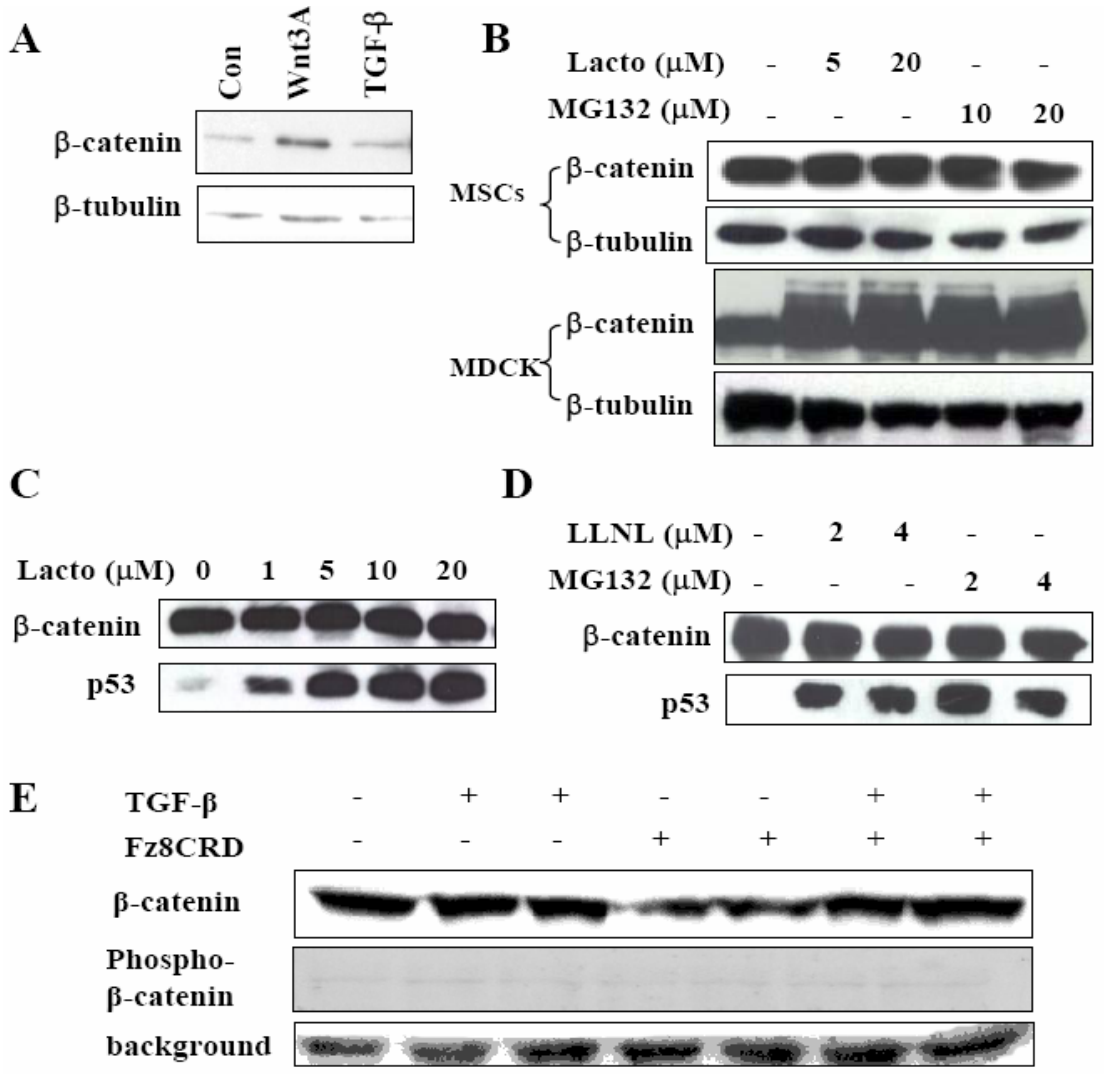

Figure 4. TGF-β induces β-catenin nuclear translocation without affecting the steady state protein level of β-catenin (A) Total cell lysates were prepared from MSCs after treatment with TGF-β or Wnt3A for 24 hours. Western blots were probed with anti-β-catenin antibody. (B) Whole cell lysates were prepared from MSCs and C57MG cells after the cells were treated with proteosome inhibitors lacatocystin or MG132 for 6 hours. (C) and (D) Whole cell lysates were prepared from MSCs after treated with proteosome inhibitors lacatocystin, MG132 or LLNL for 6 hours. Western blot was performed to detect protein levels of β-catenin. p53 was used as the internal control for MSCs. The concentrations for proteosome inhibitors were indicated in μM. Lacto represents Lactocystin. (E) Total cell lysates of MSCs treated with TGF-β or Fz8CRD for 6 hours as indicated were probed with phospho-β-catenin antibody. Background band is loading control.

5. β-catenin nuclear translocation may be mediated by Smad3

Smad3 is the most important intracellular transducer of TGF-β signaling and it has been reported to cross-talk with the Wnt signaling pathway through Axin or TCF/LEF. To further explore the mechanism, we investigated whether Smad3 is directly involved in the process of β-catenin nuclear translocation. To do this, we evaluated the effect of Smad3 knockdown on the ability of TGF-β to induce β-catenin nuclear translocation. By introducing a Smad3-specific siRNA construct into MSCs using retroviral infection, the expression of Smad3 protein was reduced by more than 90% (Fig. 5A). Subsequently, we examined β-catenin nuclear translocation in response to TGF-β in MSCs stably expressing the Smad3-siRNA in comparison to those infected with a retroviral vector control. As shown in Fig. 5B, the cell fractionation results clearly indicate that Smad3 is required for the TGF-β-induced nuclear translocation of β-catenin, since the amount of β-catenin in the nuclear fraction was barely detectable in MSCs with reduced expression of Smad3. Importantly, under the same conditions, Wnt3A treatment could still increase the level of nuclear β-catenin (Fig. 5C), suggesting that the mechanism of Wnt3A-induced β-catenin nuclear accumulation in MSCs is distinct from that of TGF-β-induced β-catenin nuclear translocation.

The requirement of Smad3 for β-catenin nuclear translocation suggests that Smad3 may actively transport β-catenin into the nucleus. Previous reports have shown that Smad3 can interact with β-catenin and its binding partners Axin and CKIε (Labbe et al. 2000; Furuhashi et al. 2001; Waddell et al. 2004). Thus, it is possible that Smad3 and β-catenin co-exist in a complex in the cytoplasm, then translocate into the nucleus simultaneously upon TGF-β stimulation. Consistent with this postulation, we found that Smad3 and β-catenin were indeed in the same complex in MSCs, since endogenous β-catenin and Smad3 could be co-immunoprecipitated by an anti-Smad3 antibody and this interaction was minimally affected by TGF-β (Fig. 5C). This result has prompted us to propose that Smad3 may act as a chaperone for the nuclear translocation of β-catenin, because it is well established that after ligand-induced phosphorylation, Smad3 forms a complex with Smad4 and translocates into the nucleus. Thus, the constitutive interaction between Smad3 and β-catenin may allow β-catenin to be shuffled into nucleus following TGF-β stimulation of MSCs.

Furthermore, endogenous GSK-3β was immunoprecipitated by an anti-Smad3 antibody, suggesting that GSK-3β interacts with Smad3 in MSCs under physiological conditions. Interestingly, this association is diminished with TGF-β treatment for two hours (Fig. 5D). Attempts to detect association between Smad3 and Axin or CKIα were unsuccessful, possibly because of the low levels of endogenous Axin or CKIα. Nevertheless, taken together with previous findings that both Smad3 and β-catenin can interact with Axin/CKIα and that association between Smad3 and Axin/CKIε decreases in response to TGF-β in cell types other than MSCs (Furuhashi et al. 2001; Waddell et al. 2004), these results support a model in which the nuclear translocation of β-catenin in response to TGF-β can be directly linked to changes in the composition and dynamics of a protein complex possibly containing β-catenin, Smad3, GSK-3β, Axin and CKIε.

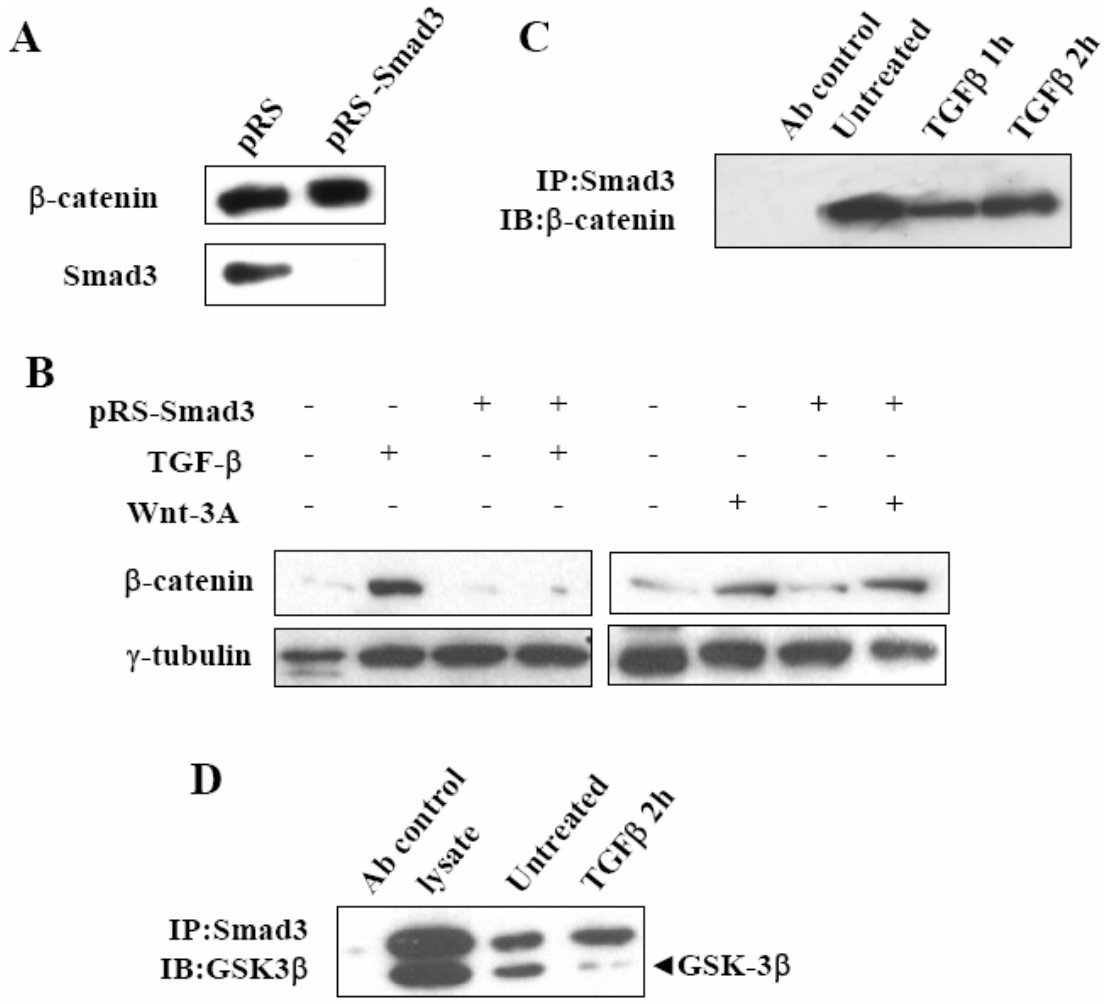

Figure 5. β-catenin nuclear translocation may be mediated by Smad3 (A) MSCs were infected with control (pRS) or Smad3 siRNA (pRS-Smad3) expressing retrovirus and selected by puromycin. Whole cell lysates from stable cell populations were blotted with an anti-Smad3 rabbit polyclonal antibody. Equal loading was confirmed by the presence of β-catenin. (B) Nuclear fractions from pRS or pRS-Smad3 retroviruse-infected MSCs untreated or treated with TGF-β for 2 hours or Wnt3A for 6 hours were blotted with the anti-β-catenin antibody. (C) Whole cell lysates from MSCs untreated or treated with TGF-β for 1 or 2 hours were subjectedto immunoprecipitation with an anti-Smad3 polyclonal antibody. An anti-Flag antibody was used as the Ab control for the immunoprecipitation. The Western blots were carried out using antibodies against β-catenin. (D) Immunoprecipitation was performed as in (C) with lysates from MSCs untreated or treated with TGF-β for 2 hours. GSK-3β was detected by a mouse monoclonal antibody. IP: Immunoprecipitation, IB: Immunoblotting.

6. LEFΔC acts as a dominant-negative inhibitor by retaining β-catenin in the cytosol

In previous results, we have described the biological function of TGF-β in MSCs. TGF-β stimulates the proliferation of MSCs, an activity that is in contrast to the potent anti-proliferative effect of TGF-β on many other cell types (Massague 1998) (Fig. 1A). TGF-β also affects the differentiation program of these MSCs, measured by the production of alkaline phosphatase (ALP) (Fig. 1D). In this chapter, we tested the postulation that nuclear translocation of β-catenin is directly linked to the TGF-β-mediated regulation of proliferation and osteogenic differentiation of MSCs. To begin, we evaluated the effects of nuclear accumulation of β-catenin on the activities of MSCs by the introduction of a mutant form of β-catenin into those cells via retroviral infection. Interestingly, we found that the mutant β-catenin we used almost completely localized in the nucleus of MSCs, in contrast to the previously reported predominant cell-cell junction localization of this mutant at the plasma membrane (Barth et al. 1999). Similar to the proliferative effect of TGF-β, expression of this β-catenin mutant in MSCs leads to a significant increase in proliferation when compared to control cells (Fig. 2-2C). Similarly consistent with the anti-osteogenic effect of TGF-β in MSCs, expression of this β-catenin mutant caused a substantial decrease in ALP staining (Fig. 2-2D). These results suggest that nuclear-localized β-catenin can exert biological effects on MSCs similar to those induced by TGF-β, strongly supporting the postulation that there is a direct correlation between the activation of the Smad3/β-catenin-mediated TGF-β signaling pathway and the elicitation of unique biological responses in MSCs.

To further test this hypothesis, we next attempted to reduce the expression of β-catenin by employing the siRNA strategy that was reported in a previous study (van de Wetering et al. 2003). However, this approach was unsuccessful due to the relatively high stability of β-catenin protein in MSCs (Fig. 4). Consequently, we resorted to the use of a C-terminal truncation mutant of LEF1, LEF1ΔC, which lacks the HMG box and the nuclear localization sequence B box (Prieve et al. 1996) (Fig. 6A). As a transcription factor, the wild type LEF1 is known to reside on specific promoter sequences of Wnt-responsive genes and form a complex with β-catenin via a domain located in the N-terminal region of LEF1 to activate transcription after β-catenin translocates into the nucleus (Logan and Nusse 2004). In addition, the HMG box of LEF1 was shown to mediate the interaction between LEF1 and Smad3 in the context of synergistic activation of specific target genes by the two transcription factors (Labbe et al. 2000). Thus, this mutant form of LEF1 was expected to retain β-catenin in the cytoplasm since its ability to interact with β-catenin through its N-terminal region should be unaltered, consequently acting as a dominant-negative inhibitor that prevents the nuclear translocation of β-catenin without affecting the movement of Smad3.

Consistent with this prediction, LEF1ΔC maintained its ability to interact with β-catenin in a a manner comparable to wild type LEF1, but was unable to associate with Smad3 (Fig. 6B and 6C). When LEF1ΔC was introduced into MSCs via an adenoviral delivery system (He et al. 1998b), its expression was found to be mainly restricted to the cytoplasm (Fig. 6D). In the presence of this mutant, TGF-β was unable to induce β-catenin translocation to the nucleus, in contrast to what was seen in cells infected with the control adenovirus (Fig. 6E), suggesting that LEF1ΔC does act as a dominant-negative

inhibitor of β-catenin nuclear translocation as we expected.

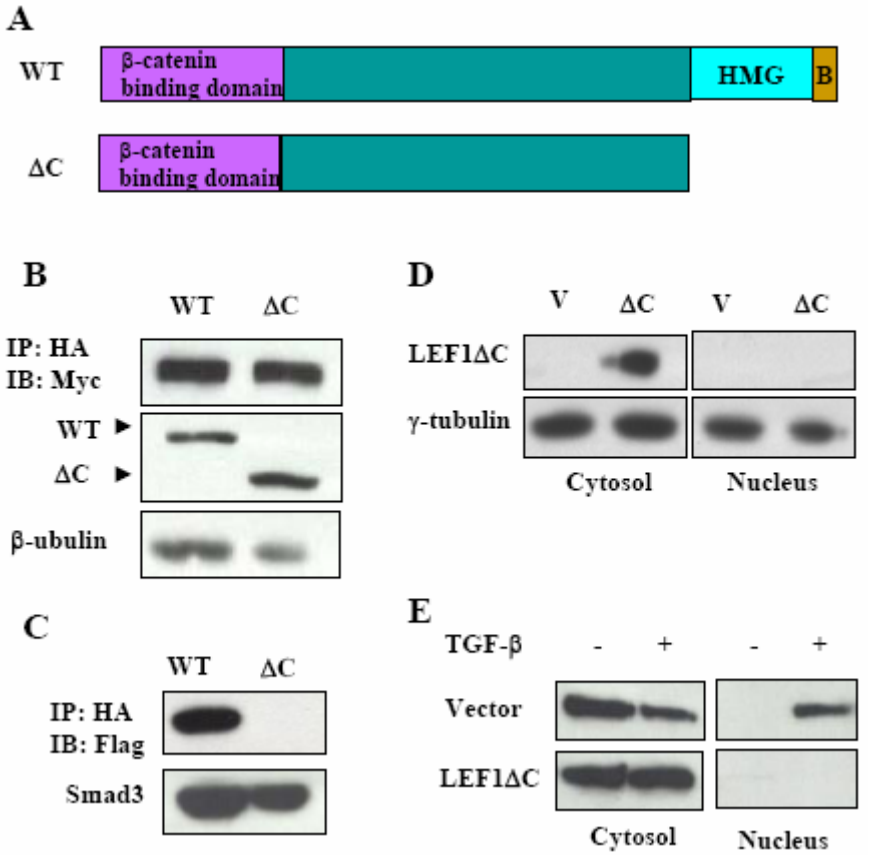

Figure 6. LEF1ΔC is a dominant negative inhibitor by retaining β-catenin in cytosol
(A) Schematic structure of wild-type LEF1 and LEF1ΔC. WT: Wild Type. ΔC: LEF1ΔC. (B) 293T cells were co-transfected with pcDNA3-Myc-β-catenin and pcDNA3-HA-LEF1 or pcDNA3-HA-LEF1ΔC, and co-immunoprecipitations were performed with anti-HA and immunoblotted with anti-Myc. (C) 293T cells were co-transfected with pRK5-Flag-Smad3 and pcDNA3-HA-LEF1 or pcDNA3-HA-LEF1ΔC, and co-immunoprecipitations were performed with anti-HA and immunoblotted with anti-Flag. (D) MSCs were infected with vector control or LEF1ΔC adenoviruses. Ectopic expression of LEF1ΔC was detected with an antiHA antibody. (E) Cytosolic and nuclear fraction of protein lysates were isolated from LEF1ΔC or vector control adenovirus-infected MSCs untreated or treated with TGF-β for 2 hours. Western blots were performed with anti-β-catenin antibody or anti-HA antibody.

7. LEF1ΔC inhibits the primary effects of TGF-β on human MSCs

To determine whether retention of β-catenin in the cytosol has any negative effect on TGF-β signaling, we examined the biological effects of TGF-β on LEF1ΔC-infected and vector control MSCs. As shown in Fig. 8A, TGF-β-induced proliferation was significantly inhibited by the expression of LEF1ΔC in MSCs, strongly suggesting that the stimulatory effect of TGF-β on MSC proliferation is mediated by this newly defined signaling pathway via induction of β-catenin translocation into the nucleus. Interestingly, the basal level of cell proliferation of MSCs was also reduced by the expression of LEF1ΔC (Fig. 8A), implicating the possible presence of an autocrine Wnt and/or TGF-β signaling pathway that functions to maintain proliferation of MSCs in culture. Additionally, we found that expression of LEF1ΔC eliminated the inhibitory effect of TGF-β on osteogenic differentiation of MSCs (Fig. 8B), further supporting the notion that rapid nuclear translocation of β-catenin is required for TGF-β to exert its primary biological effects on MSCs.

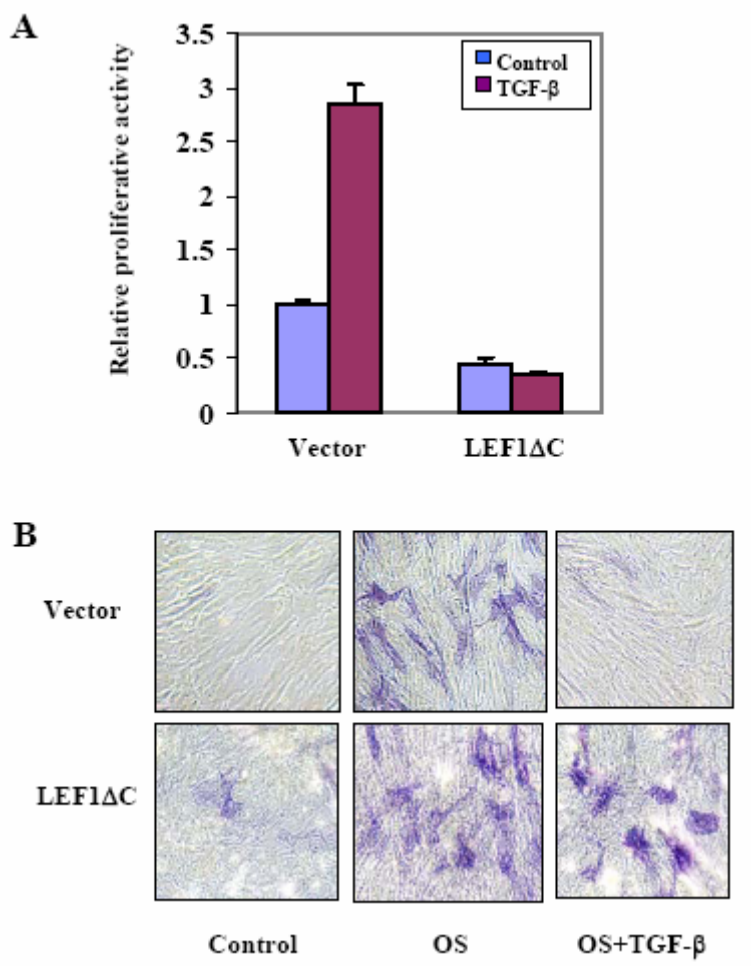

Figure 7. LEF1ΔC inhibits primary effects of TGF-β on human MSCs (A) Proliferation of human MSCs infected with the LEF1ΔC or the vector control adenoviruses was examined by 3H-thymidine incorporation after the cells were untreated or treated with TGF-β for 24 hours. Relative proliferation activities are shown and error bars were calculated for standard deviation from three duplicate experiments. (B) Alkaline phosphatase activity was probed for osteogenic differentiation after MSCs were infected with LEF1ΔC or the vector control adenoviruses and cultured in osteogenic supplementary media for 5 days.

Key Research Accomplishments

1. Identified a novel form of cross-talk of TGF-β and Wnt signaling pathways.

2. This novel cross-talk of these two pathways exerts important effects on self-renewal and differentiation of human Mesenchymal Stem Cells

3. This novel cross-talk provide a new sight towards understanding the proliferation stimulatory effects of TGF-β signaling pathway in some specific cells

Reportable Outcomes

1. A Ph.D. student graduated with support of this grant

2. A manuscript has been published.

Hongyan Jian, Xing Shen, Irwin Liu, Mikhail Semenov, Xi He and Xiao-Fan Wang. 2006. *Smad3-Dependent Nuclear Translocation of β-catenin Is Required for TGF-β1-Induced Proliferation of Bone Marrow-Derived Adult Human Mesenchymal Stem Cells*. Genes and Development. 20: 666-674

Conclusions

In this report, the functional role of a novel form of crosstalk between the TGF-β and Wnt signaling pathways in regulating human MSCs was investigated. Initially, TGF-β and Wnt were shown to exert similar effects on MSCs, suggesting that a cross-talk between these two signaling pathways may exist in control of proliferation and differentiation of these cells. In addition, it was found that TGF-β induced rapid nuclear translocation of β-catenin in MSCs in a Wnt signaling-independent fashion. The stability of β-catenin is not affected by TGF-β and nuclear localization of β-catenin does not need Wnt ligands and Frizzled receptor, but requires the activity of the TGF-β type I receptor and the presence of Smad3. Functionally, TGF-β-induced β-catenin nuclear translocation is required for the pro-proliferation and anti-osteogenic differentiation effects on MSCs by TGF-β, likely through the combined actions of β-catenin and Smad3 to regulate downstream target genes. These results provide evidence for a novel mode of cooperation between the TGF-β and Wnt signaling pathways in this specific cellular context, and suggest a potentially important role for this distinct signaling pathway in the control of self-renewal and differentiation of mesenchymal stem cells.

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Appendix

Biography

Hongyan Jian

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