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Wnt3–Frizzled 1 Chimera as a Model to Study Canonical Wnt Signaling

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ABSTRACT

Wnt proteins initiate signaling by binding to seven transmembrane spanning receptors of the frizzled (Fz) family together with the members of the low-density lipoprotein receptor-related protein (LRP) 5 and 6. A chimera of human Wnt3 and Fz1 receptor was developed that efficiently activated the TCF-luciferase reporter. Deletion of the cytoplasmic tail and point mutations in the PDZ binding region in the chimera resulted in the loss of Wnt signaling, suggesting a critical role for the Fz cytoplasmic region in Wnt signaling. The Fz CRD is also critical for Wnt signaling, as a deletion of 29 amino acids in the 2nd cysteine loop resulted in the total loss of TCF-luciferase activation. DKK-1 protein blocks upregulation of the TCF-luciferase reporter by the Wnt3–Fz1 chimera, suggesting involvement of LRP in Wnt3–Fz1 signaling. Expression of a Wnt3–Fz1 chimera in C3H10T1/2 cells resulted in the upregulation of alkaline phosphatase activity and inhibition of adipocyte formation, demonstrating that the Wnt3–Fz1 chimera is a potent activator of differentiation of C3H10T1/2 cells into osteoblasts and an inhibitor of their differentiation into the adipocyte lineage. In summary, the Wnt–Fz chimera approach has the potential to better our understanding of the mechanism of Wnt action and its role, particularly in stem cell differentiation. In addition, this methodology can be utilized to identify inhibitors of either Wnt, Fz or interactors of the canonical pathway, which may have potential therapeutic value in the treatment of cancers and other diseases. J. Cell. Biochem. 109: 876–884, 2010.

KEY WORDS: Wnt3–Fz1 CHIMERA; Wnt3; Fz1; STEM CELL DIFFERENTIATION; OSTEOBLAST; ADIPOCYTE

Wnts are secreted glycoproteins that play an essential role during animal development, as well as in the maintenance of tissues [Wodarz and Nusse, 1998; Huelsken and Birchmeier, 2001; Miller, 2002]. These proteins initiate signaling by binding to members of the Frizzled (Fz) family of seven transmembrane spanning receptors and a low-density lipoprotein-related protein (LRP) [Huelsken and Birchmeier, 2001; Miller, 2002]. Receptor activation leads to one of several signaling pathways depending upon the Wnt, Fz and the cell type involved. The canonical, or Wnt/β-catenin, pathway starts with the activation of disheveled (Dsh) that leads to the inhibition of glycogen synthase kinase-3β and subsequent stabilization of β-catenin. β-catenin is then translocated to the nucleus and, along with the T-cell factor/lymphoid enhancer factor (TCF/LEF) and other transcription factors, interacts with the target genes and regulates their transcription [Wodarz and Nusse, 1998; Huelsken and Birchmeier, 2001; Sharpe et al., 2001; Miller, 2002]. Wnt also activates noncanonical pathways, which include the G protein-mediated or Wnt/calcium pathway [Kuhl et al., 2000] and the Dsh-mediated c-JunNH2-terminal kinase pathway [Huelsken and Birchmeier, 2001; Miller, 2002; Weston and Davis, 2002].

The interaction of Wnt and its receptors is highly regulated. A wide variety of secreted proteins have been shown to modulate Wnt signaling by interacting with either Wnt or Fz. These include secreted Fz-related proteins (sFRPs), Wnt inhibitory factors (WIF), and Cerebrus that bind Wnts [Kawano and Kypta, 2003]. Members of the Dickkopf (DKK) family modulate Wnt signaling by interacting with both LRP and kremen receptors [Glinka et al., 1998].

The role of Wnt signaling has been established using several biological systems including genetic studies. These studies have shown that Wnt signaling directs cell fate determination by mediating the activity of organizers during embryonic development and influences morphogenesis by functioning to establish the planar cell polarity [Peifer and Polakis, 2000]. Wnts also play important roles in the development of several tissues and organs, including the reproductive tract, limbs, central nervous system, mammary gland, brain and kidney [Smalley and Dale, 1999]. In addition, alteration in the Wnt pathway that results in inappropriate activation has been described in a variety of human tumors [Polakis, 2000]. More recently, the Wnt signaling has been linked to the regulation of bone mineral density [Boyden et al., 2002; Bodine et al., 2004].

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A fusion protein of Wnt and Fz has been used to examine Wnt signaling [Holmen et al., 2002, 2005; Povelones and Nusse, 2002; Cong et al., 2004]. In the present study, we show that a Wnt3–Fz1 chimera of human origin is highly efficient in activating a TCF-luciferase reporter and has the potential to be a model to study the canonical Wnt pathway. The Wnt3–Fz1 chimera fails to activate cellular Fz receptors but acts intermolecularly, with the cellular response attributed primarily to Wnt3–Fz1 chimera function. The chimera requires an intact Fz CRD, as well as interaction with LRP for its signaling. Utilizing an adenovirus recombinant expressing the Wnt3–Fz1 chimera, we demonstrate the importance of canonical Wnt signaling in the differentiation of stem cells into the osteoblast lineage. The Wnt–Fz chimera approach has the potential to better our understanding of the mechanism of Wnt action and its role, particularly in stem cell differentiation. In addition, this methodology can be utilized to identify inhibitors of either Wnt, Fz or interactors of the canonical pathway, which may have potential therapeutic value in the treatment of cancers and other diseases.

MATERIALS AND METHODS

ISOLATION OF Wnt3–Fz1 CHIMERA PLASMID

The Wnt3–Fz1 chimera contains N-terminal Wnt3 with a HA tag, a 32 amino acid glycine spacer and a mature Fz1 receptor sequence. The plasmid was generated in steps, as follows: The glycine spacer was first synthesized using PCR amplification of a purified oligo with the sequence ACCGGTACCGGGCCCGGAGGCGGGGGCGGA-GGGGGCGGCGGGGGCGGAGGGGGCTCCACCGGTCCCGAATTCAAA with the following primer pairs: 5′-ACCGGTACCGGGCCCGGA 3′-TTTGAATTCGGGACCGGTGGATCCCCCT and 5′-ACCAGATCTGGG-CCCGGAGGC and 3′ primer same as above. The purified PCR products were digested with ASP718 and *Bam*HI and *Bgl*II and *Eco*RI, respectively, and the gel-purified fragments were ligated into Asp718 and *Eco*RI site of pcDNA3.1(+) vector. The Wnt3 ORF was PCR amplified using 5′ primer ATAGCTAGCCCACCATGGAGCCC-CACCTGCTCGGGCTG and 3′ primer TTTCTAGAGTTAAAGCT-TAGGCCCTGGACCCAAAGAAG and using WN3-HA tagged plasmid as the template (Upstate Biotechnology, Lake Placid, NY). The purified PCR product was digested with *Nhe*I and *Hin*dIII and cloned into corresponding sites in the glycine spacer plasmid. The Fz1 ORF without the signal sequence was amplified from Fz1 plasmid using the following primers: 5′-TAAACCGGTGGGC-CAGGCCAGGGGC and 3′ primer-GGGCCCTCTAGACTCGAGTCA-GAC. The purified PCR amplified product was digested with *Age*I and *Xba*I and was cloned into the corresponding sites in the Wnt3–glycine spacer plasmid to generate the Wnt3–Fz1 chimera plasmid.

The Wnt3–Fz1 chimera with deletion of the cytoplasmic region was generated by PCR amplification of the carboxyl region of Fz1 with the following primers: 5′-GATGGATCCAAGACCGA-GAAGCTGGA and 3′ primer ATTTCTAGAATTAGGGTGACCA-GATCCCAGAAGCCCGAC and cloning the *Fsp*I and *Xba*I fragment into the corresponding site of the Wnt3–Fz1 chimera plasmid. The deletion plasmid has a proline residue in place of glycine and has a unique *Bst*EII restriction site, which was subsequently used in the isolation of cytoplasmic mutants of Wnt3–Fz1 chimera. The mutations within the cytoplasmic portion of Fz1 were generated by PCR amplification of the cytoplasmic region with 5′ PCR primers with *Bst*EII restriction site and desired changes in the codon sequence for amino acid changes and the 3′ primer with the *Xba*I site and cloning the PCR amplified product into *Bst*EII and *Xba*I site of Wnt3–Fz1 del cytoplasmic plasmid. The schematics of the Wnt3–Fz1 chimera and its mutant are shown in Figure 1.

MUTATIONS IN THE CRD DOMAIN OF Wnt3–Fz1 CHIMERA

A unique *Eco*RV site in the 2nd cysteine loop of the CRD domain was generated by PCR amplification of the CRD domain using the following primers: 5′ primer GGAGGGGGATCCACCGGTGGGCCA and 3′ primer CGCGATATCCGTGCACAGCGGGAT and 5′-CTGTG-CACGGATATCGCGTAC and 3′-CCGGGTAGCTGAAGCGCCGCATG primers. The PCR products were digested with *Bam*HI–*Eco*RV and *Eco*RV and *Dra*III, respectively, and the two PCR fragments were ligated to the *Bam*HI and *Dra*III fragment of the Wnt3–Fz1 chimera plasmid. The 29 amino acid deletion mutant was generated by PCR amplification of the Fz CRD domain using the following primers: 5′-ACGGATATCGTGAAAGTGCAGTGTTCCGCTG and 3′ primer CCGGGTAGCTGAAGCGCCGCATG, and the *Eco*RV and *Dra*III fragment was cloned into the *Eco*RV–*Dra*III fragment of the modified Wnt3–Fz1 chimera. The nucleotide sequences of all of the plasmids were verified by sequencing.

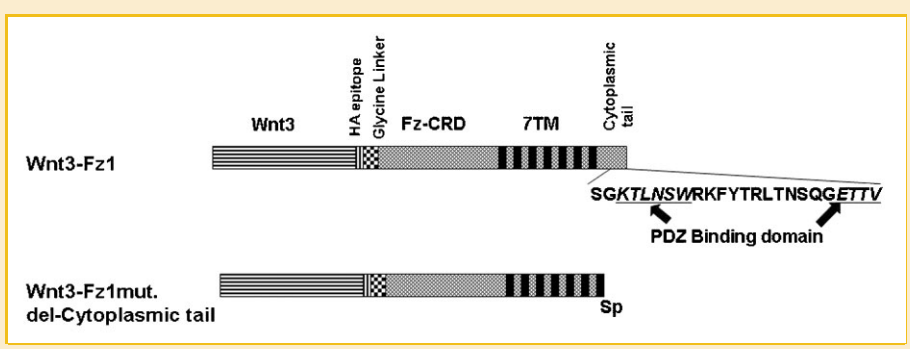

Fig. 1. Schematics of Wnt3–Fz1 chimera and its mutant with the deletion in the cytoplasmic tail. The amino acid sequence of cytoplasmic tail is shown with the potential PDZ binding sites in italics and underlined.

Ad5 RECOMBINANT VIRUS EXPRESSING Wnt3–Fz1 CHIMERA

The transcription unit of the chimera protein containing the CMV promoter and Wnt3–Fz1 fusion protein reading frame and its mutants as a *Mlu*I–*Xba*I fragment were cloned into the corresponding sites of modified pENTR 1A vector (Invitrogen, Grand Island, NY) containing SV40 PolyA signal sequence. In vitro recombination of the above plasmids with the pAD PL/Dest vector (Invitrogen) resulted in plasmids suitable for the generation of recombinant adenovirus. The plasmids were digested with the *Pac*I enzyme to release the vector, and the purified DNAs were transfected into 293 A cells using lipofectamine (Invitrogen). The cells were overlaid with agarose, and the plaques were isolated and amplified. The viruses were plaque purified, and virus stocks were prepared and titred in 293 A cells [Bhat et al., 2004].

CELL LINE AND TRANSFECTION

The osteosarcoma cell line U2OS (ATCC) was maintained in growth media consisting of McCoy's 5A medium (Invitrogen) containing 10% fetal calf serum (Hyclone, Logan, UT), 2 mM Glutamax-1 (Invitrogen) and 1X penicillin and streptomycin (Invitrogen) and incubated at 37°C with 5% CO_2/95% humidified air. For transfection studies, the cells were plated in a 96-well tissue culture plate in growth media without antibiotics and incubated overnight in the incubator. The growth medium was removed, and the cells were washed once with OPTI-MEM I medium (Invitrogen) and were then fed with 100 μl of OPTI-MEM I. The cells were transfected with the following DNA's using lipofectamine following the manufacturer's recommended protocol (Invitrogen). For each transfection, the following DNA's were diluted together in OPTI-MEM I medium: 100 ng of 16XTCF-luciferase, 20 ng of Wnt3 (Upstate Biotechnology), 1–5 ng of Wnt3–Fz1 chimera or its mutants and 25 ng of β-galactosidase (Clontech, Palo Alto, CA) along with 0.4 μl of lipofectamine 2000 (Invitrogen, Carlsbad, CA) in a total volume of 50 μl. The DNA–lipofectamine mixture was then added to each well and the plates were incubated in a 37°C incubator for 4 h. The medium was then removed and the cells were washed with 150 μl of phenol red-free RPMI 1640 medium (Invitrogen), refed with 100 μl of RPMI medium supplemented with 2% fetal calf serum, 2 mM Glutamax-1 and 1% penicillin–streptomycin, and incubated in a 37°C incubator overnight. The next day, the cells were washed twice with 150 μl/well of PBS without Ca++ and Mg++ (Invitrogen) and then lysed with 50 μl/well of cell culture lysis reagent (Promega, Madison, WI). The cell lysates were assayed for luciferase (Promega) and β-galactosidase (Tropix, Bedford, MA) activity using a microlumatPLUS luminometer (EG&G Berth hold). The luciferase activity was normalized with β-gal to offset the transfection efficiency and the data was analyzed using the JMP program (SAS Institute). Activation of Wnt signaling is presented as fold activation over the control containing TCF-luciferase reporter [Bhat et al., 2007].

Wnt–Fz CHIMERA VIRUS INFECTION TO STUDY WNT SIGNALING

For infection studies, the cells were plated in a 96-well tissue culture plate in growth media and incubated overnight in the incubator. The growth medium was removed, and the cells were infected with 10 PFU/cell of Ad5 Wnt3 and 50 PFU/cell of 16XTCF-luciferase in 50 μl of media containing 2% serum. After 1 h incubation, the virus inoculum was removed and the cells were fed with 100 μl of the growth media and incubated in a 37°C incubator overnight. The next day, the cells were washed twice with 150 μl/well of PBS without Ca++ and Mg++ (Invitrogen) and then lysed with 50 μl/well of cell culture lysis reagent (Promega,) and assayed for luciferase activity as described above.

MEMBRANE BIOTINYLATION AND IMMUNODETECTION

U2OS cells were plated at 34,000 cells/cm^2 in T75 flasks in McCoy's 5A medium (Invitrogen, NY) containing 10% FBS (Hyclone), 1× Glutamax-1 Supplement (2 mM L-alanyl–L-glutamine; Invitrogen), and 1× penicillin/streptomycin solution (Invitrogen). After 24 h in culture, the medium was removed, and the cells were infected with either Ad5 Wnt3/Fz1 or its mutants at 100 MOI for 1 h at 37°C. After 1 h of infection, the virus inoculum was removed, and the cells were washed once with serum-free McCoy's 5A medium, refed with fresh McCoy's 5A medium containing 2% FBS, 1× Glutamax-1 Supplement, and 1× penicillin/streptomycin, and incubated at 37°C, 5% CO_2 overnight. The cell surface proteins were biotin labeled using a Cell Surface Protein Biotinylation and Purification Kit (Pierce, Rockford, IL), following the manufacturer's protocol. Samples (10 μl) were resolved by SDS–PAGE on a NuPAGE 4–12% Bis–Tris gel (Invitrogen) under reducing conditions. Following transfer onto a nitrocellulose membrane, Wnt3/Fz1 was immunodetected using a monoclonal anti-Wnt3 antibody (Zymed Laboratories, Inc., South San Francisco, CA; Cat. # 39-0300). Peroxidase-conjugated AffiniPure donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) was used as the secondary antibody. Immunopositive bands were visualized by chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

DIFFERENTIATION OF C3H10T1/2 CELLS INTO OSTEOBLASTS AND ADIPOCYTES

C3H10T1/2 mesenchymal stem cells were plated at 31,600 cells/cm^2 in T225 flasks in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) containing 10% heat inactivated fetal bovine serum (Invitrogen), 4,500 mg/L glucose, 1× Glutamax-1 Supplement (2 mM L-alanyl–L-glutamine; Invitrogen), 1 mM sodium pyruvate (Invitrogen), and 1× penicillin/streptomycin solution (Invitrogen) (Growth Medium). The cells were incubated overnight at 37°C inside a 5% CO_2/95% humidified air incubator. After 24 h in culture, the medium was removed, and the cells were infected with Ad5 Wnt3/Fz1, Ad5 Wnt3/Fz1 cytoplasmic tail deletion, or Ad5 CMV-β-gal (infection control) at 400 PFU/cell in growth medium for 1 h at 37°C. A mock infection was also performed using virus-free growth medium. After 1 h of infection, the virus inoculum was removed, and the cells were washed once with serum-free DMEM, refed with fresh growth medium, and incubated at 37°C for 4 h. After the 4-h recovery period, the infected and mock-infected cells were trypsinized, counted, and plated in 24-well tissue culture plates at 40,000 cells/cm^2 in growth medium containing 50 μg/ml L-ascorbic acid phosphate (Wako, Richmond, VA), 10 mM B-glycerol phosphate (Sigma, St. Louis, MO), and 100 nM menadione sodium bisulfite (Vitamin K3; Sigma) (differentiation medium). The cells were incubated at 37°C inside a 5% CO_2/95% humidified air

incubator. The differentiation medium was replaced every 3–4 days. For alkaline phosphatase assay, the cells were washed with PBS and lysed and the cell lysates were assayed for total protein and alkaline phosphatase activity. For adipogenic differentiation, the cells were infected and plated as above and the cells were treated with adipogenic media containing insulin (10 μg/ml,) dexamethasone (1 μM) and IBMX (0.5 mM). After 3 days, the media was replaced with adipogenic progression medium (growth media with 10 μg/ml insulin), with subsequent progression media replacement every third day. Fifteen days post-infection, the cells were stained with Oil-Red-O stain.

RESULTS

THE Wnt3–Fz1 CHIMERA IS MORE EFFICIENT THAN Wnt3 IN ACTIVATING CANONICAL Wnt SIGNALING

U2OS cells contain all of the components necessary for canonical Wnt signaling and express low levels of both frizzled receptors and Wnt proteins. Canonical Wnt signaling in this study is measured using an optimized TCF-luciferase reporter containing 16 copies of the TCF element upstream of a minimal tk promoter driving the luciferase reporter gene [Bhat et al., 2007]. The transfection of U2OS cells with Wnt cDNA and TCF-luciferase reporter results in about an 8- to 10-fold increase in luciferase activity compared to control, indicating the presence of functional frizzled receptors in U2OS cells (Fig. 2). Transfection of U2OS cells with the Fz1 expression plasmid resulted in about a threefold increase in luciferase activity, suggesting the presence of low levels of expression of Wnt proteins. Co-transfection of Wnt3 and Fz1 plasmids resulted in the synergistic activation of the TCF reporter, suggesting that both the endogenous and expressed Fz1 receptors are being utilized in the activation of the TCF-luciferase reporter. Transfection of the Wnt3–Fz1 chimera resulted in about 50-to 60-fold activation of the TCF-luciferase reporter and the response obtained with the transfection of 1 ng of the Wnt3–Fz1 chimera plasmid is nearly 4–5 times better than the response obtained with 20 ng of the Wnt3 expression plasmid (Fig. 2). The results clearly demonstrate that the Wnt3–Fz1 chimera is much more efficient than Wnt expression alone in activating canonical Wnt signaling. A dose response study with the Wnt3–Fz1 chimera has shown optimal signaling at 1 ng/well, and an increase in the amount of plasmid DNA did not increase the response further; rather, the response decreased with 10–20 ng of transfected DNA. For the rest of the experiments, 1 ng/well of the Wnt–Fz1 chimera was used.

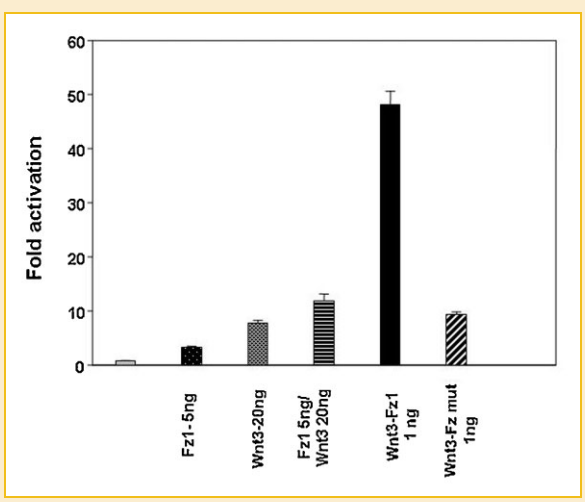

Fig. 2. Wnt3–Fz1 chimera is efficient in activating Wnt signaling in U2OS cells. Fz1, Wnt3, Wnt3–Fz1 chimera plasmids were transfected along with the 16XTCF-luciferase reporter plasmids into U2OS cells. After 20 h, the luciferase and β-galactosidase activities in the cell lysates were measured and the results were normalized with β-gal and presented as fold activation over control containing 16XTCF-luciferase reporter.

THE CYTOPLASMIC DOMAIN IS CRITICAL FOR Wnt SIGNALING

In order to determine the role of the cytoplasmic domain of Fz1 in Wnt signaling, a mutant of Wnt3–Fz1 with deletion of the cytoplasmic tail was generated and tested for its ability to activate the TCF-luciferase reporter (Fig. 3). The deletion of the cytoplasmic portion in the Wnt3–Fz1 chimera resulted in about an eight-fold decrease in activity compared to the Wnt3–Fz1 chimera clearly indicating that the cytoplasmic tail plays a critical role in the Fz1-mediated Wnt signaling. A single amino acid change from glycine to proline in the amino-terminal portion of the cytoplasmic tail, which generated the unique *Bst*EII restriction site, was used to generate the Wnt3–Fz1 cytoplasmic mutants, and resulted in a marginal increase in activity. It has been shown that the Fz1 cytoplasmic tail contains two PDZ binding domains, the N-terminal KTXXXW which is conserved in all Frizzled receptors and a C-terminal ETTV binding domain [Umbhauer et al., 2000; Wong et al., 2003]. Several mutants of the Wnt3–Fz1 chimera with mutations in either or both of the PDZ binding domains were generated and assayed for their ability to activate canonical Wnt signaling, and the results are presented in Figure 3. Mutation of ETTV to GAAA in the Wnt3–FZ1 chimera did not affect its activity, whereas the mutation of KTXXXW resulted in a substantial loss of activity. The individual mutation of either KT to AA or the combined mutation into AAXXXA resulted in a 50% loss of activity, indicating that the KTXXXW binding domain in the Fz1 cytoplasmic tail is critical for the activation of Wnt signaling. Mutation of both PDZ binding domains resulted in 80% loss of activity, suggesting that signaling involving both PDZ binding domains has a synergistic effect.

THE Fz1 CRD DOMAIN IS CRITICAL FOR Wnt SIGNALING

Interaction of Wnt with the CRD domains of Fz has been well documented and is shown to be critical for Wnt signaling. Mutation studies with the Fz homologous secreted frizzled related protein 1 (SFRP-1) CRD domain have shown that the 2nd loop of the CRD is critical for the Wnt antagonist function of SFRPs. In order to address the role of the Fz1 CRD domain in Wnt signaling, a deletion of 29 amino acids within the 2nd cysteine loop was generated and tested for its ability to activate the TCF-luciferase reporter. The Wnt3–Fz1 chimera with deletion in the CRD failed to activate the TCF-luciferase reporter (Fig. 4a). The lack of activation is not due to a change in the membrane receptor levels, as both the wild-type (wt) and mutant Fz receptors are expressed in comparable levels (Fig. 4b).

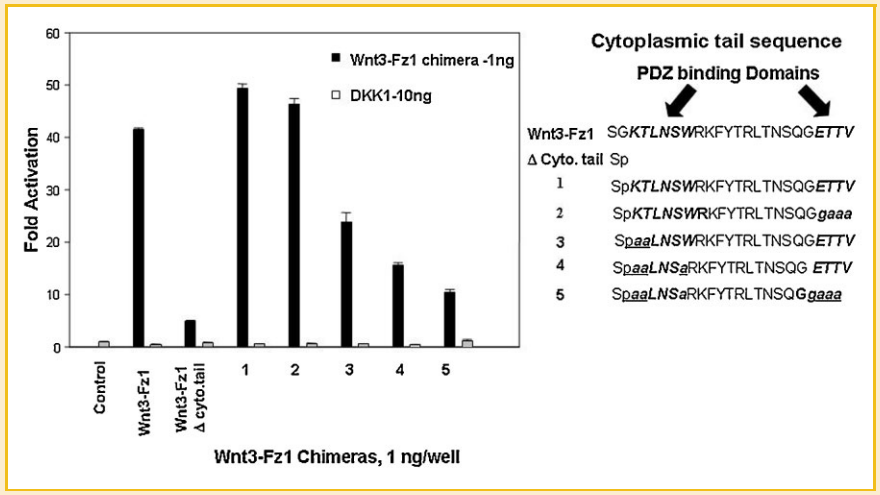

Fig. 3. Cytoplasmic tail of Wnt3–Fz1 chimera is critical for Wnt signaling. Wnt3–Fz1 chimera and its mutants were transfected along with the 16XTCF-luciferase and β-galactosidase plasmids into U2OS cells and the transfection results were normalized and presented as fold activation over reporter control.

These results clearly demonstrate the critical role of the CRD for Wnt signaling.

DKK-1 TOTALLY ABOLISHES THE ACTIVITY OF Wnt3–Fz1 CHIMERA

We have generated a replication defective adeno recombinant expressing the Wnt3–Fz1 chimera, and transduction of cells with the Wnt3–Fz1 chimera and the 16XTCF-luciferase reporter resulted in a 25- to 30-fold increase in TCF-luciferase activity (Fig. 5). DKK-1 and SFRPs antagonize Wnt signaling by interfering with the interactions of Wnt with LRP and with the Fz receptor, respectively. The addition of DKK-1-rich conditioned media totally abolished the Wnt signaling activity of the Wnt–Fz chimera. Although the data clearly suggests that the Wnt3–Fz1 chimera interacts with LRP to mediate Wnt signaling, SFRP-1 protein fails to inactivate the Wnt–Fz1 chimera action. Since Wnt3 and Fz are in close proximity in the chimera, the SFRP-1 protein may be unable to dissociate the close intermolecular interaction of Wnt with the Fz CRD in the chimeric molecule.

THE Wnt3 PORTION OF THE CHIMERA INTERACTS INTERMOLECULARLY WITH THE CRD OF THE Fz1 PORTION OF THE CHIMERA TO ACTIVATE CANONICAL Wnt SIGNALING

The Wnt3 portion of the Wnt3–Fz1 chimera is at the N-terminus and has the potential to interact intermolecularly with either the Fz1 CRD or with cellular Fz receptors. A co-culture assay was used, wherein cells infected with either the Wnt3–Fz1 chimera, or Wnt3, or mock-infected cells, were cultured with TCF-luciferase-expressing cells for 24 h, followed by the measurement of luciferase activity using the total lysate. Co-culturing Wnt3-expressing and TCF-luciferase reporter expressing cells resulted in the upregulation of luciferase activity, whereas co-culturing mock-infected cells or cells expressing Wnt3–FZ1 chimera with the TCF-luciferase-expressing cells failed to activate Wnt signaling (Fig. 6). These results indicate that Wnt3 in the chimera acts in cis and interacts with the Fz1 portion of the chimera to activate canonical Wnt signaling and fails to activate the endogenous cellular Fz1 receptor. These results imply that the observed response upon expression of Wnt3–Fz1 is solely due to the intermolecular interaction of Wnt with the Fz1 portion of the chimera protein.

THE Wnt3–Fz1 CHIMERA IS A POTENT ACTIVATOR OF MESENCHYMAL STEM CELL DIFFERENTIATION

The C3H10T1/2 cell line is a murine embryonic mesenchymal cell line, which retains the potential to differentiate into osteoblasts. Expression of the Wnt3–Fz1 chimera results in increased alkaline phosphatase activity as early as day 1, which peaks on day 2 with an approximate 12-fold increase in activity compared to that measured in uninfected, Ad5 β-gal infected control cells or in cells expressing the Wnt3–Fz1 mutant with the deletion of the cytoplasmic tail (Fig. 7a). In contrast, expression of the Wnt3–Fz1 chimera results in the inhibition of the differentiation of the cells into adipocytes (Fig. 7b). Expression analysis of key adipogenic transcription factors by qPCR showed three- and sixfold decreases in c/EBP α, β and PPARγ mRNA levels, respectively in Wnt3–Fz1 chimera-expressing C3H10T1/2 cells (data not shown). These results suggest that the Wnt3–Fz1 chimera is a potent activator of the differentiation of C3H10T1/2 cells into osteoblasts and an inhibitor of differentiation into the adipocyte lineage.

DISCUSSION

Wnt–Fz fusion protein has been shown to activate canonical Wnt signaling, and it has been proposed that the main function of Wnt is to nucleate the formation of a physical complex between LRP and a frizzled molecule [Holmen et al., 2002; Povelones and Nusse, 2002]. Previous studies have generally used xenopus Wnt and various Fz receptors [Holmen et al., 2002]. In the present work, we have used human Wnt3 linked to the human Fz1 receptor and show a robust upregulation of Wnt signaling with a 30- to 50-fold increase in luciferase activity. The ability of DKK-1 protein to block signaling of the Wnt3–Fz chimera validates the interaction of LRP with the

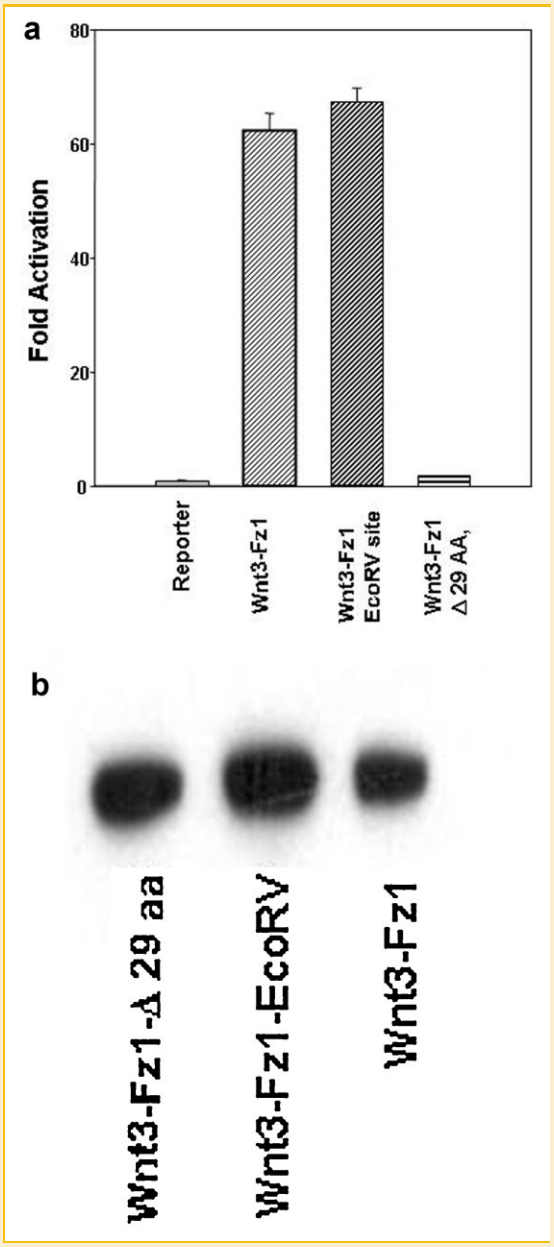

Fig. 4. a: The CRD domain of Fz1 is essential for Wnt3–Fz1 chimera signaling. Wnt3–Fz1 chimera and a mutant plasmid with a deletion of 29 amino acids in the CRD domain were transfected along with the 16XTCF-luciferase and β-galactosidase plasmids into U2OS cells and the transfection results were normalized and presented as fold activation over reporter control. b: Cell surface expression of Wnt3–Fz1 chimera and its mutants. Cell surface proteins in U2OS cells expressing Wnt3–Fz1 chimera or its mutant proteins were biotinylated and resolved on SDS–PAGE and transferred to nitrocellulose membrane. Wnt3–Fz1 chimera was immuno-detected using Wnt3-specific monoclonal antibody.

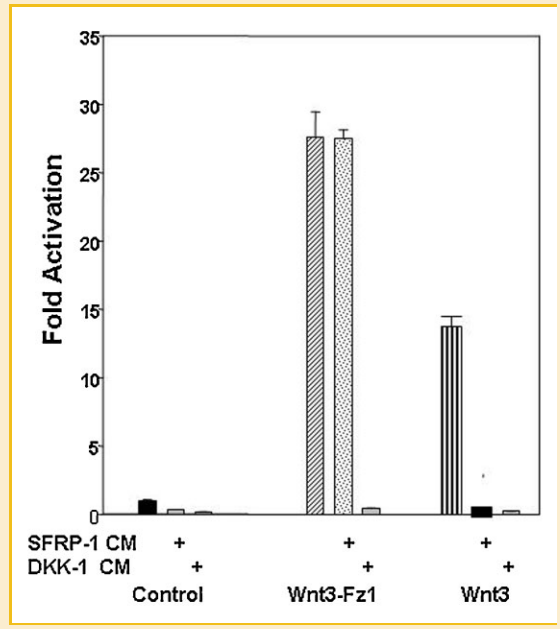

Fig. 5. Wnt3–Fz1 chimera activation of canonical Wnt signaling is blocked by DKK1. U2OS cells were infected with Ad5 16XTCF-luciferase virus alone or along with Ad5 Wnt3, Ad5 Wnt3–Fz1 viruses for 1 h. The infected cells were treated with growth media containing SFRP-1 or DKK1 proteins. After 20 h, the cells were lysed and assayed for luciferase activity and the results were presented as fold activation over reporter control.

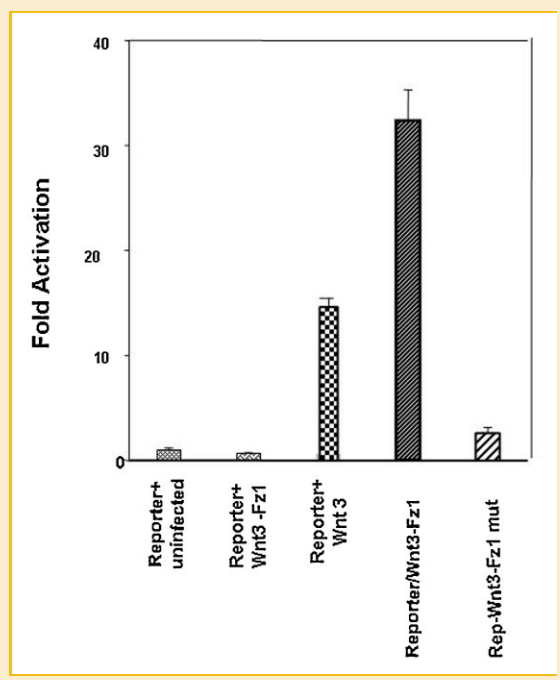

Fig. 6. Wnt3–Fz1 chimera activates canonical Wnt signaling in cis but fails to act in trans on neighboring cells. U2OS cells in 35 mm tissue culture dishes were infected with Ad5 16XTCF-luciferase reporter virus, Ad5 Wnt3, Ad5 Wnt3–Fz1 chimera and its mutant viruses separately or together. After 1 h, the cells were washed, trypsinized and co-cultured in a 96-well plate as indicated in the figure. The luciferase activity was presented as fold activation over reporter control.

chimera protein. Wnt3 in the chimera failed to interact with cellular Fz receptors; however, it appears to have a strong interaction with the CRD of Fz1 that is not disrupted by sFRP-1. These results clearly suggest that canonical signaling ultimately involves the formation of a complex between the Wnt, LRP and Fz molecules, and thus the

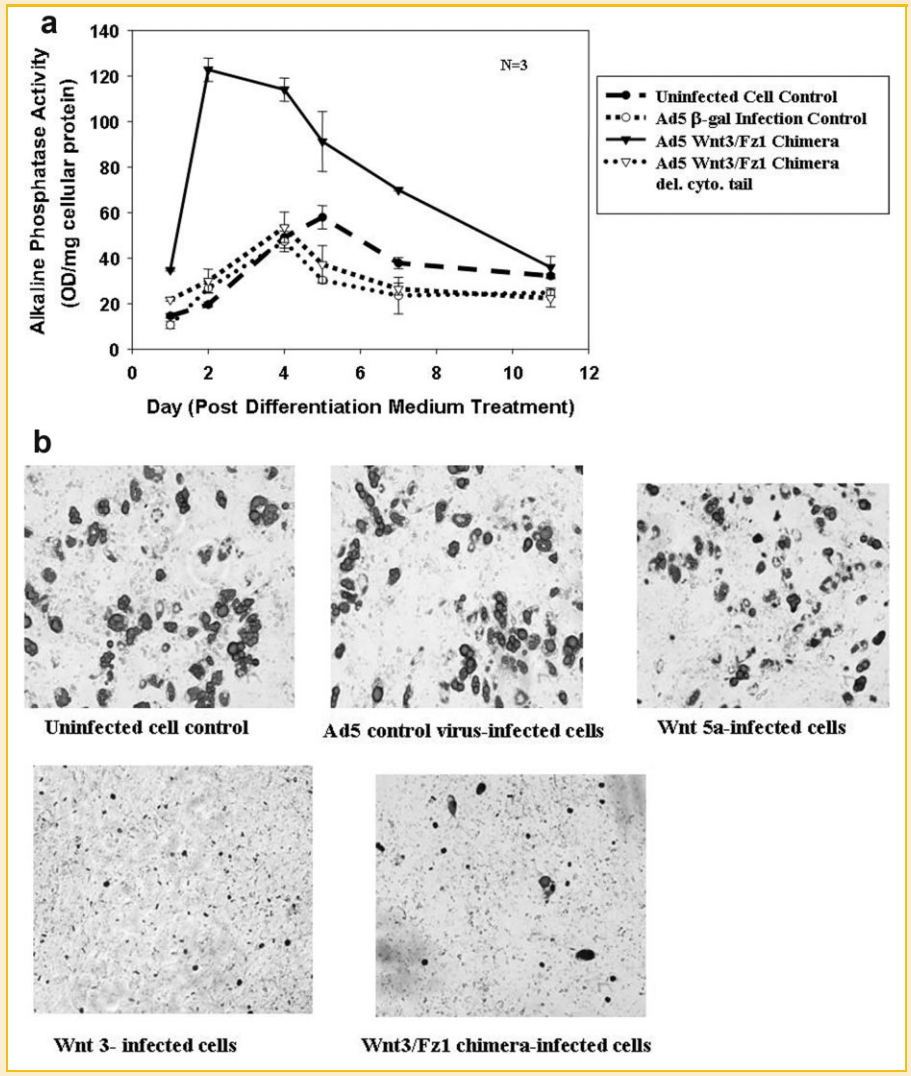

Fig. 7. a: Wnt3–Fz1 chimera is a potent activator of C3H10T1/2 cell differentiation into osteoblasts. C3H10T1/2 cells were infected with viruses for 1 h. The cells were washed, trypsinized, and plated in 96-well plates and fed with differentiation media containing β-glycerophosphate, ascorbic acid, and Vit.K3. The alkaline phosphatase activity was normalized with the total cellular protein. b: Wnt3–Fz1 chimera expression inhibits differentiation of C3H10T1/2 cells into adipocytes. C3H10T1/2 cells were infected with viruses and plated into 24-well dishes as above. The cells were treated with differentiation media containing insulin, dexamethasone, and IBMX. After 3 days the cells were fed with adipogenic progression media containing insulin. Fifteen days post-infection, the cells were stained with Oil-Red O stain.

chimeric approach can be used as a model to better study canonical Wnt signaling. The Fz receptor CRD and cytoplasmic domains play key roles in Wnt signaling as shown by several previous studies. Development of replication-defective adenoviruses expressing a Wnt–Fz1 chimera and the 16× TCF-luciferase reporter has enabled us to study Wnt signaling in several cell lines that are difficult to transfect.

Numerous studies suggest that activation of the Wnt/β catenin pathway plays an important role in human tumorigenesis [Logan and Nusse, 2004; Luu et al., 2004; Nusse, 2005]. The over-expression of Wnt has been observed in a variety of cancer cell lines, including non small-cell lung cancer, mesothelioma, breast cancer, and sarcomas. In animal models the over-expression of Wnt leads to tumors. A Wnt-1 antisense RNA or a monoclonal anti-Wnt-1 antibody has been shown to reduce Wnt signaling and decrease tumor growth in vivo [Li et al., 1999]. Similarly, an anti-Wnt2 monoclonal antibody inhibited tumor growth in malignant melanomas [He et al., 2004]. These findings hold the promise that Wnt and Fz are potential inhibitory targets for therapeutic intervention.

In the present study, using an optimized TCF-luciferase reporter system and the expression of a Wnt–Fz chimera through replication-defective adenovirus, we demonstrate that the Wnt–Fz chimera upregulates canonical Wnt signaling by about 30- to 50-fold. This reproducible and sensitive cell-based assay can be used to identify inhibitors of Wnt signaling that target Wnts, frizzleds, and downstream components of the canonical pathway. Such an inhibitor has potential therapeutic value to a treat a variety of cancers. Similarly, several Wnts, including Wnt5a, Wnt1, and Wnt7b have been shown to be present in fibroblast-like

synoviocytes, and Wnt signaling has been implicated in the pathogenesis of rheumatoid arthritis [Sen, 2005]. Wnt and Fz receptor antagonists, or small molecule inhibitors of Wnt–Fz signaling, may therefore be useful for therapeutic intervention in refractory rheumatoid arthritis.

Mesenchymal stem cells (MSCs) have the ability to self-renew and differentiate into cells of various mesenchymal lineages including osteogenic, adipogenic, and chondrogenic cells [Kleber and Sommer, 2004; Ling et al., 2009]. Several studies have demonstrated that Wnts stimulate osteoblast precursor growth and subsequent differentiation into osteoblasts [Bodine and Komm, 2006; Kang et al., 2007]. Wnt signaling is also known to inhibit adipogenesis [Bennett et al., 2002; Christodoulides et al., 2009]. In the present study, expression of the Wnt3–Fz chimera in C3H10T1/2 cells resulted in the inhibition of adipogenesis and activation of differentiation into cells of the osteoblast lineage. Thus, the Wnt–Fz chimeric approach in stem cell biology has the potential to be a valuable tool to better our understanding of the mechanisms for the differentiation of stem cells.

To date, 19 Wnt genes and 10 frizzled receptors have been identified in humans. One of the major unanswered questions in Wnt biology is the specificity of interaction between different ligands and Fz receptors, as well as the downstream pathways that these various ligand-receptor pairs stimulate. The implication of selectivity of the Wnt and the Fz receptor has been shown recently in both Norrie disease and familial exudative vitroretinopathy that have been linked to Norrin protein or Fz4 receptor. These studies have conclusively demonstrated that Norrin protein and Fz4 function as a ligand-receptor pair [Xu et al., 2004; Smallwood et al., 2007]. In vitro functional studies have indicated that Norrin-dependent signaling is highly selective for mFz4, since little or no activity is observed with mFz3, hFz5, mFz6, mFz7, and mFz8 [Xu et al., 2004]. The Wnt–Fz chimera approach may therefore provide a method to study this selectivity issue between Fz receptors and Wnt ligands and the subsequent downstream pathways that these receptor–ligand pairs activate in cells.

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