Inhibition of Adipocytogenesis by Canonical WNT Signaling in Human Mesenchymal Stem Cells

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Abstract

The WNT signaling pathway plays important roles in the self-renewal and differentiation of mesenchymal stem cells (MSCs). Little is known about WNT signaling in adipocyte differentiation of human MSCs. In this study, we tested the hypothesis that canonical and non-canonical WNTs differentially regulate *in vitro* adipocytogenesis in human MSCs. The expression of adipocyte gene *PPARγ2*, *lipoprotein lipase*, and *adipsin* increased during adipocytogenesis of hMSCs. Simultaneously, the expression of canonical *WNT2*, *10B*, *13*, and *14* decreased, whereas non-canonical *WNT4* and *11* increased, and *WNT5A* was unchanged. A small molecule WNT mimetic, SB-216763, increased accumulation of β-catenin protein, inhibited induction of *Wnt4* and *11* and inhibited adipocytogenesis. In contrast, knockdown of β-catenin with siRNA resulted in spontaneous adipocytogenesis. These findings support the view that canonical WNT signaling inhibits and non-canonical WNT signaling promotes adipocytogenesis in adult human marrow-derived mesenchymal stem cells.

Keywords

Wnt; human mesenchymal stem cells; adipocytogenesis; β-catenin; SB-216763; gene silencing

Introduction

Adult human mesenchymal stem cells (hMSCs), also known as marrow stromal cells, have the capacity to differentiate into adipocytes, osteoblasts, and chondrocytes [1-3]. WNT signaling regulates many processes during embryonic development and adult homeostasis [4], as well as in bone formation, remodeling, and repair [5, 6]. At least 19 types of Wnts, several families of secreted antagonists, and multiple receptors have been identified [7]. Two distinct Wnt signaling pathways have been described: the canonical pathway and the noncanonical pathway [4]. The canonical Wnt is not only a general stem cell growth factor but can also influence cell lineage decisions in certain stem cell types [8, 9]. Cell fate determination changes are often regulated by finely-tuned alterations in the canonical Wnt

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There are no conflicts associated with this research.

pathway [10]. In this regard, it has been demonstrated that canonical Wnt signaling is critically involved in activities of hMSC [11]. In previous studies, we [12-14] and others [16] showed evidence that Wnt/β-catenin signaling is involved in stimulation of chondrocytogenesis and inhibition of osteoblastogenesis and adipocytogenesis of human marrow stromal cells.

In humans, the proportions of fat, hematopoietic marrow and trabecular bone in the bone medullary cavity are affected by age or by osteoporosis [17]. In patients with unusually high bone mass, activation of LRP5, a WNT co-receptor, resulted in the inhibition of adipocyte differentiation in hMSCs [18]. Further, Wnt10b, a canonical Wnt, inhibits adipogenesis and stimulates osteoblastogenesis of murine 3T3-L1 preadipocytes [19]. Recently, the non-canonical Wnt4 and Wnt5a were shown to play crucial roles in murine adipogenesis as positive regulators [20]. Kang *et al.* reported that canonical Wnt signaling stimulates osteoblastogenesis of murine ST2 cells by suppressing CCAAT/enhancer-binding protein α and peroxisome proliferator-activated receptor γ (PPARγ) [21].

Although there is a growing body of information available about the mechanisms of adipocyte differentiation from studies with murine preadipocytes, little is known about the Wnt expression profile during human adipocytogenesis. We recently reported that there are age- and gender-dependent variations in constitutive expression of the *WNT* genes in hMSCs [22]. This study tests the hypothesis that canonical and non-canonical Wnts regulate adipocytogenesis in human cells. *WNT* genes were monitored during adipocytogenesis of hMSCs and the effects of modulating β-catenin with a small molecule agonist (SB-216763) or with targeted gene silencing were evaluated.

Materials and methods

Reagents

SB-216763 (Tocris Cookson Inc.) was dissolved in DMSO at 50 mM concentration and stored at −20°C. The stock solution was diluted with DMSO and the same volumes of DMSO were used as vehicle control.

Cell culture

Marrow was obtained as discarded material from patients undergoing total hip replacement [3, 22]. Samples from thirteen subjects, 6 women and 7 men, age from 36 to 85 years old, were included in this study. Not all specimens could be included in every experiment because of the surgical schedule and numbers of cells needed for each assay. Low density mononuclear cells were isolated by density centrifugation with Ficoll/Histopaque 1077 (Sigma, St Louis, MO, USA). Resident adipocytes were removed as a floating layer in the supernatant fraction. This procedure enriches for undifferentiated cells and includes a population of non-adherent hematopoietic cells as well as a fraction capable of adherence and differentiation into adipose and skeletal cells. Non-adherent cells were removed 24 hours after seeding. The adherent marrow stromal cells (MSCs) were expanded in phenol red-free α-MEM medium (Gibco BRL), 10% heat-inactivated fetal bovine serum (FBS-HI; Gibco BRL), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). Medium was replenished twice each week. The hMSCs were subcultured at a ratio of 1:5 when they attained approximately 80% confluence. Cells from passage 2 were used in these experiments.

A line of hMSCs, KM101 [15, 23] was used for some experiments. KM101 cells were maintained in Iscove's modified Dulbecco's medium (IMDM; Gibco BRL) with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The medium was replenished twice each week until the cells reached near-confluence.

For some experiments, medium was replaced with fresh medium supplemented with 1% FBS-HI with or without SB-216763. After 6 hours, cells were harvested for Western immunoblotting. After longer intervals, cells were harvested for gene expression analysis or enumeration of adipocytes.

Conditions for adipocytogenic differentiation

For adipocyte differentiation, upon confluence of hMSCs in 100-mm dishes or 12-well tissue culture plates, medium was changed to α-MEM, 1% FBS-HI with supplements (1 μM dexamethasone, 0.5 mM 1-methyl-3-isobutylxanthine, and 10 μg/ml insulin), 100 U/mL penicillin, and 100 μg/mL streptomycin, referred to as adipocytogenic medium, as previously described [12, 24]. Eighteen days after treatment, lipid accumulation in adipocytes was visualized by staining with oil red-O as follows: cells were fixed in 10% formalin for 1 hour and stained for lipid with 0.3% oil red-O for 15 minutes. After rinsing thrice with ddH_2O, the red-staining cells in six random areas of 1-mm^2 were enumerated for each well and presented as an average ± standard deviation for 3-6 replicate wells.

RNA isolation and RT–PCR

Total RNA was isolated with TRIZOL reagent (Invitrogen) at intervals following transfer to adipocytogenic medium. Two μg of total RNA was reverse transcribed into cDNA with M-MLV (Promega) following the manufacturer's instructions. Concentrations of cDNA and amplification conditions were optimized to reflect the exponential phase of amplification. One-twentieth of the cDNA was used in each 50 μL PCR reaction (30–40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min) as described [12, 13, 24]. The gene-specific human primers were: *PPARγ2* [25]: forward: 5′-ATTCTCCTATTGACCCAGAAAGCG-3′, reverse: 5′-AGCTTTATCTCCACAGACACGACATT-3′, *lipoprotein lipase* (*LPL*) [24]: forward: 5′-GAGATTTCTCTGTATGGCACC-3′, reverse: 5′-CTGCAAATGAGACACTTTCTC-3′, *adipsin* [26]: forward: 5′-CAAGCAACAAAGTCCCGAGC-3′ reverse: 5′-CCTGCGTTCAAGTCATCCTC-3′, and *GAPDH*: forward: 5′-GGGCTGCTTTTAACTCTGGT-3′, reverse: 5′-TGGCAGGTTTTTCTAGACGG-3′. The gene-specific primers for human *WNTs* had been designed for previous studies [22, 27]. PCR products were separated by 2% agarose gel electrophoresis and expression levels were measured by semi-quantitative RT-PCR. Images of bands were captured with KODAK Gel Logic 200 Imaging System and measured by KODAK Molecular Imaging Software (KODAK, Molecular Imaging Systems, New Haven, CT, USA). Quantitative data were expressed by normalizing the densitometric units to *GAPDH* (internal control).

Western immunoblotting

*After 6 hours treatment with SB-216763 or DMSO control, human marrow stromal cells were harvested with lysis buffer containing 150 mM NaCl, 3 mM $NaHCO_3$, 0.1% Triton x-100 and a mixture of protease inhibitors (Roche Diagnostics, CA) as previously described [12]. Cells were scraped from dishes and were homogenized in lysis buffer with a Kontes' Pellet Pestle. Insoluble cellular materials were removed by centrifugation at 16,000 g. Protein concentration was determined with the BCA system (Pierce, Rockford, IL, USA). Proteins were resolved by electrophoresis on 4-12% SDS-PAGE (NuPAGE Bis-Tris gel; Invitrogen) and were transferred onto polyvinylidene fluoride membranes (Amersham Biosciences). The membranes were blocked with 5% nonfat milk in PBS buffer containing 0.1% Tween-20 (PBSMT) for 2–3 h at room temperature and incubated with primary antibodies overnight at 4°C: anti-β-catenin (E5, Santa Cruz Biotechnology) and anti-β-actin (Sigma). After removal of unbound primary antibodies by three 10-minute washes with PBS buffer containing 0.1% Tween-20 (PBST), the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature and washed

thrice for 10 minutes with PBST. The second antibody anti-mouse IgG-HRP was from Amersham, and anti-rabbit IgG-HRP was from Santa Cruz Biotechnology. The antibody-associated protein bands were revealed with the ECL-plus Western immunoblot system (Amersham Biosciences).

Transient transfection of β-catenin siRNA

Transient transfection of β-catenin siRNA (Stealth RNAi duplex siRNA, Invitrogen) or control siRNA (SiRNA-A, Santa Cruz Biotech., Inc.) into hMSCs was performed by electroporation with the Human MSC Nucleofector Kit (Lonza) according to the manufacturer's instruction and as described [13]. In brief, hMSCs were harvested by trypsinization, and resuspended at one million cells in 100 μL of nucleofector solution for human MSCs with 100 pmole of β-catenin siRNA or control siRNA. Electroporation was performed in a Nucleofector™ II with program U-23 provided by Lonza/Amaxa Biosystems. Immediately after electroporation, the cells were transferred to 35- or 60-mm dishes in MEM-α with 10% FBS-HI. After confluence, cells in 60-mm dishes were prepared for Western immunoblot. Cells in 35-mm dishes were cultured for 14 days in expansion medium.

Statistical analyses

All experiments were performed three times, with 3 to 6 replicate wells per treatment. Data are presented as means ± standard error (SE). Datum that was more than $\pm 5 \times SD$ from the mean of the rest of the samples was excluded as an outlier. Quantitative data were analyzed with either the non-parametric Mann-Whitney test or unpaired Student's two-tailed t-test for independent samples. A value of $p < 0.05$ was considered significant.

Results

Expression of signature genes during adipocyte differentiation of hMSCs

Human MSCs were cultivated in α-MEM with 1% FBS-HI and adipocytogenic supplements. Adipocyte signature genes, *PPARγ2, LPL*, and *adipsin* were examined at intervals with RT-PCR. Time course analysis indicated that expression of *PPARγ2* and *LPL* was undetectable during the first 6 hour period in adipocytogenic medium and became detectable at 1 day (Figure 1). The expression of *PPARγ2*, *LPL*, and *adipsin* increased with time thereafter.

Expression of WNT genes during adipocyte differentiation of hMSCs

The expression of *WNT* genes was determined with RT-PCR in hMSCs undergoing adipocytogenesis at intervals to 10 days (Figure 1). The earliest change after transfer to adipocytogenic medium was an increase in non-canonical *WNT11*. There was a later upregulation of *WNT4*. In contrast, there were decreases in the expression of canonical *WNT* genes, *WNT2*, *10B*, *13*, and *14*. The expression levels of *WNT3*, *5A*, and *WNT7B* were unchanged during the 10 day experimental period. Compared with dramatic reductions in expression of *WNT2*, *10B*, *13*, and *14*, there was a smaller and later decrease in expression of *WNT5B*. The expression of *WNT10B* was inversely correlated with *PPARγ2* expression (r=−0.938, *P*= 0.018). The expression level of *WNT3A* was below detection through the evaluation period. *WNT6* was expressed at levels too low for assessing variations. The expression of *WNT16B* in hMSCs appeared bimodal, with an increase from 0 to 24 hours, and decrease thereafter in adipocytogenic medium (Data not shown).

SB-216763 mimics WNT signaling pathway by accumulation of β-catenin in hMSCs

The line of KM101 human marrow stromal cells and hMSCs were analyzed for accumulation of β-catenin, a key member of the canonical Wnt signaling pathway, in the

absence and presence of SB-216763, a small molecule WNT mimic. As shown in a representative result from two independent experiments, six hours of treatment with SB-216763 increased β-catenin in KM101 cells at concentrations at or greater than 5 μM (Figure 2A). Similarly, 5 μM SB-216763 increased cellular β-catenin in hMSCs (Figure 2B); that dose was used for subsequent experiments.

SB-216763 blocked induction of adipocyte genes in hMSCs

The effects of 5μM SB-216763 on induction of adipocyte gene expression in hMSCs were determined at intervals during culture in adipocytogenic medium (Figure 3A). There was a time-dependent increase in expression of *PPARγ2*, *LPL*, and *adipsin* in the absence of SB-216763, similar to the findings shown with another sample in Figure 1. In cells treated with 5 μM SB-216763, however, the expression of *PPARγ2* was not detected at any time during the 10-day experiment. The expressions of *LPL* and *adipsin* were reduced or eliminated by 5 μM SB-216763. Reproducibility of the effect of SB-216763 was assessed with hMSCs from a series of 6 subjects after 7 days in adipocytogenic medium (Figure 3B, 36-85 years old, 4 women and 2 men). In these hMSCs, SB-216763 significantly inhibited expression of *PPARγ2* (6% of control, p=0.044), *adipsin* (51%, p=0.018), and *LPL* (11%, p=0.013) (paired t-test) (Figure 3 C).

SB-216763 inhibited WNT4and WNT11 induction in hMSCs

To determine whether activation of the canonical WNT signaling pathway alters expression of non-canonical *WNT* genes, we analyzed expression of *WNT4*, *WNT5*, and *WNT11* at day 7 in adipocytogenic medium in the presence and absence of SB-216763. The expression of *WNT4* was significantly reduced by SB-216763 (27% of DMSO vehicle control, p=0.031, Wilcoxon matched-pairs signed-ranks test, Figure 3B, D). *WNT11* was reduced by SB-216763 to 40% of control (p=0.031) (Figure 3B, E). There were no significant effects of SB-216763 on expression of *WNT5A* in the series of 6 samples (Figure 3B).

SB-216763 inhibited adipocytogenesis in a dosage and duration-dependent way

Human marrow stromal cells were used to determine the effects of different concentrations of SB-216763 on adipocyte differentiation (Figure 4). Generation of oil red-O-positive cells after 18 days of culture was inhibited significantly by 0.037μM SB-216763 (54.0% of control, *p*<0.0001) (Figure 4B). The number of adipocytes was decreased further with higher concentrations of SB-216763. At the concentration of 5 μM SB-216763, adipocyte differentiation was blocked completely (Figure 4A, B).

Reproducibility of the inhibitory effect of 5 μM SB-216763 on adipocyte differentiation was assessed with hMSCs from 6 subjects (57-82 years old, 2 women and 4 men). There was a range in the numbers of adipocytes generated in cultures of hMSCs from different subjects (4.9 to 59.3 per mm^2), without an apparent effect of age or gender. There were no oil-red-O-positive cells in cultures treated with 5μM SB-216763.

The duration of exposure to SB-216763 necessary to inhibit adipocyte differentiation was assessed (Figure 4C). The number of adipocytes generated 18 days after transfer to adipocytogenic medium was similar in controls and in hMSCs that were exposed to 5 μM SB-216763 for only the first 1 or 2 days. When exposure duration was between 3 and 7 days, the number of adipocytes was between 23 and 28% of controls. Continuous exposure to SB-216763 for the 18 days of the experiment resulted in complete inhibition of adipocytogenesis.

Knockdown of β-catenin resulted in spontaneous adipocytogenesis in hMSCs

To further assess the role of β-catenin in adipocyte differentiation of hMSCs, we transfected β-catenin siRNA or control siRNA into hMSCs. Western immunoblot verified that β-catenin protein was absent in cells transfected with 100 pmole siRNA per million cells, but was present in cells transfected with control siRNA (Figure 4D). Knockdown of β-catenin with siRNA resulted in spontaneous adipocyte differentiation of hMSCs in basal medium (Figure 4E). After 14 days, there were 6.8 ± 1.5 adipocytes per mm^2 in β-catenin siRNA hMSCs (n=4 dishes), compared with the control group (0.2 ± 0.5, n=5) (p=0.015, Mann-Whitney test). These data further support the conclusion that β-catenin inhibits differentiation of hMSCs into adipocytes.

Discussion

Adipocyte differentiation consists of a complex series of events in which cellular and extracellular factors interact to induce an undifferentiated marrow stromal cell or pre-adipocyte to develop into an adipocyte. PPARγ is a member of the nuclear hormone receptor superfamily and is expressed at high levels specifically in adipose tissue and is a central regulator of adipocyte gene expression and differentiation [28, 29]. Studies with animal cells established the Wnt/β-catenin signaling pathway as an important regulator of adipocyte differentiation [30, 31]. These studies with human marrow-derived mesenchymal stem cells show that the canonical WNT signaling pathway inhibits adipocyte differentiation *in vitro*. First, during adipocyte differentiation, canonical *WNT2*, *10B*, *13*, and *14* genes were down-regulated in hMSCs. Second, activation of Wnt/β-catenin signaling with highly selective inhibitor of GSK-3β, SB-216763, inhibited adipocytogenesis of hMSCs. Third, knockdown of endogenous β-catenin with siRNA resulted in spontaneous adipocyte differentiation. These lines of evidence indicate that canonical WNT/β-catenin pathway inhibits adipocytogenesis in human MSCs. While the expression of canonical *WNT2*, *10B*, *13*, and *14* was downregulated in hMSCs undergoing adipocyte differentiation, there was increased expression of *WNT11* and *4*. These results suggest that in human cells, canonical *WNT* genes may be inhibitors of adipocyte differentiation and non-canonical *WNTs*, in particular *WNT4* and *11* may be enhancers of adipocyte differentiation. A previous analysis of constitutive expression of *WNTs* in human MSCs revealed an age-related decline in a number of canonical *WNTs*, but that *WNT4* was unique in displaying an age-related increase in cells from men [22]. It is possible that *WNT* expression plays a role in age-related lineage restriction in bone cell progenitors. Adult human MSCs from discarded surgical tissue provide an opportunity to unravel the mechanisms of canonical and non-canonical Wnt interactions in adipocyte differentiation and effects of clinical factors, such as age, diabetes, and use of anti-diabetic drugs, on adipocyte differentiation.

These data with human MSCs are similar to some aspects of differentiation reported with murine preadipocyte 3T3-L1 cells; *wnt10b* was described as a potent inhibitor of murine adipocytogenesis [32], and *wnt4* was described as a promoter of murine adipocytogenesis [20]. There is no retrievable literature on the role of Wnt11 in adipocytogenesis, but it was the Wnt that displayed the earliest change, an increase, and prior to detection of PPARγ2 upregulation. Whereas non-canonical *wnt5a* promotes murine adipocytogenesis [20], it appeared in this study to be unchanged during adipocytogenesis of hMSCs and upon treatment with SB-216763. Bilkovski *et al*. reported that non-canonical Wnt5a maintained osteoblast potential and inhibited adipocytogenic differentiation in hMSCs that were isolated from umbilical cord blood [48]. The difference in roles of Wnt5a in Bilkovski's study and ours may be due to different biological behaviors in their neonatal cells and adult marrow-derived hMSCs used herein. For example, Jager *et al*. summarized the evidence that cord blood-derived MSCs have a niche-specific phenotype, are constitutively osteoblastic independent of the usual stimuli, and have greater osteoblast potential than do adult hMSCs

[33]. It is likely that different Wnts may be involved in constitutive lineage potential in cord blood and adult marrow-derived hMSCs. The lack of an effect of SB-216763 on *WNT5A* while it downregulated *WNT 4* and *11* also suggests that *WNT5A* is unlikely to be a key regulator of adipocytogenesis of adult human MSCs.

In studies with the murine preadipocyte cell line 3T3-L1, *Wnt5b* was reported to be upregulated during adipogenesis and overexpression of *Wnt5b* stimulated adipogenesis [34]. A recent study with 3T3-L1 cells indicated that Wnt5a was more abundantly expressed than Wnt5b, but both were down-regulated upon induction of adipogenic differentiation in this cell line [35]. Our present study with human cells shows that *WNT5A* was unchanged and *WNT5B* was decreased modestly in adipocyte differentiation, whereas *wnt5a* and *5b* play stimulatory roles in mouse cells. Our results suggest that in human marrow stromal cells, *WNT5B* may be an inhibitor of adipocytogenesis, but the exact roles of *WNT5B* in human adipocytogenesis remain to be elucidated. Moreover, there may be differences in regulation of adipocytogenesis in human marrow-derived cells, compared with adipose tissue from normal subjects or from subjects with type 2 diabetes mellitus [36].

The studies with SB-216763 indicate cross-talk between canonical and non-canonical regulators of adipocyte differentiation. Inhibition of adipocytogenesis with SB-216763 was accompanied by striking decreases in *WNT4* and *WNT11* expression. Research with murine cells also reveals crosstalk [37] and multiple mechanisms [38] regulating adipocytogenesis.

Wnts signaling occurs by activating membrane receptors of the Frizzled (Fz) family in a complex with members of the low-density-lipoprotein-related protein (LRP) family; the complex promotes the stability and nuclear localization of β-catenin by either degradation of Axin or inhibitory of GSK3β activity; thereafter, β-catenin activates transcription in conjunction with co-transcription factors Lefs/Tcfs [4, 39, 40]. Activating Wnt signaling pathway with other agents such as LiCl or GSK3 inhibitor CHIR 99021in 3T3-L1 pre-adipocytes was shown to block adipocyte differentiation of these cells [19, 32]. Studies from our lab [12] and from De Boer *et al.* [41] with human MSCs indicated that LiCl inhibited adipocyte differentiation. LiCl is not only an inhibitor of GSK-3β [19, 32]; LiCl enhances phospholipase C activity [42] and inhibits casein kinase-2 and MAPK activated protein kinase-2 [43]. Coghlan *et al.* developed SB-216763, a highly selective, cell permeable small molecule inhibitor of GSK-3β, which does not inhibit the activity of the kinases required for insulin signaling and cell survival [44]. SB-216763 has been used to manipulate β-catenin signaling [44-47]. The Western immunoblot results presented here show that treatment with SB-216763 resulted in the accumulation of β-catenin in both a line of hMSCs, KM101, and in freshly isolated human MSCs. This study shows that activation of WNT signaling by SB-216763 inhibited the induction of *PPARγ2* and adipocyte differentiation in hMSCs. The maximum effective concentration of SB-216763 is known to depend on the cell type [43]. This study shows that hMSCs were sensitive to 0.037 μM SB-216763 and that 5 μM blocked adipocytogenesis, in a reproducible manner with hMSCs representing different ages and both genders.

Targeted knock-down of β-catenin in hMSCs with the gene silencing approach resulted in spontaneous generation of lipid-filled adipocytes. This observation adds evidence to the hypothesis that canonical Wnt signaling is important for constitutive suppression of adipocytogenesis.

In summary, these results suggest that in adult marrow-derived human mesenchymal stem cells, canonical *WNTs*, specifically *WNT2*, *10B*, *13*, and *14*, may all be constitutive inhibitors of adipocyte differentiation and that non-canonical *WNTs*, in particular *WNT11* and *4*, may be enhancers that are upregulated during adipocyte differentiation. Activation of

Wnt/β-catenin signaling with highly selective inhibitor of GSK-3β, SB-216763, inhibited adipocytogenesis of hMSCs and knockdown of β-catenin with siRNA stimulated adipocyte differentiation in hMSCs. Further, these studies indicate cross-talk between canonical and non-canonical regulators of adipocyte differentiation. These findings support the view that canonical and non-canonical WNT signaling pathways regulate adipocytogenesis in human marrow-derived mesenchymal stem cells.

Acknowledgments

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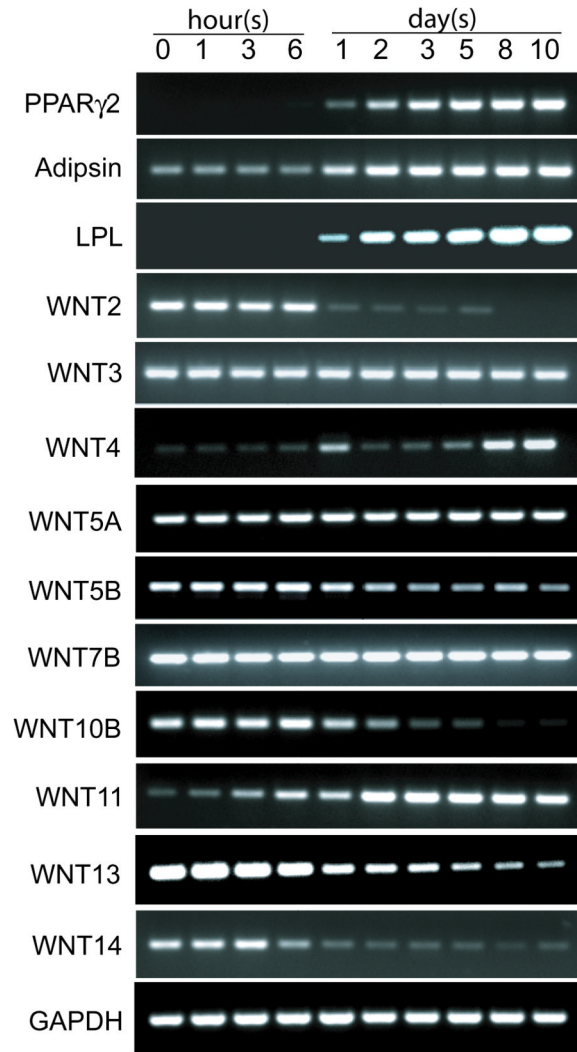
Figure 1.
Modulation of expression of adipocyte marker genes (PPARγ2, adipsin, and lipoprotein lipase, LPL) and *WNT* genes in representative hMSCs (60-year-old woman) at intervals after transfer to adipocytogenic medium. Agarose gel electrophoretograms show effects of time on RT-PCR products.
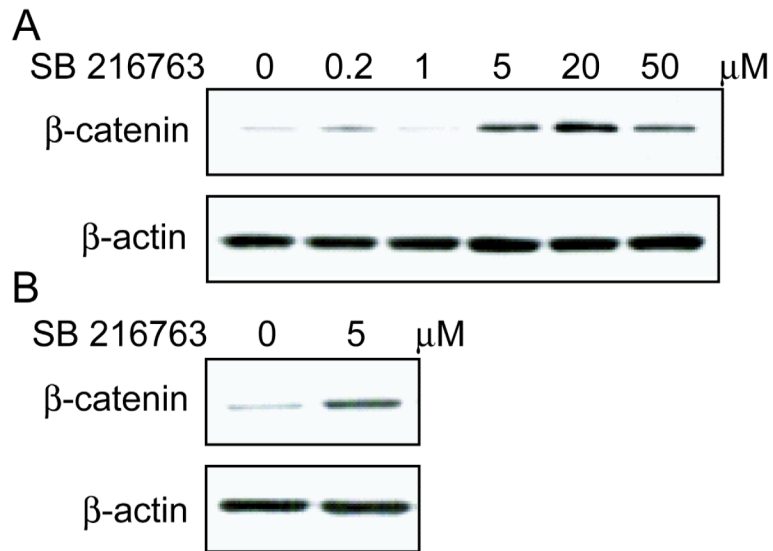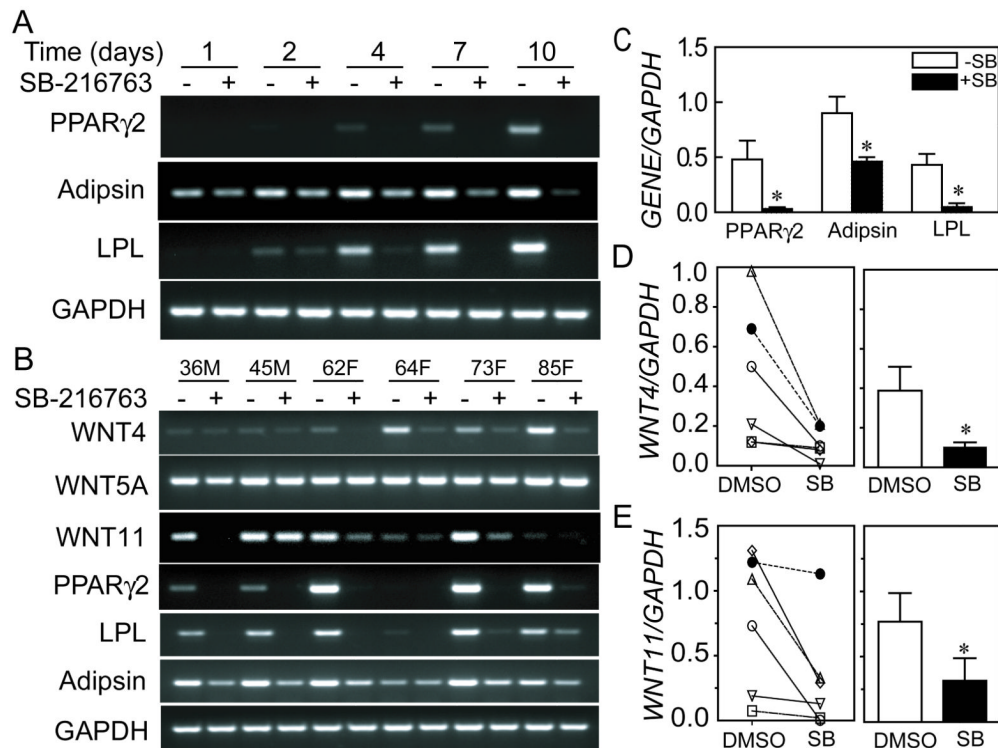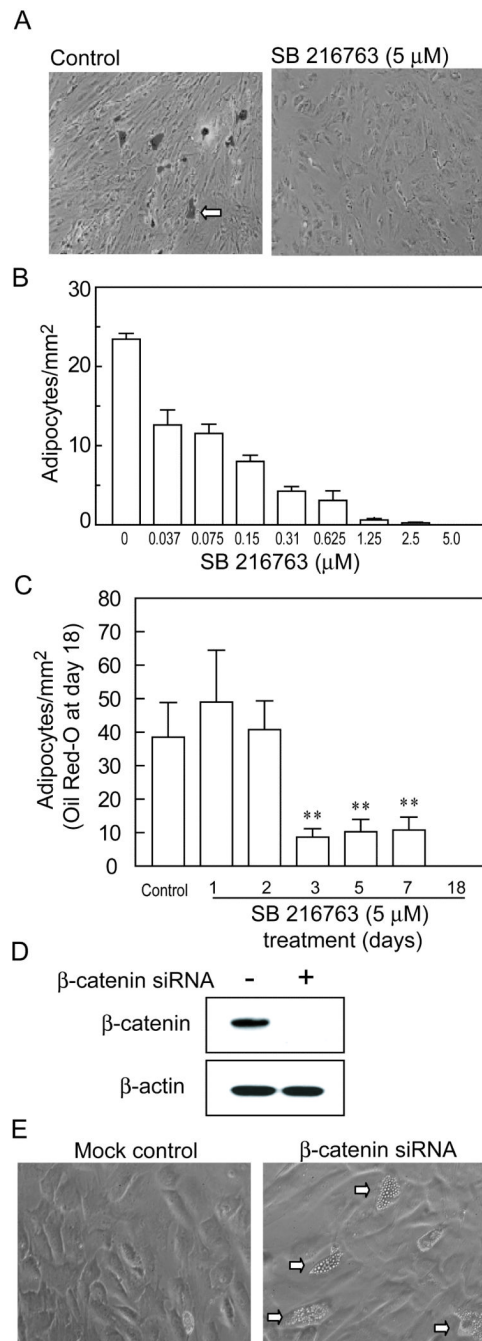
Figure 2.
Effects of SB-216763 on β-catenin levels in hMSCs. (A) There was a dose-dependent effect of SB-216763 on β-catenin in KM101 cells. (B) SB-216763 (5 μM) increased β-catenin in hMSCs (61-year-old man). Protein was extracted from cells after 6 hours incubation with or without SB-216763 and subjected to immunoblot for β-catenin and β-actin.

Figure 3.
Effects of SB-216763 on adipocyte differentiation and expression of *WNT4* and *WNT11* in hMSCs. (A) Expression of adipocyte signature genes in hMSCs was inhibited by SB-216763 after transfer to adipocytogenic medium. Agarose gel electrophoretogram shows PCR products for hMSCs (36-year-old man) after indicated days in adipocytogenic medium in the absence or presence of 5 μM SB-216763 (B) Expression of *WNT4, WNT11* and adipocyte signature genes was inhibited by 5 μM SB-216763 in hMSCs from 6 subjects, evaluated 7 days after transfer to adipocytogenic medium. There was little or no effect on *WNT5A*. Each pair of lanes indicates the age in years and the gender (M, male; F, female) of the subject from whom the cells were obtained. (C) Expression of adipocyte signature genes was significantly inhibited by 5 μM SB-216763. Data are shown for 6 samples as the mean and standard error (SE) for each gene (* $p < 0.05$, paired t-test). (D) Expression of *WNT4* relative to *GAPDH* is shown (left panel) for each specimen in DMSO vehicle control and 5 μM SB-216763, and (right panel) as the group means and standard error (*p=0.031, Wilcoxon matched-pairs signed-ranks test). (E) Expression of *WNT11* relative to *GAPDH* is shown (left panel) for each specimen in DMSO vehicle control and 5 μM SB-216763, and (right panel) as the group means and standard error (*p=0.031, Wilcoxon matched-pairs signed-ranks test).

Figure 4.
Effects of SB-216763 and β-catenin siRNA on adipocytogenesis in hMSCs. (A)
Photomicrographs of cultures of representative hMSCs (47-year-old man) without and with
5μM SB-216763 for 18 days show oil red-O-positive cells (arrow) in control, but not in
treated cultures. (B) Cultures of hMSCs (47-year-old man) were treated with different
concentrations of SB-216763 for 18 days. Bars indicate the numbers of oil red-O-positive
cells per mm^2, presented as mean ± S.D. of 4 replicate wells. (C) Cultures of hMSCs (60-
year-old man) were treated with 5 μM SB-216763 for the first 1, 2, 3, 5, 7, days or the entire
18 days. Bars indicate the numbers of oil red-O-positive cells per mm^2, enumerated at day
18, presented as mean ± S.D. of triplicate wells. Asterisks indicate significance (**p<0.01)

of treatments compared with control (no exposure to SB-216763). (D) Western immunoblot shows that β-catenin protein in hMSCs was absent in cells transfected with β-catenin siRNA, but not control siRNA. (E) Phase-contrast photomicrograph shows that there was spontaneous generation of lipid-containing adipocytes (arrows) in control medium in hMSCs transfected with β-catenin siRNA, but not in control hMSCs.