The roles of Wnt antagonists Dkk1 and sFRP4 during adipogenesis of human adipose tissue-derived mesenchymal stem cells

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Abstract. *Objectives:* The canonical Wnt signalling pathway performs an important function in the control of adipogenesis. However, the mechanisms and mediators underlying these interactions have yet to be defined in detail. Thus, this study was performed in order to elucidate the roles of the Wnt family during adipogenic differentiation of human adipose tissue-derived mesenchymal stem cells (hAMSCs). *Materials and methods:* We assessed several members of the Frizzled (FZD) family, the receptors of Wnts, inhibitors including the secreted frizzled-related protein (sFRP) family and Dickkopfs (Dkks), and the downstream factor, β -catenin. Expressional levels of adipogenic markers regulated by the small interfering RNA of Dkk1 (siDkk1) and sFRP4 (sisFRP4) were assessed using real-time quantitative PCR and Western blot analysis. *Results:* The mRNA level of Dkk1 was expressed abundantly in the early stages of adipogenesis and decreased rapidly during the late stages of adipogenesis. However, sFRP4 mRNA was up-regulated gradually during adipogenic differentiation in hAMSCs. Expression of FZD1, FZD7 and β -catenin were reduced during adipogenic differentiation. Transfection of hAMSCs with siDkk1 or sisFRP4 partially inhibited differentiation of hAMSCs into adipocytes and restored levels of β -catenin. *Conclusions:* We determined that Dkk1 was up-regulated transiently in the early stages of adipogenesis, and that sFRP4 levels increased gradually during adipogenesis *via* inhibition of Wnt signalling. Collectively, these results show that Dkk1 and sFRP4 perform an important function in adipogenesis in hAMSCs.

INTRODUCTION

Increases in numbers of fat cells are observed in cases of severe human obesity, and obesity is a prevalent health hazard in industrialized countries (Kuczmarski *et al.* 1994), associated closely

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with a number of pathological disorders, including non-insulin-dependent diabetes, hypertension, cancer and atherosclerosis (Bray 2004; Naaz *et al.* 2004). With regard to these health implications, the need to develop novel and effective strategies for the control of obesity has become increasingly acute. Understanding signals that regulate the balance proliferation of existing pre-adipocytes and differentiation of new adipocytes may constitute a novel process for prevention of obesity and its related complications. However, in order to develop better control strategies, an increased understanding of the molecular mechanisms that initiate differentiation of pre-adipocytes and stem cells into adipocytes in humans remains necessary.

Mesenchymal stem cells (MSCs) are thought to be multipotent cells that possess the ability to differentiate into cells of multiple tissue lineages, including chondrocytes, osteocytes, adipocytes, myocytes and neuronal cells (Prockop 1997; Pittenger *et al.* 1999; Deans & Moseley 2000; Woodbury *et al.* 2000). In addition, MSCs can be readily acquired from a variety of sources (Erices *et al.* 2000; In 't Anker *et al.* 2003; Katz *et al.* 2005; Yen *et al.* 2005) and are currently being used in the development of gene therapy and tissue engineering (Hamada *et al.* 2005; Risbud & Shapiro 2005). The results of several recent studies have indicated that human adipose-derived stem cells are multipotent and are capable of undergoing *in vitro* differentiation into adipocytes as well as other mesenchymal cell lineages (Halvorsen *et al.* 2001; Sen *et al.* 2001). Human adipose tissue-derived mesenchymal stem cells (hAMSCs), therefore, are a special cell model with which human adipogenesis can be studied and human molecular regulation of hAMSCs has yet to be thoroughly characterized, as compared with murine adipogenesis.

Differentiation of stem cells into adipocytes is regulated by a variety of endocrine and paracrine factors, although the manner in which these factors perform remains poorly understood. Recently, several reports have suggested that Wnt is one of the principal factors (Ross *et al.* 2000; Bennett *et al.* 2003; Kanazawa *et al.* 2005).

The Wnt family includes more than 20 cysteine-rich secreted glycoproteins (Miller 2002) that function in both autocrine and paracrine manners to direct pattern specification during embryogenesis and adult tissue remodelling (Wodarz & Nusse 1998; Logan & Nusse 2004). In addition, these Wnt family members perform important roles in covering generation of cell polarity, specification of cell fate, and regulation of proliferation and differentiation (Moon *et al.* 2004; Yu *et al.* 2006). In the canonical pathway, Wnts bind to the Frizzled-LRP5-LRP6 receptor complex, and Disheveled (Dvl) is activated, subsequently inhibiting phosphorylation of β -catenin, which accumulates within the cytoplasm then translocates to the nucleus, where it binds to the lymphoid enhancer factor/T-cell-specific transcription factor (LEF/TCF) family of transcription factors and induces target gene expression (Li *et al.* 1999). Wnt signalling is controlled by soluble extracellular antagonists, including secreted Frizzled-related proteins (sFRPs), Wnt inhibitory factor-1 (WIF-1), Cerberus and Dickkopfs (Dkk) (Logan & Nusse 2004). sFRPs, WIF-1 and Cerberus function as competitive inhibitors of the Frizzled receptors via sequestration Wnt factors and can therefore block both canonical and non-canonical Wnt pathways. Dkk1, by way of contrast, binds to Wnt co-receptors LRP 5 and 6, thereby blocking canonical Wnt signalling (Bafico *et al.* 2001; Mao *et al.* 2001). With regard to adipogenesis, it has been demonstrated that Wnt 5b may promote adipogenesis in 3T3-L1 cells, at least in part by antagonizing the canonical Wnt/ β -catenin pathway (Kanazawa *et al.* 2005), and Wnt 10b has been shown to prevent differentiation of 3T3-L1 cells via inhibition of the expression of adipogenic transcription factors CCAAT/enhancer binding protein- α (C/EBP α) and peroxisome proliferator-activated receptor gamma (PPAR γ) (Bennett *et al.* 2003). In addition, suppression of Dkk1 and sFRP2 expression by the vitamin D receptor (VDR) may up-regulate canonical Wnt signalling pathway and inhibit adipogenesis of murine bone marrow stromal cells (Cianferotti & Demay 2007). The VDR inhibits adipogenesis of bone marrow-derived MSCs by suppressing

expression of *Dkk1* and *sFRP2* in the murine canonical Wnt signalling pathway. The coordinated regulation of *Dkk1* and its receptors, additionally, might facilitate pre-adipocyte differentiation via inhibition of Wnt signalling in humans (Christodoulides *et al.* 2006). Indeed, these results indicate that the Wnt signalling pathway, and in particular, Wnt antagonists play a potentially crucial role in adipogenesis.

In the current study, we have demonstrated for the first time that *Dkk1* and *sFRP4*, both extracellular Wnt antagonists, may facilitate human adipogenesis of hAMSCs, in part, via inhibition of canonical Wnt signalling.

MATERIALS AND METHODS

Isolation and culture of hAMSCs

Human adipose tissue-derived mesenchymal stem cells were isolated from freshly excised human mammary fat tissue, which was obtained at the time of surgery from women undergoing reduction mammoplasty for cosmetic reasons, and all procedures were previously approved by the institutional review board of Seoul National University (IRB No. 0611/001-001). For primary hAMSC cultures, the adipose tissues were washed with equal volumes of phosphate-buffered saline (PBS), and tissues were minced and digested for 2 h with collagenase type I (1 mg/mL) at 37 °C. These were washed with PBS and centrifuged 5 min at 192 *g* in order to obtain a pellet. The pellet was then filtered through 100- μ m nylon mesh to remove any cell debris and then incubated overnight at 37 °C with 5% humidified CO₂ in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS). After 24 h, unattached cells and residual non-adherent red blood cells were removed by washing with PBS, and the cell medium was exchanged with K-NAC medium (Lin *et al.* 2005). K-NAC medium used was modified MCDB 153 medium (Keratinocyte-SFM, Gibco-Invitrogen Corporation, Paisley, UK) supplemented with N-acetyl-L-cysteine (NAC; Sigma A8199, St. Louis, MO, USA) (2 mM) and L-ascorbic acid 2-phosphate (Asc 2P; Sigma A8960) (0.2 mM); calcium concentration in the medium was 0.09 mM. Growth factors and hormones added to this medium were recombinant epidermal growth factor (5 ng/mL), bovine pituitary extract (50 μ g/mL), insulin (5 μ g/mL) and hydrocortisone (74 ng/mL). Media were changed at 48 h intervals until the cells became confluent. When the cells had achieved more than 90% confluence, they were generated for trypsinization and storage in liquid nitrogen or for subculturing.

Flow cytometry analysis

The specific surface antigens of hAMSCs in the cultures of passages 3–5 were characterized by flow cytometry analysis. Cells in culture were trypsinized and stained with fluorescein isothiocyanate (FITC) – or phycoerythrin (PE)-conjugated antibodies against CD90, CD105, CD34, CD45, CD14, CD29 and HLA-DR (BD Bioscience, San Diego, CA, USA). Thereafter, cells were analysed using a Becton Dickinson flow cytometer (Becton Dickinson, San Jose, CA, USA).

In vitro differentiation assay for hAMSCs

Human adipose tissue-derived mesenchymal stem cells were initially cultured and propagated in K-NAC medium with 5% FBS and then changed to adipogenic medium (DMEM supplemented with 5% FBS, 1 μ M dexamethasone, 10 μ M insulin, 200 μ M indomethacin, 0.5 mM isobutylmethylxanthine), osteogenic medium (DMEM supplemented with 5% FBS, 50 μ M L-ascorbate-2-phosphate,

0.1 μ m dexamethasone and 10 mM glycerophosphate) or chondrogenic medium (α -modified minimum essential medium supplemented with 2% FBS, 10 ng/mL transforming growth factor- β 1, 50 μ M L-ascorbate-2-phosphate, 6.25 μ g/mL insulin) for 3 weeks. Chondrogenic differentiation was induced via the micromass culture technique (Denker *et al.* 1995). Adipogenic differentiation was evaluated using Oil Red O staining as an indicator for intracellular lipid accumulation. After being photographed, elutes of Oil Red O were obtained from the cultures using 100% isopropyl alcohol, and were quantified with an ELISA plate reader (EL800, Bio-Tek Instruments Inc., Winooski, VT, USA) at OD500. Osteogenic differentiation was noted by positively staining with von Kossa stain, which is specific for calcium. Chondrogenic differentiation was assessed via toluidine blue staining. In order to induce neurogenesis, the hAMSCs were exposed to neuronal pre-induction media consisting of 10% FBS and 1 mM β -mercaptoethanol in DMEM. After 24 h, the cells were washed three times with PBS and then incubated for 5 h in 100 μ M butylated hydroxyanisole and 1% dimethyl sulfoxide in DMEM, in accordance with established protocols (Woodbury *et al.* 2000), then analysed by immunocytochemical staining with antibodies for neuronal class III β -tubulin (Tuj-1), glial fibrillary acidic protein (GFAP), and microtubule-associated protein 2 (MAP2).

Immunofluorescence

Cells were fixed for 20 min in 4% paraformaldehyde and permeabilized for 10 min with 0.2% Triton X-100 (Sigma). Then they were pre-incubated for 1 h with normal goat serum (10%) (Zymed Laboratories Inc., San Francisco, CA, USA). Cells were subsequently stained with antibodies against GFAP (1 : 200; Chemicon, Temecula, CA, USA), MAP2 (1 : 200; Chemicon), and Tuj-1 (1 : 200; Chemicon) followed by 1 h of incubation with anti-Alexa 488- or anti-Alexa 594 (1 : 1000)-labelled secondary antibody (Molecular Probe, Eugene, OR, USA). Nuclei were stained with Hoechst 33238 dye (1 μ g/mL; 10 min) following incubation with secondary antibody. Images were captured using a confocal microscope (Nikon, Eclipse TE200, Tokyo, Japan).

Nuclear extracts

Preparation of nuclear extracts was conducted using Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) in accordance with the manufacturer's recommendations. In brief, cells were washed with 4 mL of ice-cold PBS/phosphatase inhibitors, lysed in 500 μ L hypotonic buffer and then centrifuged for 30 s at 14 000 g at 4 °C. Cell pellets then were re-suspended in 50 μ L complete lysis buffer and centrifuged for 10 min at 14 000 g at 4 °C, and supernatants (nuclear fraction) were saved.

Western blot analysis

Cells were lysed with buffer (150 mM NaCl, 20 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid) containing protein inhibitors (1 g/mL aprotinin, 1 μ M leupeptin, 1 mM phenylmethylsulphonyl fluoride), and protease inhibitors (1 mM NaOV₃, 1 mM NaF). Collected proteins were separated using 10–15% SDS-PAGE, transferred to nitrocellulose, incubated with antibody to β -catenin (1 : 1000; Cell Signaling, Beverly, MA, USA), lamin A (1 : 1000; Abcam, Cambridge, UK), FABP4 (1 : 1000; Abcam), PPAR γ (1 : 1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), β -actin (1 : 10 000; sigma) and C/EBP α (1 : 1000; cell signalling), and detected via chemiluminescence.

RNA interference

Transfection of siRNA into the cells was conducted when they had reached 70% confluence. The small interfering RNAs of Dkk1 (siDkk1, J-003843-11), sFRP4 (sisFRP4, J-011388-07) and

non-targeting control (siControl #1, D-001810-01) were purchased from Dharmacon (Chicago, IL, USA). Experiments were conducted using DharmaFECT1 (Dharmacon) as transfection agent and siRNA at a concentration of 100 nmol/L. For mRNA or Western blot analysis, cells were transfected with target gene siRNA or control non-targeting siRNA using DharmaFECT1. After 24 h, medium was changed and the cells were treated with or without adipogenic medium.

MTT proliferation assay

The effect of the siDkk1 transfection on hAMSC proliferation was measured by MTT assay, based on the ability of live cells to convert tetrazolium salt into purple formazan. The cells were seeded in 6-well microplates and incubated overnight. Then they were transfected with siDkk1 or siControl for 24 h. After culture for the indicated periods (Fig. S1, Supporting Information), 100 μ L of MTT stock solution (5 mg/mL; Sigma) was added to each well and further incubated for 4 h at 37 °C. Supernatants were removed and 500 μ L of dimethyl sulfoxide was added to each well to dissolve the water-insoluble purple formazan crystals, and then transferred into 96-well microplates for reading. Absorbance at a wavelength of 540 nm was measured with the EL800 microplate reader (Bio-Tek Instrument Inc.).

Reverse transcriptase (RT)-PCR and real-time quantitative PCR

Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA). In brief, 500 ng of total RNA was transcribed into cDNA using AMV reverse transcriptase (Takara, Ohtsu, Japan). The polymerase chain reaction (PCR) was conducted in a 20- μ L reaction mixture containing 1 μ L of cDNA templates and 0.5 μ M oligonucleotide primers. Primers were designed using Primer3 input software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/primer3_www.cgi) and specificity of each primer was controlled with BLAST software. Primer sequences and length of the PCR products are provided in Table 1. Real-time quantitative PCR was conducted using an SYBR green PCR kit (Applied Biosystems, Foster City, CA, USA) and validated with an Applied Biosystem 7500 (Applied Biosystems) real-time quantitative PCR system. Primers employed in this study are provided in Table 2. The comparative method of relative quantification ($2^{-\Delta\Delta C_t}$) was utilized to calculate expression levels of each target gene (normalized to β -actin).

Statistical analysis

All experiments were conducted at least in triplicate ($n = 3$), and results are expressed as the mean $\pm$ SD. Statistical analysis was conducted via analysis of variance, followed by Duncan's multiple range tests.

RESULTS

Characterization of hAMSCs in culture

Human adipose tissue-derived mesenchymal stem cells were cultured in DMEM with 10% FBS, and the majority of the cells remained in suspension, including cell aggregates and erythrocytes. In order to remove suspended cells, the culture was washed three times with PBS, after which a few attached single cells or cell clumps were observed. In order to facilitate proliferation and extended lifespan of the hAMSCs, media were exchanged with K-NAC medium with 5% FBS. In K-NAC medium, attached cells actively proliferated and reached approximately 90%

Table 1. Primers used for PCRs and products length (bp)

Gene	Primers	bp
C/EBP α	F: CACCTGCAGTTCCAGATCG R: GTACTCGTTGCTGTTCTGTCCAC	243
C/EBP β	F: ACTTCTACTACGAGGCGGACTG R: GAGAAGAGGTCGGAGAGGAAGT	286
C/EBP δ	F: GACTCAGCAACGACCCATACC R: TGCTCAGTCTTTTCCTCTTAT	317
PPAR γ	F: AGACATTCCATTACACAAGAACAGA R: TGAAGTCCATAGTGAAATCCAGAA	225
sFRP1	F: GGTCATGCAGTCTTCGGCT R: TCCTCAGTGCAAACCTCGCTG	206
sFRP2	F: ACCGAGGAAGCTCCAAAGGTAT R: TCATCTCCTCACAGGTGCACTG	259
sFRP3	F: CTCATCAAGTACCGCCACTCGTG R: CCGGAAATAGGTCTTCTGTGTAGCTC	210
sFRP4	F: TGGTGGATGTAAAAGAGATCTTCA R: CTCTCTTCCCACTGTATGGATCTT	220
Dkk1	F: GTCCAAGATCTGTAAACCTGTCCT R: AGCCTAGAAGAATTACTGGCTTGA	162
Dkk3	F: CCATCCATGTGCACCGAGAAATTCAC R: TCCCAGCAGTGCAGCGGCGGCAGC	216
GAPDH	F: TCATTGACCTCAACTACATGGTTT R: TGAGGCTGTTGTCATACTCTCAT	324

F, forward; R, reverse.

confluence in 75-cm² flasks. Cell phenotype was fibroblast-like or spindle-shaped morphology. The hAMSCs employed in this study were obtained from third to fifth passages (Fig. 1a).

In order to characterize the hAMSCs, we initially analysed surface antigen characteristics of isolated hAMSCs by flow cytometry. These results indicated that the surface antigens common to MSCs, including CD29, CD105 and CD90 (Pittenger *et al.* 1999; Dennis & Charbord 2002) were expressed in the hAMSCs. However, haematopoietic surface markers, including CD14, CD34, CD45, CD133 and HLA-DR, were absent (Fig. 1f). Furthermore, in order to confirm multilineage differentiation ability, the hAMSCs were induced into osteocytes, chondrocytes, adipocytes and neuronal cells, indicating that they were capable of multilineage differentiation as shown by the results of von Kossa, toluidine blue and Oil Red O staining, respectively (Fig. 1b–d). hAMSC-differentiated neuron-like cells also stained positively for the neuronal markers of MAP2, GFAP and Tuj-1. These results indicated that the isolated cells had the previously reported properties of MSCs (Pittenger *et al.* 1999).

Expression of early and late adipogenesis-related genes during adipogenic differentiation in hAMSCs

In order to distinguish between early and late stages of adipogenesis in hAMSCs, we assessed the expression of several adipogenic markers (C/EBP α , PPAR γ and FABP4: late adipogenic differentiation stage markers, C/EBP β and C/EBP δ : early adipogenic differentiation stage markers) during adipogenic differentiation (Morrison & Farmer 1999), by RT-PCR. Levels of mRNA expressions of the C/EBP α , PPAR γ and FABP4 genes were shown to have gradually

Table 2. Primers used for real-time quantitative PCRs and products length (bp)

Gene	Primers	bp
sFRP4	F: CTGCCCCATCAAGATGTTCT R: ATCATCCTTGAACGCCACTC	53
Dkk1	F: CCTTGGATGGGTATTCCAGA R: CCTGAGGCACAGTCTGATGA	103
PPAR γ	F: TTCAGAAATGCCTTGCAGTG R: CCAACAGCTTCTCCTTCTCG	84
C/EBP α	F: TGGACAAGAACAGCAACGAG R: TTGTCACTGGTCAGCTCCAG	130
β -Catenin	F: GCCGGCTATTGTAGAAGCTG R: GAGTCCCAAGGAGACCTTCC	152
LRP5	F: GCCCTACATCATTCGAGGAA R: GGGTGGATAGGGGTCTGAGT	139
LRP6	F: CCCATGCACCTGGTTCTACT R: CCAAGCCACAGGGATACAGT	114
FZD1	F: GTGAGCCGACCAAGGTGTAT R: AGCACAGCACTGACCAAATG	93
FZD4	F: TGGGCACTTTTTCGGTATTC R: TGCCCACCAACAAAGACATA	82
FZD6	F: CGATAGCACAGCTGCAATA R: ACGGTGCAAGCCTTATTTTG	87
FZD7	F: CGCCTCTGTTCGTCTACCTC R: CCATGAGCTTCTCCAGCTTC	126
β -Actin	F: GGCATCCTCACCCTGAAGTA R: AGGTGTGGTGCCAGATTTTC	82

F, forward; R, reverse.

increased after 48 h of adipogenic differentiation, whereas those of C/EBP β and C/EBP δ were temporarily increased from 3 to 48 h and then declined (Fig. 2a). We also demonstrated, by Western blot analysis, that the protein level of FABP4 was higher after 48 h of adipogenic induction (Fig. 2b). Collectively, our results indicated that the early adipogenic differentiation stage progressed until 48 h of adipogenic differentiation and the late differentiation stage occurred after 48 h of adipogenic differentiation.

Expression of Wnt antagonist during adipogenic differentiation in hAMSCs

It has been demonstrated that Wnt signalling performs an important function in adipogenesis and that the Wnt antagonist Dkk1 can act, in part, as a regulator of adipogenesis in human pre-adipocytes. We hypothesized that the other Wnt antagonists might also participate in adipogenesis of hAMSCs. In order to confirm this hypothesis, we assessed Wnt antagonist mRNA levels by RT-PCR. Dkk1 mRNA expression was up-regulated transiently in early adipogenesis; however, sFRP4 mRNA expression increased gradually during adipogenesis in hAMSCs. sFRP1, sFRP2, sFRP3 and Dkk3 did not produce any significant differences during the adipogenic induction period (Fig. 3).

Expression of Wnt receptors during adipogenic differentiation in hAMSCs

As Wnt antagonists have been shown to utilize the connection of receptors, such as FZD1, FZD4, FZD6, FZD7, LRP5 and LRP6 to inhibit Wnt signalling (Heller *et al.* 2003; Logan & Nusse

Figure 1. Characterization of isolated hAMSC. (a) Phase-contrast microscopy of the spindle-shape morphology of hAMSCs in the third passage. (b) Osteogenic differentiation of hAMSC: hAMSCs were cultured for 3 weeks in osteogenic induction medium, and calcium deposits were visualized via von Kossa staining. (c) Chondrogenic differentiation of hAMSC: hAMSCs were cultured for 3 weeks in chondrogenic medium, and were stained with toluidine blue. (d) Adipogenic differentiation of hAMSC: hAMSCs were cultured for 3 weeks in adipogenic induction medium. Lipid droplets were visualized by oil red O staining. Bar represents 100 μ m. (e) Neurogenic differentiation: immunostaining results for MAP2, GFAP and Tuj-1. Bar represents 20 μ m. (f) FACS analysis of hAMSC: the results showed that hAMSCs expressed CD90, CD105 and CD29 but did not express CD14, CD133, CD34, CD45 and HLA-DR. The experiments were repeated three times.

2004), and thus we assessed the expression of these receptors during adipogenic differentiation in hAMSCs. Figure 4 demonstrates that there was little change in the expression of LRP6 and FZD6 mRNA over 7 days of adipogenesis. However, the mRNA expression level of FZD1 and FZD7 was up-regulated during the early stage of adipogenesis for up to 48 h and down-regulated thereafter. The expression level of FZD4 and LRP5 mRNA continuously increased throughout the entire period of adipogenesis in hAMSCs.

Knockdown of Dkk1 or sFRP4 inhibit adipogenic differentiation in hAMSCs

In order to evaluate whether Dkk1 and sFRP4 regulate adipogenesis of hAMSCs, we attempted to determine whether siRNA transfection specific for Dkk1 or sFRP4 could interrupt their adipogenesis. Since Dkk1 may affect proliferation of MSCs (Gregory *et al.*, 2003), we conducted proliferation assay using MTT. The data showed no significant difference between siControl and

Figure 2. mRNA expression of several adipogenesis-related genes were evaluated by RT-PCR during adipogenic differentiation in hAMSCs. (a) RT-PCR results: C/EBP α , C/EBP β , C/EBP δ , PPAR γ and FABP4 mRNA expression during adipogenesis. hAMSCs were induced to differentiate and total RNA was isolated from the cell extracts at the indicated time points. GAPDH was utilized as a loading control. (b) Western blotting results: FABP4 protein expression during adipogenesis. Cells were induced to differentiate and the whole cell lysates were acquired at the indicated time points. β -Actin was utilized as a loading control. Twenty-five micrograms of protein was loaded into each well. The results were representative of at least two independent experiments. Zero hour indicates the onset of differentiation; 3 h, 6 h, 12 h, 24 h, 48 h, 1 week and 2 weeks, respectively, indicate 3 h, 6 h, 12 h, 24 h, 48 h, 1 week, and 2 weeks' post-induction of differentiation.

Figure 3. Expression of Wnt antagonist during adipogenesis in hAMSCs. The expression pattern along the differentiation process of the indicated gene. hAMSCs were induced to differentiate and the total RNA was isolated from cell extracts at the indicated time points. Gene expression was evaluated via RT-PCR. GAPDH was utilized as a loading control. The experiments were repeated three times.

siDkk1 at 100 nM dose (Fig. S1). As is shown in Fig. 5a, a 7-day treatment with adipogenic induction media induced adipogenesis in the control group and the control siRNA-transfected group. However, transfection with siDkk1 or siSFRP4 was shown to reduce the degree of adipocytic differentiation, as assessed by changes in cell morphology and lipid droplets visualized by Oil

Figure 4. Expression of Dkks and sFRPs receptors is regulated during human adipogenesis. Total RNA was employed for real-time quantitative PCR analysis of LRP5, LRP6, FZD1, FZD4, FZD6 and FZD7. Normalization was conducted with primers specific for β -actin. The indicated values are expressed as the means $\pm$ standard deviation for three independent experiments.

Red O staining. Quantification of Oil Red O staining demonstrated diminutions of approximately 1.5-fold and 2-fold in lipid accumulation in the siDkk1- and siFRP4-transfected cells, respectively, as compared to the siControl-transfected cells (Fig. 5b). We also assessed the effects of Dkk1 or sFRP4 on expression of adipocyte markers utilizing real-time quantitative PCR. Utilizing mRNA prepared from siRNA-non-transfected cells or siRNA-transfected cells prior to and after induction of adipogenic differentiation, we conducted real-time quantitative PCR analysis for C/EBP α and PPAR γ , two primary transcription factors for adipogenesis. At 7 days post-induction, hAMSCs transfected with siDkk1 had reduced mRNA levels of C/EBP α (2-fold) and PPAR γ (1.3-fold) as compared to the siControl group. Transfection of hAMSCs with siFRP4 could also reduce expression levels of C/EBP α and PPAR γ mRNAs by 3.5-fold and 2-fold, respectively, as compared to the siControl group (Fig. 6a). In an effort to verify the changes in C/EBP α and PPAR γ expression in terms of the protein level, we conducted Western blot analysis. As had been expected, expression levels of C/EBP α and PPAR γ proteins were also down-regulated via siDkk1 or siFRP4 transfection as compared to the siControl group (Fig. 6b). Collectively, adipogenic differentiation of hAMSCs and expression of adipocyte markers, C/EBP α and PPAR γ , were suppressed by the siRNA inhibition of Dkk1 or, to a greater extent, sFRP4.

Dkk1 and sFRP4 correlate with the inhibition of canonical Wnt signalling during adipogenic differentiation in hAMSCs

In these studies, we noted that Dkk1 and sFRP4, two inhibitory ligands against Wnt signalling, were expressed during adipogenesis and performed a regulatory function in the later stages of differentiation of hAMSCs into adipocytes. In order to determine whether Wnt signalling was affected by inhibition of ligands mentioned above, we conducted real-time quantitative PCR for β -catenin, a downstream effector of canonical Wnt signalling. The expression level of β -catenin mRNA and its protein, also substantially decreased, up to day 7 of the adipogenic induction period, as is shown in Fig. 6. Transient inhibition of Dkk1 or sFRP4 with the respective specific siRNA was shown to restore β -catenin expression (Fig. 6). These data indicated that Dkk1 and sFRP4 could facilitate adipogenic differentiation in hAMSCs by the inhibition of canonical Wnt signalling.

Figure 5. Knockdown of *Dkk1* or *sFRP4*, inhibited adipogenic differentiation of hAMSCs. (a) The cells were transfected with siDkk1, siFRP4 or siControl. After transfection, the cells were induced to differentiate for seven days and were stained with Oil Red O staining to detect lipid accumulation. The images are presented at $\times 100$ magnification. Bar represents 100 μm . (b) Oil Red O staining of accumulated lipids was quantified via measurements of OD500. The indicated values are expressed as the means $\pm$ standard deviation for three independent experiments. Asterisks indicate statistically significant differences ($*P < 0.05$).

DISCUSSION

As *in vitro* systems, various pre-adipose cell lines and primary cultures of adipose-derived stromal vascular cells have been successfully employed for adipogenic differentiation studies (Kanazawa *et al.* 2005; Choi *et al.* 2007). In addition to these models, embryonic stem cells have been demonstrated to differentiate into mature adipocytes *in vitro* (Dani *et al.* 1997; Li *et al.* 2007). In this study, we demonstrated that hAMSCs are capable of differentiating into adipocyte, chondrocyte, osteocyte and neuronal-like cells; also, we demonstrated *in vitro* regulation of adipogenesis, using a hAMSC model. We assessed the time-course differentiation procedure biochemically via quantitative expression analysis of mRNAs encoding for PPAR γ and C/EBP α , two well-described adipocyte differentiation markers. hAMSCs underwent terminal differentiation after 7 days of adipogenic induction. In addition, we were able to distinguish the early adipogenic differentiation stage up to 48 h post-induction on the basis of the elevated expression of the pre-adipocyte markers, C/EBP β and C/EBP δ . The late differentiation stage occurred after 48 h of adipogenic differentiation.

While evidence has been discovered to show that the Wnt signal pathway is crucial to murine adipogenesis (Ross *et al.* 2000; Bennett *et al.* 2003; Kawai *et al.* 2007), only a few recent studies have demonstrated that Wnt signalling may contribute to adipogenic differentiation in murine bone marrow stromal cells (Cianferotti & Demay 2007; Shang *et al.* 2007). In particular, the specific roles of Wnt signalling in adipogenic differentiation in human adult stem cells have yet to be thoroughly elucidated.

Canonical Wnt signalling is negatively regulated by Dkks and sFRPs. These proteins directly bind to Wnts (sFRPs), or to FZD receptors (sFRPs), or to interact with LRP6 (Dkks). Inhibitors such as sFRPs that bind to Wnts or FZD receptors harbour the ability to blunt all Wnt-mediated pathways (Jones & Jomary 2002), whereas Dkks only suppress the canonical pathway (Zorn 2001).

Among Wnt antagonists, we observed in this study that *Dkk1* and *sFRP4* were expressed abundantly during hAMSC adipogenesis. We also demonstrated that the expression of *Dkk1* was transiently up-regulated during the early stages and *sFRP4* increased gradually during the late stages of adipogenic differentiation in hAMSCs. These results are correlated with the inhibition of canonical Wnt signalling, as demonstrated by inhibition of β -catenin expression. We also observed the coordinated down-regulation of the receptors FZD1 and FZD7, representative transmembrane receptors of the Wnt ligands, which was likely to potentiate the effect of *sFRP4* on the inhibition of Wnt signalling. In addition, up-regulation of LRP5 was observed, which was also thought to be likely to potentiate the ability of *Dkk1* to inhibit Wnt signalling. These results indicate that *Dkk1*, *sFRP4* and their receptors are reciprocally regulated during adipogenic differentiation in hAMSCs.

We also determined that knockdown of *Dkk1* and *sFRP4* could modulate adipogenic differentiation as demonstrated by its ability to inhibit morphological changes and lipid accumulation in hAMSCs after transfection with si*Dkk1* or si*sFRP4*. This appeared likely to occur due to rescue from inhibition of canonical Wnt signalling, as this was accompanied by recovery of nuclear β -catenin levels. Specific differentiation into a variety of target tissues is regulated by a series of signalling pathways, of which canonical Wnt signalling is believed to be a crucial regulator (Kleber & Sommer 2004; Reya & Clevers 2005). Moreover, recent studies have shown that Wnt signalling controls specific differentiation into adipocytes (Ross *et al.* 2000; Christodoulides *et al.* 2006). This implies that Wnt signalling performs a crucial role in the determination of mesodermal cell fate (Ross *et al.* 2000). Other reports have shown that

activated β -catenin prevents adipogenic differentiation and induces early osteoblast differentiation in C3H10T1/2 MSC cells (Bain *et al.* 2003; Mbalaviele *et al.* 2005). In this study, we have demonstrated that the activation of Wnt signalling via knockdown of Dkk1 and sFRP4 inhibited adipogenesis in hAMSCs; hence, Wnt signalling may direct mesenchymal stem cell fate towards adipogenesis, and this process appears to be regulated by Dkk1 and sFRP4. In addition, Wnt signalling may influence proliferation, survival and migration of adult stem cells. Thus, the biological roles of Wnt signalling in adult stem cells should be further clarified in order to avoid possible detrimental adverse effects in stem cell-based treatment (Luo *et al.* 2007).

In summary, we assessed the effects of Wnt signalling on adipogenic differentiation of hAMSCs. To the best of our knowledge, this is the first study to demonstrate that Dkk1 and sFRP4, both of which are extracellular Wnt antagonists, may contribute to adipogenic differentiation via inhibition of Wnt signalling in hAMSCs. These experiments provide preliminary insights into the regulators of adipogenesis in humans. Moreover, molecular regulation of adipogenesis in hAMSCs may provide us with a better understanding of obesity and may guide development of novel treatments.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. MTT proliferation assay in hAMSCs transfected with siDkk1 or siControl.

Cell proliferation was measured by a MTT assay. hAMSCs were transfected with siDkk1 or siControl. MTT assay was conducted (a) immediately after transfection or (b) after adipogenic induction for 2 days following transfection. Numbers represent the absorbance (optical density) at 540 nm. Values indicated are given as means $\pm$ SD for three independent experiments. The difference between si-DKK1- and siControl-transfected groups was not statistically significant.

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