Adult human mesenchymal stem cells from bone marrow stroma (hMSCs) differentiate into numerous mesenchymal tissue lineages and are attractive candidates for cell and gene therapy. When early passage hMSCs are plated or replated at low density, the cultures display a lag phase of 3–5 days, a phase of rapid exponential growth, and then enter a stationary phase without the cultures reaching confluence. We found that as the cultures leave the lag phase, they secrete high levels of dickkopf-1 (Dkk-1), an inhibitor of the canonical Wnt signaling pathway. The addition of recombinant Dkk-1 toward the end of the lag period increased proliferation and decreased the cellular concentration of β-catenin. The addition of antibodies to Dkk-1 in the early log phase decreased proliferation. Also, expression of Dkk-1 in hMSCs decreased during cell cycle arrest induced by serum starvation. The results indicated that high levels of Dkk-1 allow the cells to reenter the cell cycle by inhibiting the canonical Wnt/β-catenin signaling pathway. Since antibodies to Dkk-1 also increased the lag phase of an osteosarcoma line that expressed the gene, Dkk-1 may have a similar role in some other cell systems.

Human bone marrow contains two main populations of stem cells: hematopoietic stem cells usually identified by CD 34 progenitor cells and a population of CD 34− cells of mesenchymal origin. The population of human nonhematopoietic mesenchymal stem cells or marrow stromal cells (hMSCs) can differentiate into numerous mesenchymal tissue lineages including osteoblasts, chondrocytes, adipocytes, and neural precursors (1–8). hMSCs are easily obtained from bone marrow aspirates and are readily separated from hematopoietic cells by virtue of their adherence to tissue culture plastic (1). Under the appropriate conditions, hMSCs can be propagated manifold in vitro while retaining their multipotentiality, a feature that makes them attractive candidates for stem cell and gene therapy (2, 5, 9–12). Although some of the in vitro growth characteristics of hMSCs have been documented, the molecular mechanisms by which hMSCs regulate their own growth in culture are poorly understood. In particular, there is no apparent explanation for the observation that when early passage hMSCs are replated at low density, they display a lag period of 3–5 days, followed by a phase of rapid exponential growth, and then enter a stationary phase without reaching confluence (8, 11, 13).

Preliminary observations (15) suggested to us that conditioned medium from cultures of hMSCs increased the rate of proliferation when added to freshly plated cultures of hMSCs. In the experiments described here, we demonstrate that hMSCs in the early log phase of growth synthesize and secrete dickkopf-1 (Dkk-1), an inhibitor of the canonical Wnt pathway (16–18). The Wnt signaling pathway controls patterning and cell fate determination in the development of a wide range of organisms, from Drosophila to mammals (19). The signaling can occur by at least three different pathways (20). In the canonical pathway, Wnt ligands bind to the transmembrane receptor frizzled and the co-receptor lipoprotein-related proteins 5 and 6 (LRP-5/6). Activation of frizzled recruits the cytoplasmic bridging molecule, disheveled, so as to inhibit glycogen synthetase kinase 3. Inhibition of glycogen synthetase kinase 3 decreases phosphorylation of β-catenin, preventing its degradation by the ubiquitin-mediated pathway (20, 21). The stabilized β-catenin translocates to the nucleus, where it complexes with transcription factors that promote either differentiation or proliferation (22, 23). Also, the β-catenin stabilizes adherens junctions (24). The binding of Dkk-1 to LRP-5/6 and to the associated protein kremen dissociates LRP-5/6 from frizzled and thereby prevents the formation of a functional Wnt receptor complex (25–29). The decrease in Wnt signaling destabilizes β-catenin and inhibits Wnt-induced transcriptional regulation. This in turn can influence cell fate and cell growth and may predispose cells to apoptosis (17, 30). Signaling through Wnt ligands has been shown to have a role in differentiation of neural systems (31, 32), skeletal muscle (33), cardiac cells (34), endoderm (35), cartilage (36), and limbs (37). The effects of Wnt signaling, however, are complex in part because some members of the family of Wnt ligands can trigger signaling through noncanonical pathways involving Jun/Jun kinase (JNK) or calcium ion regulation (20). For example, alternative signaling through the canonical Wnt/β-catenin and the noncanonical Wnt/Ca2+ pathways in Xenopus produced antagonistic regulation of convergent extension movements during gastrulation. Also, alternative signaling through Wnt and two different frizzled receptors in Drosophila drove either epithelial planar polarity or morphogenesis by activating different regions of the same dishesved
bridging molecule (39). In addition, a second dickkopf-2 (Dkk-2) activated instead of inhibited Wnt/β-catenin signaling in Xenopus (40) through a pathway that was independent of disheveled (41).

The results presented here demonstrate that after stationary phase, when hMSCs are replated at clonal densities, synthesis of the Wnt inhibitor Dkk-1 allows cells to reenter the cell cycle by inhibiting the canonical Wnt/β-catenin signaling pathway.

EXPERIMENTAL PROCEDURES

Tissue Culture—Bone marrow aspirates of about 2 ml were drawn from healthy donors ranging in age from 19 to 49 years under an Institutional Review Board-approved protocol. Plastic adherent nucleated cells were collected from the aspirate and cultured as previously described (8, 14). After 14 days in culture, adherent cells were recovered from the monolayer by incubation with 0.25% (w/v) trypsin and 1 mM EDTA (Fisher) for 5–7 min at 37 °C and replated at a density of 100 cells/cm². The cells were then cultured for various times with changes of medium every 2–3 days. Cells were radiolabeled at the indicated intervals by the addition of new medium containing 5 μCi of 111In-labeled methionine (Amersham Biosciences). The cultures were allowed to incorporate the label for 48 h, followed by recovery of the cells and media. The labeling and inhibitor experiments were repeated with hMSCs from three donors. Nondishydrated cells were purchased from Sigma. Cell lines MG-63, SAOS, JAR, and JEG-3 were acquired from the American Type Culture Collection (Manassas, VA) and were expanded in accordance with their protocols. For experiments, the cells were cultured in hMSC complete medium (α-minimal essential medium, 20% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine).

Preparation of Labeled Media and Cell Extracts—To remove unwanted cells and debris, the media were filtered through a 0.22-μm pore size membrane (Millipore Corp., Bedford, MA). To remove unincorporated [35S]methionine, the medium was dialyzed against 10 volumes of PBS (Sigma) using a tangential flow filtration system fitted with 150-cm² polyvinylidene difluoride 5-kDa filters (Millipore). The samples were then cultured for various times with changes of medium every 2–3 days. Cells were radiolabeled at the indicated intervals by the addition of new medium containing 5 μCi of 111In-labeled methionine (Amersham Biosciences). The cultures were allowed to incorporate the label for 48 h, followed by recovery of the cells and media. The labeling and inhibitor experiments were repeated with hMSCs from three donors. Nondishydrated cells were purchased from Sigma. Cell lines MG-63, SAOS, JAR, and JEG-3 were acquired from the American Type Culture Collection (Manassas, VA) and were expanded in accordance with their protocols. For experiments, the cells were cultured in hMSC complete medium (α-minimal essential medium, 20% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine).

Electrophoretic Analysis and Western Blotting—Unless otherwise stated, electrophoresis was carried out using commercial reagents and systems (Novex; Invitrogen). Two μl of medium were added to 5 μl of SDS-PAGE sample buffer and 1 μl of 2-mercaptoethanol (Sigma). The samples were heated at 100 °C for 2 min and electrophoresed on a 4–12% NuPAGE bis buffer gel using the Tropixflow filtration system fitted with 150-cm² polyvinylidene difluoride 5-kDa filters (Millipore). Cells were counted with a hemacytometer, followed by lysis in PBS containing 0.01% (w/v) SDS (Sigma). The cell lysates were dialyzed against 1000 volumes of 1× PBS for 24 h using 3500-Da limiting dialysis cassettes (Pierce). Radioactivity was assayed by liquid scintillation counting using 30% scintillant (Scintisafe; Fisher).

For autoradiographic analysis, filters were air-dried and exposed to autoradiography film (Kodak Biomax MR; Sigma). After a 2-day exposure, the film was automatically developed using a commercial instrument and reagents (AGFA Corp.). For immunoblotting, filters were blocked in PBS containing 0.1% (v/v) Tween 20 (Sigma) for 1 h. For detection of β-catenin, blots were probed with an anti-β-catenin monoclonal antibody at a dilution of 1:1000 (clone 4B12; Chemicon International, Temecula, CA). For detection of Dkk-1, blots were probed in 1 μg/ml of anti-Dkk-1 polyclonal antibody (see below) followed by an anti-rabbit peroxidase-conjugated monoclonal antibody (clone RG 96; Sigma). For detection of the polyhistidine tag, blots were probed with a peroxidase-conjugated monoclonal antibody at a dilution of 1:1000 (clone M2143; Chemicon International). For detection of actin, blots were probed with a monoclonal antibody at a dilution of 1:1000 (clone AC-10; Sigma). For detection of GAPDH, blots were probed with a monoclonal antibody at a dilution of 1:1000 (clone 6C5; Chemicon International). All unconjugated monoclonal antibodies were detected with an anti-mouse horseradish peroxidase-conjugated rabbit serum (Sigma), and unless otherwise stated, polyclonal antisera were detected by either goat anti-rabbit or rabbit anti-goat horseradish peroxidase conjugates (Sigma). Positive bands were detected by chemiluminescence in accordance with a previously described procedure (42) using an imaging system to detect and quantify the signal (Typhoon Imaging System; Amersham Biosciences). Equal sample loading for Western blots was confirmed by Bradford protein assay (Sigma) and measuring cell number. Equal transfer of the membrane was confirmed by Ponceau S staining (Sigma). Western blots and autoradiographic analyses were repeated using hMSCs from at least two donors. Blots were stripped using the Blot Restore kit (Chemicon International).

Electroelution and Tryptic Fingerprinting of Bands—200 μl of 5-fold concentrated radiolabeled medium were separated by electrophoresis on a 4–20% polyacrylamide Tris-glycine preparative gel (Invitrogen). 15 fractions were laterally electroeluted into 1 ml of 100 mM ammonium bicarbonate (pH 8.0) using a whole gel eluter system (Bio-Rad). The fractions were analyzed by SDS-PAGE followed by autoradiography and rotary evaporation (Savant AES 2010 Rotary Evaporation System; Savant Inc., Holbrook, NY). Samples were proteolytically digested in 50-μl reactions containing 100 mM ammonium bicarbonate (pH 8.0) in the presence of 5 ng of agarase-coupled trypsin (Sigma). The reaction was incubated at 37 °C for 16 h followed by removal of the trypsin by centrifugation. Analysis by mass spectrometry was carried out using commercial instruments and reagents (Ciphergen Biosystems Inc., Freemont, CA). Aliquots (2 μl) of digested samples were mixed with 2 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid in acetonitrile. The mixture was air-dried onto silica-coated aluminum mass spectrometry chips and analyzed using a PBS II SELDI-TOF chip according to the manufacturer's protocol (Amersham Biosciences). A 3-ml aliquot of 5000-Da limiting dialysis cassettes (Pierce) was used to analyze eluted samples from three donors.

Antibody Production and Purification—A 15-residue peptide corre-}

sponding to a sequence in the second cysteine-rich domain of Dkk-1 (17), ARHFWSKICKPVKLE, was synthesized and conjugated to keyhole limpet hemocyanin (Sigma). The conjugated peptide was used to immunize two New Zealand White rabbits. Antibodies were purified from 20-ml aliquots of postimmune serum by affinity chromatography against the immunizing peptide. Briefly, 5 mg of peptide at a concen- tration of 1 mg/ml in 100 mM bicarbonate, pH 9.4, was coupled to a 1-ml N-hydroxysuccinimide-activated Sepharose column (Amersham Biosciences) for 16 h at a flow rate of 1 ml min⁻¹. The column was then blocked with 500 mM Tris-HCl (pH 8.0) and washed with PBS. For antibody purification, 50 ml of a 5 mg ml⁻¹ solution of postimmune rabbit serum was cycled through the peptide-coupled col- umn for 5 h. The column was then washed with 50 ml of PBS following elution of the polyclonal antibodies in 0.5-ml fractions with 100 mM glycine, pH 2.0. The fractions were adjusted to pH 7.4 with 100 mM Tris-HCl and then visualized by SDS-PAGE prior to use. Using a similar protocol, Dkk-1 was immunoaffinity-purified from 50 ml of conditioned medium by affinity chromatography using antibody-coupled N-hydroxysuccinimide-activated Sepharose 4B (Amersham Biosciences). Blots were stripped using the Blot Restore kit (Chemicon International). For detection of the polyhistidine tag, blots were probed with a peroxidase-conjugated monoclonal antibody at a dilution of 1:1000 (clone H9262; Chemicon International, Temecula, CA). For detection of Dkk-1, blots were probed in 1 μg/ml of anti-Dkk-1 polyclonal antibody (see below) followed by an anti-rabbit peroxidase-conjugated monoclonal antibody (clone RG 96; Sigma). For detection of the polyhistidine tag, blots were probed with a peroxidase-conjugated monoclonal antibody at a dilution of 1:1000 (clone M2143; Chemicon International). For detection of actin, blots were probed with a monoclonal antibody at a dilution of 1:1000 (clone AC-10; Sigma). For detection of GAPDH, blots were probed with a monoclonal antibody at a dilution of 1:1000 (clone 6C5; Chemicon International). All unconjugated monoclonal antibodies were detected with an anti-mouse horseradish peroxidase-conjugated rabbit serum (Sigma), and unless other- wise stated, polyclonal antisera were detected by either goat anti-rabbit or rabbit anti-goat horseradish peroxidase conjugates (Sigma). Positive bands were detected by chemiluminescence in accordance with a pre-
into 20 mm ammonium carbonate at pH 8.7. The pure, dialyzed protein was dried by rotary evaporation (Savant AES 2010 Rotary Evaporation System) in 10-μg aliquots and stored at −80 °C. For tissue culture studies, each aliquot was resuspended in 1 ml of α-minimal essential medium containing 10% (v/v) FCS.

**Analysis of Colony Size and Proliferation—** hMSCs were plated at about 0.6 cells/cm² and incubated in complete medium for 17 days. For direct visualization of colonies, a 5% (v/v) solution of crystal violet in methanol (Sigma) was added to tissue culture dishes previously washed twice with PBS. After 20 min, the plates were washed with distilled water and air-dried. Stained colonies with diameters of 2 mm or greater were counted. For assay of proliferation, cells were also quantified by fluorescent labeling of nucleic acids (CyQuant dye; Molecular Probes, Inc.). hMSCs were plated at 100 cells/cm² and allowed to grow for 4 days. The cells were washed with PBS, and medium was added containing the appropriate concentration of Dkk-1 and FCS. The cells were recovered by trypsinization as described above. Fluorescence analysis was carried out using a microplate fluoro-

**Quantitative RT-PCR Analysis—** Extraction of total mRNA was carried out from 1 million cells (High Pure; Roche Diagnostics). A one-tube RT-PCR (Titan; Roche Diagnostics) was employed for the synthesis of cDNA and subsequent amplification. The following primers were designed for amplification: ccacctgtatggtggggcggaga (sense) and tctggaggttagttctctgaacctgg (antisense) (for Dkk-1); ctctgatggtggctgctg (sense) and tctgaaatgtggtttttgctgcg (antisense) (for Dkk-1); and ccctggacgagcggagatag (sense) and ggtttagtgtctctgacaagtgtggaa (antisense) (for Dkk-1). Reactions were carried out on a thermal cycler (Applied Biosystems 9700; PerkinElmer Life Sciences) to the following parameters: initial cDNA synthesis, 50 °C for 45 min; denaturation, 95 °C for 1 min; annealing, 52 °C for 1 min; and extension, 72 °C for 1 min for 28 cycles. Following this, total RNA extracted from human mesenchymal progenitor cells for Dkk-3 (Clonex, Gaithersburg, MD) and human placental RNA for other Dkk cDNAs (Stratagene, La Jolla, CA). The primers ggtagcctttctagcgttc (sense) and atagtggagccagctagcag (antisense) were used to amplify Wnt-5a signal. The following oligonu-

**Cell Cycle Analysis—** Cells were seeded into 146-cm² tissue culture plates at an initial seeding density of 100/cm². After 4 days, the medium was replaced with fresh medium with or without FCS, and the cultures were incubated for a further 24 h. Cells were harvested by trypsinization and washed once with PBS, and then cell pellets were frozen at −80 °C. For analysis, ~500,000 cells were incubated in a preparatory labeling reagent containing propidium iodide, detergent, and RNase (New Concept Scientific, Niagara Falls, NY). Fluores-

**Microarray Transcript Analysis—** Experimental procedures for GeneChip analysis were performed according to the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). In brief, 8 μg of total RNA was used to synthesize double-stranded DNA (Superscript Choice System; Invitrogen). The DNA was purified by phenol/chloroform extraction and concentrated by ethanol precipita-

**In vitro transcription—** was performed to produce biotin-labeled cRNA using a BioArray HighYield RNA transcription labeling kit (Enzo Diagnostics, Farmingdale, NY). Biotinylated cRNA was cleaned with the RNeasy minikit (Qiagen, Valencia, CA) and quantified. 25 μg of biotinylated cRNA was fragmented to 50–200 nucleotides and hybridized for 16 h at 45 °C to HG-U133A array, which contains sequences corresponding to ~22,200 human genes. After washing, the array was stained with streptavidin/phycoerythrin (Molecular Probes). Staining signal was amplified by biotinylated anti-streptavidin (Vector Labora-

**Extraction of Cytoskeletal Fractions—** Triton-insoluble fractions were prepared in accordance with Ko et al. (46). Briefly, one-half million cells were suspended in 1 ml of ice-cold PBS containing a mixture of protease inhibitors (Roche Diagnostics) with 1% (v/v) Triton X-100 (Sigma). Lysis was allowed to proceed for 10 min on ice followed by a 60-μg centrifugation at 8000 × g for 15 min and resuspended in 1 ml × 1 SDS-PAGE loading buffer. The concentration of protein was measured by Bradford assay (Sigma) prior to immunoblotting.

**Immunocytochemistry—** hMSCs in tissue culture dishes were fixed with 4% (v/v) paraformaldehyde (U.S. Biochemical Corp.) for 10 min at 4 °C and washed with PBS (Fisher). Sections (30 × 60 μm) of the dishes containing the adherent cells were excised using a scapel under wet condition and placed on a 8-well multiplate (Nalge Nunc). The samples were blocked in PBS containing 0.4% (v/v) Triton X-100 (Sigma) and 5% (v/v) goat serum (Sigma) for 30 min at room temperature. Following two washes, the samples were incubated for 16 h at 4 °C followed by washing in PBS. The samples were then incubated for 1 h in a 1:900 dilution of Alexa-Fluor 546-conjugated secondary antibody (Molecular Probes). Slides were washed and mounted with medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories). Immu-

**Nuclear Extraction—** The extraction of nuclei was carried out using a method involving Triton X-100 extraction and centrifugation in the presence of divalent cations. All reagents were purchased from Sigma unless otherwise stated. Briefly, hMSCs were cultured as described above in the presence of 0.1 μg/ml recombinant Dkk-1 or vehicle, recovered by trypsinization, and washed twice in cold PBS. Cells were then resuspended in 400 μl of DNA-prep stain containing propidium iodide, detergent, and then resuspended in cold nuclei buffer (320 mM sucrose, 5 mM MgCl₂, 10 mM HEPES, pH 7.4, 1% Triton X-100), vortexed for 10 s, and incubated on ice for 10 min. Intact nuclei were isolated by centrifugation at 2000 × g for 10 min and resuspended in cold nuclei wash buffer (320 mM sucrose, 5 mM MgCl₂, 10 mM HEPES, pH 7.4). Nuclei were then incubated for 1 h at room temperature in blocking buffer (wash buffer containing 5% (v/v) goat serum) and then immunolabeled with mouse anti-human β-catenin (Chemicon) at 1:1000 dilution for 1 h at room temperature. The nuclei were then washed twice in nuclei wash buffer. The antibody was detected by a secondary incubation with Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes) at 4 μg/ml for 1 h. Cells were then washed twice in PBS and mounted onto a hemacytometer and inspected by phase-contrast microscopy and epifluorescence using an upright fluorescent microscope (Eclipse 800; Nikon). Similar distributions for β-catenin were demonstrated using hMSCs from three donors.

**In vivo transcription—** was performed to produce biotin-labeled cRNA using a BioArray HighYield RNA transcription labeling kit (Enzo Diagnostics, Farmingdale, NY). Biotinylated cRNA was cleaned with the RNeasy minikit (Qiagen, Valencia, CA) and quantified. 25 μg of biotinylated cRNA was fragmented to 50–200 nucleotides and hybridized for 16 h at 45 °C to HG-U133A array, which contains sequences corresponding to ~22,200 human genes. After washing, the array was stained with streptavidin/phycoerythrin (Molecular Probes). Staining signal was amplified by biotinylated anti-streptavidin (Vector Labora-

**Cell Cycle Analysis—** Cells were seeded into 146-cm² tissue culture plates at an initial seeding density of 100/cm². After 4 days, the medium was replaced with fresh medium with or without FCS, and the cultures were incubated for a further 24 h. Cells were harvested by trypsinization and washed once with PBS, and then cell pellets were frozen at −80 °C. For analysis, ~500,000 cells were incubated in a preparatory labeling reagent containing propidium iodide, detergent, and RNase (New Concept Scientific, Niagara Falls, NY). Fluores-

W. Telford, personal communication.
cent activated cell sorting was carried out using an automated instrument (Epics XL; Beckman Coulter), and data were analyzed using ModFit LT 3.0 software (Verity Software House, Topsham, ME).

RESULTS

Conditioned Medium Increases Proliferation of hMSCs—Initial studies with hMSCs (Fig. 1a) demonstrated that the growth of early log phase cultures of hMSCs was arrested for 12 h after replacement of conditioned medium with fresh medium. By adding conditioned medium from rapidly dividing hMSCs, the delay in proliferation was decreased. The results therefore suggested that the cultures of hMSCs must reestablish a critical concentration level of one or more secreted factors to reenter cell cycle.

Analysis of Secreted Proteins by $[^{35}S]$Methionine Labeling—To identify newly synthesized proteins in the medium, hMSCs were plated at a density of 100 cells/cm² and allowed to grow in medium containing 20% (v/v) FCS. Cells were labeled in the presence of 5 μCi ml⁻¹ of $[^{35}S]$methionine for 48-h periods between days 5 and 7, days 10 and 12, or days 15 and 17. The early log phase of growth at days 5–7 was accompanied by the largest incorporation of radiolabel and the largest secretion of labeled protein (Fig. 1b). The most abundant labeled proteins were 185 and 100 kDa (Fig. 1b).

Western blotting and immunoprecipitation demonstrated that these proteins were fibronectin and laminin, respectively (data not shown). An additional doublet of labeled protein was detected at 30–35 kDa (Fig. 1b), a region that contained relatively little unlabeled protein (Fig. 1c). The radiolabeled 30–35-kDa band was eluted from the gel (Fig. 1d) and examined by tryptic fingerprinting. 13 tryptic peptides were detected by surface-enhanced laser desorption/ionization mass spectrometry. The data were analyzed by the Pepmapper algorithm (43) with appropriate settings for detection of oxidized methionine and acryl-cysteine modifications. 7 of the 13 peptides were identical within 0.5 Da to tryptic peptides from Dkk-1 (Fig. 1i). The remaining six peptides corresponded to tryptic peptides from bovine prothrombin, also detectable in the appropriate fraction of control medium not conditioned by hMSCs (data not shown).

A rabbit polyclonal antibody was produced against a peptide corresponding to a 15-residue-long sequence in the second cysteine-rich domain of Dkk-1 (17) and used to probe Western blots of medium obtained from rapidly expanding hMSCs. The antibody detected a band of 30 kDa that was present in conditioned medium (Fig. 1e) but was absent in unconditioned me-
Fig. 2. Recombinant Dkk-1 enhances proliferation in hMSCs. a (top), SDS-PAGE analysis of 5 μg of recombinant Dkk-1 in reducing (R) and nonreducing (NR) conditions. Following silver staining, the presence of monomeric (1), dimeric (2), trimeric (3), and multimeric forms (x) are detectable in the nonreduced form. The monomeric component of the sample is estimated to be ~40%. Western blots of the recombinant protein probed with an anti-polyhistidine monoclonal antibody and the anti-Dkk-1 polyclonal antiserum are also shown (a, bottom). b, effect of Dkk-1 on the lag phase of hMSCs. Conditioned medium of log phase cells was replaced with fresh medium containing vehicle, 0.1 μg ml⁻¹ (top), or 0.01 μg ml⁻¹ (bottom) recombinant Dkk-1. Cells from three wells were recovered by trypsinization and analyzed by fluorescence incorporation assay. Results are expressed as a mean of three values, and error bars represent S.D. values. Data were analyzed by two-tailed Student’s t test, and data with p values <0.05 and <0.01 are denoted by a single or double asterisk, respectively.

dium (Fig. 1f). The intensity of the silver-stained band (Fig. 1e) suggested that the concentration of Dkk-1 in medium from rapidly expanding cultures of hMSCs was up to 50 ng ml⁻¹. Also, a small amount of Dkk-1 was recovered from conditioned medium by immunoaffinity chromatography using the same antibody (Fig. 1g). Western blotting of cells recovered from early log phase (5-day) and late stationary phase (15-day) cultures further indicated that Dkk-1 was expressed at a high levels during log phase and significantly down-regulated as the cultures became stationary (Fig. 1h).

Expression of Recombinant Dkk-1 in E. coli—To prepare recombinant Dkk-1, the cDNA encoding the entire coding region of Dkk-1 was cloned into the bacterial expression vector, pET 16b. The clone was constructed to encode an in-frame hexahistidine tag at the amino terminus for protein purification. Recombinant Dkk-1 was recovered in insoluble inclusion bodies from the bacteria. The protein was solubilized, refolded, and purified. The yield of protein was relatively low, at ~100 μg of soluble protein per liter of culture. Assays by SDS-PAGE under reducing and nonreducing conditions indicated that about 60% of the protein had concatamerized through intermolecular disulfide bond formation (Fig. 2a). Circular dichroism indicated that significant portions of the protein adopted β-sheet and α-helical structures (not shown), a conclusion that agreed with the theoretical prediction of the secondary structure by the PHDsec algorithm (47). The recombinant Dkk-1 could be detected by the anti-Dkk-1 antiserum and a monoclinal antibody against the hexahistidine tag (Fig. 2a).

Effect of Recombinant Dkk-1 on hMSC Proliferation—To test the hypothesis that Dkk-1 increased proliferation of hMSCs, its effects on rate of growth were assayed. The hMSCs were plated at a density of 100 cells/cm² in 6-well plates (10-cm² surface area/well). After 4 days, when the cells were in early log phase of growth, the conditioned medium was removed and replaced with fresh medium containing either vehicle, 0.1 μg ml⁻¹ Dkk-1, or 0.01 μg ml⁻¹ Dkk-1. Fluorescence assays for cell number indicated that the recombinant Dkk-1 initially increased proliferation (Fig. 2b). The effect of Dkk-1 persisted for 30 h at 0.1 μg ml⁻¹ (Fig. 2b, top), whereas the effects of Dkk-1 were only significant for about 15 h when 10-fold less Dkk-1 was added (Fig. 2b, bottom), suggesting that the molecule had a short half-life.

To test the effect of recombinant Dkk-1 on the colony-forming potential of hMSCs, 100 hMSCs were plated onto a 176-cm² tissue culture dish and allowed to grow 2.5 weeks in the presence of vehicle, 0.1 μg ml⁻¹, or 0.01 μg ml⁻¹ of recombinant Dkk-1. After staining with crystal violet, visible colonies above 2 mm in diameter were counted (top). Colonies were measured and categorized based on diameter in millimeters (lower). Results are expressed as a mean of three values, and error bars represent S.D. values. Data were analyzed by two-tailed Student’s t test, and data with p values <0.05 and <0.01 are denoted by a single or double asterisk, respectively.
The error bars observations further, falling as hMSCs became confluent (Fig. 3). After 10 days (late log), and not detectable at 15 days (stationary phase; Fig. 3, d). In untreated early log phase cultures, β-catenin was distributed throughout the cytoplasm and the plasma membrane at sites of cell-cell contact (Fig. 4b, i and ii). In many instances of cell-cell contact, there appeared to be a gradient of β-catenin distribution throughout the cytoplasm, with most concentration proximal to the contact site (Fig. 4b, i and ii). In stationary cultures, the distribution of β-catenin was similar, but the concentration at cell contacts was more apparent (Fig. 4b, iii and iv). As expected, the addition of medium containing 0.1 μg ml\(^{-1}\) Dkk-1 produced a clearance of the β-catenin molecules in the stationary phase were extensively redistributed from the cytoplasmic pool to the detergent-insoluble actin-rich cytoskeletal fraction (Fig. 3e), suggesting that β-catenin contributed to the formation of actin-associated intracellular adherens junctions. Actin and GAPDH were also assayed to serve as fractionation controls. It is noteworthy that actin almost exclusively partitioned to the insoluble fraction of the cell lysate, but GAPDH, which has been reported to exist in the cytoplasm and associated with microtubules (48), was almost completely cytosolic.

Recombinant Dkk-1 Decreases the Intracellular Concentration of β-Catenin—In further experiments, the effects of recombinant Dkk-1 on β-catenin levels in hMSCs were investigated. As expected, treatment of stationary phase cultures of hMSCs with 0.1 μg ml\(^{-1}\) recombinant Dkk-1 reduced the levels of β-catenin (Fig. 4a).

(a)  RT-PCR assay of Dkk-1, LRP-6, and Wnt-5a mRNA levels in hMSCs. The resultant fragments were analyzed by agarose gel electrophoresis followed by ethidium bromide staining.  
(b)  Microarray and RT-PCR Assays for Dkk-1 and LRP-6—Microarray assays on 5-day (early log), 10-day (late log), and 15-day (stationary) cultures demonstrated that several components of the canonical Wnt signaling pathway were expressed, including Dkk-1; Wnt-5a; α-catenin; β-catenin; frizzled 1, 4, 6, and 7; disheveled; glycogen synthetase kinase 3 β; and glycogen synthetase kinase 3 α. To investigate the mRNA profiles more closely, a quantitative RT-PCR and a previously described ELISA-based assay was employed (45). The level of Dkk-1 mRNA was highest after 5 days in culture (early log), lower after 10 days (late log), and not detectable at 15 days (stationary phase; Fig. 3, a and b). Expression of one of the Dkk-1 receptors, LRP-6, paralleled expression of Dkk-1 with levels falling as hMSCs became confluent (Fig. 3c). Multiple attempts to amplify LRP-5 from hMSCs using different primers were unsuccessful, but the same primers gave satisfactory signals when mRNA generated from another source was assayed (Fig. 3d). Further RT-PCR assays demonstrated that there were no detectable levels of Dkk-2, Dkk-3, and Dkk-4 transcripts (Fig. 3d). Also, RT-PCR assays for Wnt-5a demonstrated that the gene was not expressed in early log cultures, expressed at moderate levels in late log cultures, and expressed at high levels in stationary cultures (Fig. 3, a and b). To explore the observations further, β-catenin levels were assayed based on the canonical Wnt pathway, leading to a destabilization of β-catenin. As expected, Western blotting demonstrated that the steady-state level of β-catenin was lower in early log phase cultures than in late log or stationary phase cultures (Fig. 3c).
cytoplasmic pool of β-catenin (Fig. 4b, iv and vi). Low power images confirmed that the effect of Dkk-1 was present throughout the monolayer (Fig. 4b, v and vi). The staining was specific for β-catenin, since extended exposure of the control slides with an appropriate concentration of isotype control did not give a fluorescent signal (Fig. 4b, vii).

Nuclear β-Catenin Is Also Reduced by Recombinant Dkk-1—Detailed inspection of stained hMSC monolayers at the late log or stationary phase of growth was hindered by the high signal in untreated cultures. This prevented accurate evaluation of nuclear levels of β-catenin, since it appeared indistinguishable from membrane-bound fractions (exemplified in Fig. 5a). To test whether the recombinant Dkk-1 directly affected nuclear β-catenin levels, late log phase cultures were treated with medium in the presence or absence of 0.1 μg ml⁻¹ Dkk-1. After an incubation period of 24 h, the cells were recovered, and the nuclei were extracted in accordance with the protocol described under “Experimental Procedures.” The extracted nuclei were stained for both β-catenin using a monoclonal antibody and an Alexa 498-conjugated secondary antibody (green) and for DNA using propidium iodide (red). Flow cytometric analysis of the extracted nuclei demonstrated that there was an overall and highly significant (p = 0.002, from 10,000 events) decrease of detectable β-catenin in the nuclei of Dkk-1-treated cells (Fig. 5b).
Furthermore, the nuclei from Dkk-1-treated cells seemed to consist of two strikingly separate subpopulations: one with relatively high levels of β-catenin, presumably derived from cells expressing lower levels of LRP-6 on the membrane, and another with lower levels of β-catenin, presumably derived from cells expressing more LRP-6. It is probable that this partitioning of the nuclei from Dkk-1-treated cells into two populations could reflect microscopic variations in cell density over the monolayer, thus affecting LRP-6 expression. The iso-type control yielded no detectable signal (data not shown). The nuclear extraction protocol was validated by microscopic inspection of wet-mounted nuclei on a hemacytometer (Fig. 5c).

Jun/JNK Signaling and Expression of VCAM-1—Since Dkk-1 binds to LRPs and the LRP complexes can regulate JNK (49), we examined the pattern of Jun/JNK signaling during the expansion of hMSCs cultures. Microarray data for mRNAs (Table I) indicated that three members of the Jun family (c-Jun, JunB, and JunD) were expressed and that the levels of two (JunB and JunD) increased as the cultures entered the stationary phase. JunD expression also increased during serum starvation of the cells. Only one of three isoforms of JNK was detected, and the level did not change as the cultures entered the stationary phase or underwent serum starvation. Assays of the proteins by Western blotting indicated that c-Jun was present but did not change during expansion (Fig. 6a), an observation that was consistent with the microarray data (Table I). However, there was an increase in c-Jun phosphorylated at serine 73 in stationary cultures that coincided with the decrease of Dkk-1. There was no increase in c-Jun phosphoryl-

**Fig. 5.** Evaluation of the levels of β-catenin in extracted nuclei from stationary phase hMSCs treated for 24 h in the presence or absence of 0.1 μg ml⁻¹ Dkk-1. a, detection of β-catenin in monolayers of stationary phase cultures of hMSCs demonstrating high levels of the protein in the untreated monolayer (lower images) but not in the Dkk-1 treated monolayer (upper images). The level of nuclear β-catenin is difficult to evaluate in the untreated monolayer due to the extensive staining even when the location of the nuclei are indicated in the absence of 4',6-diamidino-2-phenylindole signal (lower right panel). b, evaluation of nuclear β-catenin levels by fluorescence activated sorting of nuclei extracted from stationary phase hMSCs treated for 24 h in the presence (upper histogram) or absence (lower histogram) of 0.1 μg ml⁻¹ Dkk-1. The average fluorescence detected per event (10,000) from each experiment is presented on the right. Results are expressed as a mean of three values, and error bars represent S.D. Data were analyzed by two-tailed Student’s t test and data resulting in a p value of <0.005 (denoted by a triple asterisk). Below is a Western blot assay of the Dkk-1-treated and untreated nuclei from two donors. The filters were stripped and then stained with Ponceau S after detection of the β-catenin to confirm equal loading. c, microscopic examination of the stained, extracted nuclei. Images were generated, using (left to right) phase microscopy, fluorescence for propidium iodide (red), fluorescence for β-catenin staining (green), merged fluorescence signal demonstrating coincidental staining of the nuclei with propidium iodide and β-catenin, and merged fluorescence with phase images demonstrating minimal levels of unstained debris. Upon closer inspection of the nuclei, uneven localization of nuclear β-catenin was apparent (extreme right).
ated at the serine 63 (not shown). The increase in phosphorylated c-Jun was not reversed by the addition of recombinant Dkk-1. However, the addition of Dkk-1 reduced the level of endogenous Dkk-1 (Fig. 6, a and c), an observation consistent with the conclusion that the recombinant Dkk-1 was active and previous observations indicating that expression of Dkk-1 is down-regulated by inhibition of the Wnt/β-catenin pathway (16).

Since phosphorylated c-Jun increased in the stationary phase, we searched the microarray data for downstream genes regulated by c-Jun. The second highest increase in signal intensity was found for VCAM-1, a gene that is up-regulated by c-Jun and c-Fos of the AP-1 class of transcription factors (50) and that regulates bone development (51–53). Both RT-PCR (Fig. 6b) and Western blot (Fig. 6c) assays demonstrated a dramatic increase in VCAM-1 expression that coincided with the decrease in Dkk-1 and the increase in phosphorylated c-Jun. The addition of recombinant Dkk-1 to stationary cultures did not decrease the protein levels of VCAM-1 (Fig. 6c), but the levels of VCAM-1 mRNA were markedly increased by sequester-

Table I
Comparison of the steady-state mRNA levels of various forms of c-Jun, JNK, and related genes by hMSCs over time in culture by microarray transcript analysis

| Transcript Accession       | Log   | Lag   | Stat  | Sfree |
|----------------------------|-------|-------|-------|-------|
| c-Jun protooncogene        | gb:NM 002288.2 | P     | E     | E     |
| JunB protooncogene         | gb:NM 002229.1 | P     | 1.5X  | 2X    |
| JunD protooncogene         | gb:NM 005354.2 | P     | 1.5X  | 2X    |
| JNK 1 α, protein kinase     | gb:NM 002750.1 | A     | A     | A     |
| JNK 2 β, protein kinase     | gb:U35004.1  | A     | A     | A     |
| Homo sapiens mitogen-activated protein kinase JNKK 2 | gb:NM 005043.1 | A     | A     | A     |

Fig. 6. Effect of Dkk-1 on Jun kinase activity and modulation of VCAM-1 expression. hMSCs were plated at a density of 100 cells/cm² and allowed to expand to the early log phase of growth (early), the late log phase of growth (late), and the stationary (stat) phase. After each period of expansion, the hMSCs were counted to evaluate density and prepared for Western blotting or RT-PCR analysis. a, Western blot analysis for c-Jun, phospho-c-Jun, Dkk-1, GAPDH, and actin. The level of phospho-c-Jun increases during expansion. This effect is not reversed by the addition of recombinant Dkk-1. b, VCAM-1 expression is up-regulated as hMSCs enter the stationary phase of growth. RT-PCR assay for VCAM-1 expression in early log phase or stationary phase hMSCs. Multiple amplifier sets were used for amplification of the cDNA. d, the addition of 50 μg ml⁻¹ of the anti-Dkk-1 antibody to the medium increases VCAM-1 expression in early log hMSCs, and the addition of the AP-1 transcriptional inhibitor nordihydroguaiaretic acid reduces expression of VCAM-1 in stationary cultures (above). RT-PCR ELISA assay for VCAM-1 expression in early log phase hMSCs in the presence or absence of 50 μg ml⁻¹ of the anti-Dkk-1 antibody (above) and RT-PCR assay for VCAM-1 expression in stationary phase hMSCs in the presence or absence of 10 μM nordihydroguaiaretic acid (below). e, ELISA assay of the RT-PCR products generated in d. Results are expressed as a mean of three values, and error bars represent S.D. Data were analyzed by two-tailed Student’s t test. p values of <0.01 and <0.05 are denoted by double and single asterisks, respectively.
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Fig. 7. During growth arrest by serum starvation, transcription of Dkk-1 is inhibited. a, cell cycle analysis of hMSCs after 5 days in culture followed by the addition of medium containing no FCS or 20% (v/v) FCS. The relative proportions of cells in G1, S phase, and G2/M are indicated on the histograms. Phase contrast micrographs are presented with each histogram, illustrating cell density in each case. b, RT-PCR analysis of Dkk-1 transcription by hMSCs subjected to conditions described in a. c, hybridization ELISA analysis of the Dkk-1 PCR products normalized against the appropriate GAPDH control. Error bars, S.D. of the mean of three sets of data. d, analysis of β-catenin levels with or without 24 h of serum starvation. Cellular β-catenin levels were analyzed for both conditions tested by 4–12% SDS-PAGE and Western blotting. The lanes were equally loaded with 2 μg (~10,000 cells) of protein, and equal transfer was confirmed by Ponceau S staining.

Effect of anti-Dkk-1 antibodies to Dkk-1 (Fig. 6d) had no effect on stationary cultures of hMSCs that were growth-arrested by serum starvation. Hybridization ELISA of RT-PCR products indicated that Dkk-1, but not GAPDH levels, were significantly reduced under serum-free conditions that inhibit division (Fig. 7, a, b, c). In addition, β-catenin levels were increased in the growth-arrested hMSCs (Fig. 7d), possibly in response to the reduction of Dkk-1 synthesis. VCAM-1 levels remained unchanged in response to serum starvation as assayed by microarray analysis (data not shown).

Dkk-1 Expression Is Concomitant with Cell Cycle Activity—Since Dkk-1 expression was highest in hMSCs during the early log phase of growth, we tested the hypothesis that expression of Dkk-1 would decrease if the cells were growth-arrested by serum starvation. Hybridization ELISA of RT-PCR products indicated that Dkk-1 was down-regulated in the presence of conditions that inhibit division (Fig. 7, a, b, c). The addition of higher concentrations of the antiserum to Dkk-1 decreased proliferation of early log phase cultures. The antibodies had no effect on the choriocarcinoma cell line (JEG-3), which did not express detectable levels of Dkk-1 mRNA. It is also noteworthy that the JEG-3 cell line also did not exhibit the lag phase in response to a change to fresh medium.

**DISCUSSION**

The results presented here demonstrate that after early passage cultures of hMSCs are lifted and the cells are replated at low density, the cells do not leave a prolonged lag phase and reenter the cell cycle until they have synthesized Dkk-1 and the protein accumulates at sufficient high levels in the culture medium. The observations indicate that Dkk-1 inhibits positive signaling through the canonical Wnt/β-catenin pathway by binding to LRP-6 and thereby disrupting the frizzled receptor complex. The inhibition of the Wnt/β-catenin pathway decreases both the nuclear β-catenin available to regulate transcription and the cytoskeletal β-catenin available to form adherens junctions. Therefore, Dkk-1 decreases the cell-to-cell contacts that are required for differentiation of the cells (8, 14).

The evidence that Dkk-1 inhibited the canonical Wnt signaling pathway was based on several observations. Dkk-1 was a major component secreted into the medium during the early log phase. The addition of recombinant Dkk-1 to cultures increased proliferation during a second lag period that was induced by replacing conditioned medium. Also, the addition of antibodies to Dkk-1 decreased proliferation of early log phase cultures. During expansion of the cultures from the early log phase to the stationary phase, there was coincidental down-regulation of expression of both Dkk-1 and its co-receptor, and an up-regulation of Wnt-5a. At the same time, there was a marked increase in total cellular levels of β-catenin and of β-catenin in both the nuclear and cytoskeletal fractions. The addition of
recombinant Dkk-1 dramatically reduced the levels of β-catenin in the same compartments. Since positive signaling through the canonical Wnt/β-catenin pathway is generally linked to increased cell proliferation through activating c-Myc or cyclin D1 (56), the increased proliferation of hMSCs observed by inhibiting the pathway with Dkk-1 was unexpected. The observations made here, however, are consistent with the model for Wnt signaling during limb bud development proposed by Hartmann and Tabin (37), in which positive signaling through the canonical Wnt/β-catenin/Lef pathway drove chondrocytes out the cell cycle and pushed them toward differentiation and a postmitotic state.

The observations also raised the possibility that Dkk-1 down-regulates the AP-1/JNK pathway and thereby decreases expression of the cell adhesion protein VCAM-1. The levels of c-Jun remained constant as the culture passed from the early log to the stationary phase, but there was an increase in phosphorylated c-Jun that paralleled the decreased expression of Dkk-1. The increase in phosphorylated c-Jun coincided with a marked increase in expression of VCAM-1 that is positively regulated by the AP-1/JNK pathway (50). Sequestering endogenous Dkk-1 by the addition of antibodies to early log phase cultures drove chondrocytes out the cell cycle and pushed them toward differentiation and a postmitotic state.

The requirement for Dkk-1 to return hMSCs to the cell cycle is probably important for limiting replication of the cells in vivo, since, if replated at low density, the cells expand over a billion-fold over about 8 weeks (11), and such uncontrolled expansion in vivo would impose a considerable burden on the organism.

The effect of Dkk-1 in driving hMSCs into cell cycle are of interest in terms of the recent reports that two different mutations in LRP-5 that prevented Dkk-1 from inhibiting Wnt signaling caused high bone density in two unrelated kindreds (61, 62). In light of the observations made here, the effects of the mutations may be explained by increased Wnt signaling driving hMSCs out of the cell cycle and toward differentiation to osteoblast precursors. Judicious administration of Dkk-1 might be a useful means of stimulating expansion of hMSCs in vivo and thereby enhancing the tendency for the cells to home to sites of tissue damage and repair the tissues (63).

To explore the possibility that Dkk-1 might also decrease the lag phase observed in other cultured cells, we screened several malignant cell lines for expression of Dkk-1. Two osteosarcoma lines expressed the gene. Cultures of both exhibited a lag phase through a Wnt pathway may also contribute to the decreased proliferation. Wnt-5a was originally recognized as a ligand for a noncanonical Wnt signaling pathway, but the noncanonical Wnt pathways are still poorly understood. Also, a recent report indicates that Wnt-5a signaling can mimic positive signaling through the canonical pathway in one cell system (58). The expression of the Wnt-5a ligand in the stationary phase cultures is consistent with the extensive use of confluent cultures of hMSCs as feeder layers for the culture of hematopoietic stem cells and the recent reports that several Wnt ligands enhance hematopoiesis (58–60).
in culture, and the lag phase was prolonged by the addition of antibodies to Dkk-1. Therefore, synthesis of Dkk-1 may be a useful adjunct therapy for some malignancies.

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The Wnt Signaling Inhibitor Dickkopf-1 Is Required for Reentry into the Cell Cycle of Human Adult Stem Cells from Bone Marrow

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