Regulation of Mesenchymal Stem Cell Osteogenic Differentiation by Glucocorticoid-induced Leucine Zipper (GILZ)*

Received for publication, May 21, 2007, and in revised form, December 12, 2007 Published, JBC Papers in Press, December 14, 2007, DOI 10.1074/jbc.M704147200

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Mesenchymal stem cells (MSCs) can differentiate into multiple cell lineages, including osteoblasts and adipocytes. We reported previously that glucocorticoid-induced leucine zipper (GILZ) inhibits peroxisome proliferator-activated receptor γ-2 (Pparγ2) expression and blocks adipocyte differentiation. Here we show that overexpression of GILZ in mouse MSCs, but not MC3T3-E1 osteoblasts, increases alkaline phosphatase activity and enhances mineralized bone nodule formation, whereas knockdown of Gilz reduces MSC osteogenic differentiation capacity. Consistent with these observations, real-time reverse transcription-PCR analysis showed that both basal and differentiation-induced transcripts of the lineage commitment gene Runx2/Cbfa1, as well as osteoblast differentiation marker genes including alkaline phosphatase, type I collagen, and osteocalcin, were all increased in GILZ-expressing cells. In contrast, the mRNA levels of adipogenic Pparγ2 and C/ebpα were significantly reduced in GILZ-expressing cells under both osteogenic and adipogenic conditions. Together, our results demonstrate that GILZ functions as a modulator of MSCs and that overexpression of GILZ shifts the balance between osteogenic and adipogenic differentiation of MSCs toward the osteogenic pathway. These data suggest that GILZ may have therapeutic value for stem cell-based therapies of metabolic bone diseases, such as fracture repair.

Bone marrow-derived mesenchymal stem cells (MSCs)§ are pluripotent and can give rise to several distinct cell lineages, such as osteoblasts, adipocytes, chondrocytes, myocytes, and even neurons under appropriate conditions (1–4). Increasing evidence has shown that adipocytes and osteoblasts are two major pathways and that the relationship between these two is reciprocal, i.e. when the adipogenic pathway is blocked, the MSCs enter the osteogenic pathway, and vice versa (5–9). Thus, balanced MSC osteoblast and adipocyte differentiation is critical for the maintenance of healthy bone and lean body composition, and understanding of the mechanisms by which this balance is modulated will have significant medical implications in stem cell-based therapies. We reported previously that a glucocorticoid (GC)-inducible protein, called GC-induced leucine zipper (GILZ) (10), can inhibit the transcription of a key adipogenic regulator, peroxisome proliferator-activated receptor γ-2 (Pparγ2), and blocks adipocyte differentiation of 3T3-L1 cells (11). Gilz is a new member of the leucine zipper protein family and belongs to the transforming growth factor β-stimulated clone-22 (Tsc-22) family of transcription factors (12, 13). Members of this family contain three distinct domains: an N terminus TSC box, a middle leucine zipper domain, and a C terminus proline-rich domain. GILZ has been shown to interact with and inhibit the activities of the key inflammatory signaling mediators NF-κB and AP-1 (14, 15). GILZ can also interact with the mitogen-activated protein kinase family member, Raf1, resulting in inhibition of Raf-1 phosphorylation and, subsequently, inhibition of MEK/ERK-1/2 (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase) phosphorylation and AP-1-dependent transcription (12). We have reported recently that GILZ can mediate the anti-inflammatory effect of GC and inhibit inflammatory cytokine-induced cyclooxygenase-2 expression in mouse MSCs (16). In this report we address the question whether GILZ, while inhibiting adipocyte differentiation, can stimulate osteoblast differentiation of MSCs using GILZ gain-of-function and loss-of-function studies.

EXPERIMENTAL PROCEDURES

Chemicals—All chemicals, except where specified, were purchased from Sigma.

Isolation and Characterization of Mouse Bone Marrow MSCs—The bone marrow MSCs used in this study were isolated from 18-month-old male C57BL/6 mice (NIA, National Institutes of Health). All animal procedures were approved by the Institutional Committee for Animal Care and Use Committee (IACUC) at the Medical College of Georgia. In brief, the MSCs were isolated from long bones of 6 mice as a mixture population using a protocol modified from Gimble et al. (17), Peister et al. (18), and Tropel et al. (19). These MSCs, which are negative for CD-11b, CD-11c,
CD45R/B220, PDCA-1, and positive for Sca-1, are capable of undergoing osteogenic, adipogenic, and myogenic differentiation. The cells were maintained in DMEM with 10% FBS. MC3T3-E1 cells (subclone number 14) (ATCC) were maintained in α-minimal essential medium supplemented with 10% FBS.

Production of Recombinant Retrovirus and Infection—The retroviral vectors expressing GILZ or GFP were constructed in a replication defective ΔU3nlsLacZ vector (20). In brief, the LacZ sequence in the parental ΔU3nlsLacZ vector was excised by Xbal and BamHI and replaced with the full-length coding region of GILZ or Gfp cDNA. Xbal and BamHI restriction sites were incorporated into the 5’- and 3’-end of the PCR products, respectively, when GILZ and Gfp cDNA were amplified by PCR. The retroviral particles (Ret-GILZ and Ret-GFP) were produced by transfecting ΔU3-GILZ or ΔU3-GFP plasmid DNA into the retroviral packaging cell line 293GPG as described by Ory et al. (20).

For infection, 2 ml of viruses prepared as described above were added to the cells (seeded in a 60-mm dish the previous day at ~70% confluence) and incubated at 37 °C for 6 h in the presence of 8 μg/ml Polybrene. The viruses were then removed and fresh media added to the cells.

Production of Lentivirus and Infection—GILZ knockdown constructs expressing small hairpin RNA (shRNA) targeting endogenous GILZ (shGILZ) were generated in a lentivirus-based shRNA vector pLl3.7. Details of DNA construction, virus production, cell infection, and the knockdown efficiency of dexamethasone-induced endogenous GILZ were shown in our recent publication (16).

Immunofluorescence Labeling and Fluorescence Microscopy—Cells were grown in chamber slides and fixed with 3.7% paraformaldehyde (for 100 ml: 10 ml of 10× PBS, 33.4 ml of 11.1% formaldehyde (freshly prepared from its parapolymer), 0.6 ml of 30% Triton X-100, 56 ml of distilled water) for 30 min at room temperature. Cells were rinsed with PBS, blocked with 1% bovine serum albumin in PBS for 30 min at room temperature, and then incubated with polyclonal anti-GILZ antibody (11) at 1:200 dilution for at least 1 h at room temperature. After several washes, the slides were incubated with goat anti-rabbit IgG-Cy3 conjugate (Zymed Laboratories Inc.) at 1:600 dilution for 1 h at room temperature in the dark. The slides were washed three times in PBS for 5 min each and stained with 4′,6-diamidino-2-phenylindole (300 nm) to visualize the nucleus. Finally, the slides were washed, mounted with Vectashield mounting media (Vector Laboratories), and analyzed using a Nikon TE2000 fluorescence microscope equipped with COOLSNAP Monochrome Camera. Images were acquired and processed with MetaMorph Imaging System.

Western Blotting—Whole cell lysates of MSC-GILZ and MSC-GFP or MC3T3-GFP and MC3T3-GILZ cells, as well as parental MSCs treated with dexamethasone (100 nm), were collected, and equal amounts of total protein were separated on 12% SDS-PAGE. Expression of GILZ was detected using rabbit anti-GILZ antibody as described previously (16).

Osteogenic Differentiation—Cells were plated in 96-well plates in triplicate at a density of 1 × 10^4 cells/cm² the previous day and then treated with osteogenic supplements (OS) consisting of DMEM supplemented with 2% FBS, 5 mM β-glycerophosphate, and 50 μM L-ascorbic acid-2-phosphate, or cultured in DMEM with 2% FBS, as a control.

For ALP staining, cells were rinsed three times with calcium and phosphate-free saline solution, fixed in 4% paraformaldehyde for 30 min at room temperature, washed three times with double-distilled H₂O, and stained with One-Step™ NBT/BCIP solution (Pierce) for 15 min at room temperature. Cells were washed again with double-distilled H₂O and the plates were scanned using a CanoScan LiDE 80 flat-bed scanner (Canon USA Inc.).

Alizarin Red S staining and calcium mineral content quantification were performed as described by Stanford et al. (21). In brief, the cells were rinsed with calcium and phosphate-free saline solution, and fixed with ice-cold 70% ethanol for 1 h. After a brief wash with water, the cells were stained for 10 min with 40 mM Alizarin Red S solution (pH 4.2) at room temperature. The cells were rinsed five times with water followed by a 15-min wash with PBS (with rotation) to reduce nonspecific Alizarin Red S stain. Stained cultures were scanned followed by a quantitative distaining procedure using 10% (w/v) cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0), for 15 min at room temperature, and the Alizarin Red S concentrations were determined by absorbance measurement at 570 nm on Victor™ V (PerkinElmer) using an Alizarin Red S standard curve in the same solution. The values were normalized to DNA contents.

Adipogenic Differentiation—For adipogenic induction, cells were seeded in 24-well plates at a density of 30,000 cells/cm². After the cells reach confluence, they were treated with adipogenic induction medium (DMEM containing 10% FBS, 1 μM dexamethasone, 60 μM indomethacin, 10 μg/ml insulin, and 0.5 mM 3-isobutyl-1-methylxanthine) for 3 days and then switched to adipogenic maintenance medium (growth medium plus 10 μg/ml insulin) with media replaced every other day. The formation of adipocytes was monitored by the appearance of lipid droplets under a microscope.

RNA Extraction and Real-time RT-PCR—The RNA isolation, reverse transcription, and PCR analysis were performed as described previously (11). In brief, total cellular RNA was isolated using TRIzol reagent (Invitrogen Corp.), 2 μg of RNA was reverse transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems), and the mRNA levels of the indicated genes were analyzed in triplicate using SYBR Green master mixture (Applied Biosystems) and a Chromo-4 real-time RT-PCR instrument (MJ Research). The mRNA levels

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4 W. Zhang, G. Ou, M. Hamrick, W. Hill, J. Borke, K. Wenger, N. Chutkan, J. Yu, Q.-S. Mi, C. Isales, and X.-M. Shi, submitted for publication.
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were normalized to β-actin (internal control) and gene expression was presented as -fold changes (ΔΔCt method) (22, 23). The primer sequences used in the PCR are listed in Table 1.

Statistical Analysis—All experiments, except real-time RT-PCR analysis and proliferation assay, which were performed two times, were performed at least three times in triplicate. Unpaired Student’s t test (2-tailed) were used to determine the significance (p < 0.05) of the data from all experiments. -Fold differences are indicated and are presented as mean ± S.E.

RESULTS

Infection of MSCs and MC3T3-E1 Cells with GFP- and GILZ-expressing Retroviruses—MSCs, which are CD11b− CD11c− CD45R/B220− PDCA-1− Sca-1+, and are capable of undergoing osteogenic, adipogenic, and myogenic differentiation, were infected with retroviruses expressing GILZ (Ret-GILZ) or GFP (Ret-GFP), as control. The infection efficiency of cells by GFP-expressing virus was monitored directly by fluorescence microscopy (Fig. 1A, left panel). The infection efficiency of cells by GILZ-expressing virus and the expression of GILZ protein were confirmed by immunocytochemistry (Fig. 1A, right panel) and Western blot analysis of whole cell lysates using anti-GILZ polyclonal antibody (Fig. 1B). These infected MSCs express GFP or GILZ protein after extensive passages as monitored by GFP expression and immunofluorescence labeling or Western blot using GILZ antibody. Dynamic changes of endogenous GILZ in response to dexamethasone stimulation (100 nM) in parental MSCs were also examined by Western blot analysis (Fig. 1C). These results show that the MSCs can be infected efficiently (>95%) by retroviruses, and these cells express GILZ in response to dexamethasone stimulation. Similarly, the MC3T3-E1 cells were infected and the expression of GFP and GILZ confirmed by GFP expression, immunofluorescence labeling, and Western blotting (Fig. 1, D and E). These cells, referred hereafter as MSC-GFP and MSC-GILZ, or MC3T3-GFP and MC3T3-GILZ, were used in the studies described below.

Overexpression of GILZ Enhances Osteogenic Differentiation of MSCs, but Not Committed MC3T3-E1 Cells—Our previous studies showed that overexpression of GILZ inhibits its adipocyte differentiation (11), the questions we asked in this study were whether and how GILZ enhances osteoblast differentiation. To address these questions, we first examined ALP, an early stage osteoblast differentiation marker. The retrovirally transduced cells, MSC-GFP and MSC-GILZ, were cultured in OS induction media and harvested at the indicated time points for measurement of ALP activity. As shown in Fig. 2A, ALP activity increased in a time-dependent manner in both MSC-GFP and MSC-GILZ cells as the differentiation program proceeded. Interestingly, the MSC-GILZ cells showed a significantly higher ALP activity than MSC-GFP control cells at all time points examined. ALP activity decreased in both MSC-GFP and MSC-GILZ as the cells mature (day 15), which fits the well established pattern of osteoblast differentiation. To confirm this result, we also performed an ALP staining experiment and assessed surface ALP-positive cell populations. Consistent with the activity assays, the ALP staining results also showed a significant increase of ALP-positive MSC-GILZ cells after 5 days of OS treatment (Fig. 2B). To further examine the effect of GILZ on osteoblast maturation, we performed Alizarin Red S staining experiments. MSC-GFP and MSC-GILZ cells were treated for 21 days in osteogenic induction media as for ALP studies and stained with Alizarin Red S solution to visually detect the presence of mineralization. Again, the results showed a significantly higher degree of mineralization in MSC-GILZ cells than in MSC-GFP control cells (Fig. 2C). The cells cultured in regular growth media (DMEM) showed no mineralized nodule formation. A quantitative analysis of Alizarin Red S staining, by extraction of the calcified mineral from the stained monolayer at low pH, is also shown (Fig. 2D).

Because the relationship between osteogenic and adipogenic differentiation of MSCs is reciprocal, it is not possible to determine whether the observed increase in osteogenic differentia-
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Effect of GILZ overexpression on MSC osteogenic differentiation. A–D, MSC-GFP and MSC-GILZ were plated at a density of 1 × 10⁴ cells/cm² in regular growth media (DMEM) the previous day and then switched to osteogenic induction media (OS). A, at the indicated time points the cells were harvested and ALP activity was measured. B, cells were stained with ALP substrate at day 5 to detect surface ALP-positive cells. C, cells were stained with Alizarin Red S at day 21 to visualize mineralized bone matrix. D, a bar graph showing quantitative results of C, E and F; MC3T3-GFP and MC3T3-GILZ cells were differentiated and analyzed as in C and D, G. MC3T3-GFP and MC3T3-GILZ cells were treated and stained for ALP as in B. All experiments were performed a minimum of three times in triplicate except that ALP activity assays were performed two times. Experiments were performed in 96-well plates, and representative scanned images are shown. a, p < 0.05; b, p < 0.01.

FIGURE 2. Effect of GILZ overexpression on MSC osteogenic differentiation. A–D, MSC-GFP and MSC-GILZ were plated at a density of 1 × 10⁴ cells/cm² in regular growth media (DMEM) the previous day and then switched to osteogenic induction media (OS). A, at the indicated time points the cells were harvested and ALP activity was measured. B, cells were stained with ALP substrate at day 5 to detect surface ALP-positive cells. C, cells were stained with Alizarin Red S at day 21 to visualize mineralized bone matrix. D, a bar graph showing quantitative results of C, E and F; MC3T3-GFP and MC3T3-GILZ cells were differentiated and analyzed as in C and D, G. MC3T3-GFP and MC3T3-GILZ cells were treated and stained for ALP as in B. All experiments were performed a minimum of three times in triplicate except that ALP activity assays were performed two times. Experiments were performed in 96-well plates, and representative scanned images are shown. a, p < 0.05; b, p < 0.01.

In the study, to emphasize the effect of GILZ in MSC osteogenic differentiation, the dexamethasone was excluded from the induction media in our studies. We thought that the inability of GILZ knockdown to influence MSC osteogenic differentiation observed was due to the low levels of basal GILZ expression in these cells as GILZ is GC-inducible and its expression is barely detectable (Fig. 1). To test this hypothesis, we added dexamethasone (10 nm) back to the induction media in the initial differentiation stages. Interestingly, when dexamethasone was supplemented in the OS media for the first several days (2–4 days) during differentiation, the ability of mineralization decreased significantly in MSCs infected with shGILZ-1 lentiviruses (Fig. 3A, third row), a shRNA sequence that significantly reduced dexamethasone-induced endogenous GILZ expression (16). In contrast, mineralization was observed normal in MSCs infected with lentiviruses expressing shGILZ-2, a shRNA sequence that had no effect on dexamethasone-induced GILZ, or pLL3.7, a control lentivirus that contained an empty lentiviral vector. Knockdown of GILZ in MSCs seems to have no significant effect on ALP activity in the presence or absence of dexamethasone treatment as measured by ALP staining and activity assays (Fig. 3, B and C). Together, these gain-of-function and loss-of-function studies demon-
strated that overexpression of GILZ enhances, and knockdown reduces MSC osteogenic differentiation capacity. These results suggest that GILZ could be a key factor linking glucocorticoids and bone marrow stromal cell osteogenic differentiation.

Next, we examined the effect of GILZ on the expression of osteoblast lineage-associated genes. Equal amounts of total RNA isolated from MSC-GILZ and MSC-GFP cells cultured for 5 days in osteogenic induction media were reverse transcribed and the mRNA levels of the indicated genes were analyzed using SYBR Green master mixture and the primers listed in Table 1 on a Chromo-4 real-time PCR instrument. Results show that the mRNA levels of the osteogenic commitment gene Runx2/Cbfa1, as well as other differentiation markers, including Alp, type I collagen (Col1a1), and osteocalcin (Ocn), were all increased significantly in MSC-GILZ cells compared with that in MSC-GFP control cells (Fig. 4). These results demonstrated that overexpression of GILZ enhances osteogenic differentiation of MSCs by increasing the expression of the osteogenic associated genes.

**GILZ Enhances Osteogenesis through Inhibition of Adipogenesis**—Because the relationship between osteogenic and adipogenic differentiation of MSCs is reciprocal, and GILZ inhibits adipocyte differentiation (11), we then asked whether the observed enhancement of osteoblast differentiation was due to the inhibition of adipocyte differentiation. To address this question, we treated MSC-GILZ and MSC-GFP cells with either osteogenic (OS) or adipogenic (MID) induction media and examined the mRNA expression of Pparγ2 and C/ebpα by real-time RT-PCR analysis. Results show that the mRNA levels of Pparγ2 (Fig. 5A) and C/ebpα (Fig. 5B) were inhibited significantly in MSC-GILZ cells, compared with that in MSC-GFP control cells, under both osteogenic and adipogenic induction conditions. Consistent with the mRNA profiles, the MSC-GILZ cells also failed to accumulate intracellular lipid vacuoles, whereas the MSC-GFP cells differentiated normally into adipocytes filled with lipid droplets (Fig. 5C). These results support our hypothesis that the enhancement of MSC osteoblast differentiation by GILZ is achieved through inhibition of Pparγ2 expression. This conclusion is in agreement with and supported by the evidence that Pparγ2 inhibits Runx2/Cbfa1 expression and terminal osteoblast differentiation (9).

**GILZ Does Not Affect MSC Proliferation**—Osteoblast differentiation is a sequential event that initially supports cell proliferation. To determine whether the enhancement of osteogenic differentiation by GILZ overexpression was due to an increase in the rate of cell growth, we performed proliferation assays. MSC-GILZ and MSC-GFP cells were plated in triplicate in 96-well plates (1 × 10^4 cells/cm²), and cell proliferation was assayed every day for 11 consecutive days using a Cell Growth Determination kit (Sigma). As shown in Fig. 6, both MSC-GILZ and MSC-GFP cells proliferated over time but no significant difference was seen between the two cell lines. This result confirms that the enhancement of osteogenic differentiation by GILZ overexpression was not caused by an increase in cell proliferation.

**DISCUSSION**

MSCs are pluripotent and can differentiate into multiple cell lineages (3). This feature lays the foundation for their use in stem cell therapies. However, the question of how a MSC decides to which path- way it goes is largely unknown. Using GILZ gain-of-function and loss-of-function approaches, we provide evidence in this report that a GC-inducible protein, GILZ, plays an important role modulating the balance of MSC osteoblast and adipocyte differentiation.

GCs regulate a spectrum of cell functions, including growth, differenti- ation, and apoptosis (25, 26). GCs are also among the best known anti-inflammatory and immune suppressive agents and are widely used for treating diseases such as rheumatoid arthritis (27–29), pul-

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**TABLE 1**

| Genes   | GenBank™ | Forward (5’ to 3’) | Reverse (5’ to 3’) | Size(bp) |
|---------|----------|--------------------|--------------------|----------|
| Alp     | BC065175 | AACCACGACCAAGCCATTC | CCACAAAGAGGAGCTTTTG | 213      |
| Runx2   | NM_009820| CACCACTCACTACCACACG | TCAAGCTCAACACATTTT | 250      |
| Col1a1  | U93419  | CACCCCTGAGAGCTGCTTC | TTTCCTGCACATGCTGACC | 250      |
| Ocn     | U11542  | TTTTGGCTCTGCTGACTAAC | TTTTGAGGCGCTTTCTCAGG | 250      |
| C/ebpα  | M62362  | GATTAGAAGCAAACAGGA AGCACAC | TCTAGAGCTACGAGCAAGCA | 255      |
| Pparγ2  | BC021798| TTTCCTCGGAGAAACATTGAT | ACAAZAGGTGATTTGCTGACCG | 343      |
| β-Actin | NM_007393| CTGCTCGACGCACGGTATCA | GTAGAGCCGACAGCATCTCA | 190      |
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**FIGURE 4.** Real-time RT-PCR analysis of osteogenic-specific gene expression. MSC-GFP and MSC-GILZ cells were cultured as described in the legend to Fig. 2 (except that 6-well plates were used) and harvested for total RNA isolation 5 days after treatment. Equal amounts of RNA (2 μg) were reverse transcribed and the mRNA levels of the indicated genes were analyzed using SYBR Green master mixture and primers listed in Table 1. Data were normalized to β-actin and expressed as fold changes relative to the mRNA levels in untreated cells (ΔΔCt method). Experiments were repeated two times and PCR performed in triplicate. Error bars indicate S.D.  a, p < 0.05; b, p < 0.01.

monary disease (30), and asthma (31, 32), and in organ transplantation to prevent rejection (33). However, the molecular mechanisms by which GCs transduce their anti-inflammatory and immune suppressive actions are not well understood. Importantly, long term GC treatment causes, among other adverse effects, accelerated bone loss resulting in osteoporosis (29, 34–38), as well as the replacement of marrow cell populations with adipose tissues (fatty marrow) (39–41). GC in excess causes bone loss in vivo, possibly by inducing the death of osteoblasts and osteocytes (42). Paradoxically, GCs stimulate osteogenesis of primary bone marrow stromal cell cultures (43, 44). Therefore, it is clinically relevant to understand the role of GILZ in osteogenesis and its relationship with the GCs.

Because GILZ is a GC-inducible protein and its basal level is low, it is difficult to determine the role of endogenous GILZ without overexpression. To avoid the complexity of GCs and underscore the role of GILZ in osteogenic differentiation, we excluded dexamethasone from osteogenic induction media (OS), except in knockdown experiments where dexamethasone is necessary for generating endogenous GILZ. We found that the mouse MSCs can differentiate into osteoblasts without adding dexamethasone (Fig. 2). However, knockdown of GILZ significantly reduced dexamethasone enhancement of MSC osteogenic differentiation capacity (Fig. 3), suggesting that the reported positive effect of GCs on in vitro marrow stromal cell cultures (43, 44) may have a connection with GILZ. Further studies regarding the underlying mechanisms regulating GILZ stability, as well as the effect of knockdown of overexpressed GILZ (e.g. introduction of shGILZ-1 into MSC-GILZ cells) on MSC proliferation, differentiation, and apoptosis may shed new light on GC-induced bone loss.

**FIGURE 5.** GILZ inhibits MSC adipocyte differentiation. A and B, real-time RT-PCR analysis of adipogenic-specific gene expression. MSC-GFP and MSC-GILZ cells were cultured in either OS for 5 days or MID for 2 days followed by 4 days in adipogenic maintenance media (DMEM plus 5 μg/ml insulin) before total RNA was prepared. mRNA levels of Pparγ2 (A) and C/ebpα (B) were analyzed by real-time RT-PCR as described in the legend to Fig. 3. This experiment was repeated two times with the same results. Result of one representative experiment is shown. C, MSC-GFP (left) and MSC-GILZ (right) cells were induced with MID for 2 days and then switched to adipogenic maintenance media for further differentiation with media changed every other day. The cell morphology was monitored daily by microscopy for the appearance of lipid vacuoles and photographed (day 16). Although the retrovirus infection efficiency is very high, it cannot reach 100%. The adipocyte seen in GILZ-expressing virus-infected MSCs (C, right panel) is an uninfected cell. Cells cultured in regular growth media showed no lipid-droplet accumulation (not shown). This experiment was repeated at least three times.

PPARγ2 and Runx2/Cbfa1 are two key transcription factors that drive MSCs to undergo adipocyte and osteoblast differentiation, respectively. Because MSC adipocyte and osteoblast differentiation has a reciprocal relationship, and our previous studies have shown that GILZ inhibits Pparγ2 transcription (11), we conclude here that the enhancement of MSC osteogenic differentiation by GILZ occurs through the inhibition of adipogenic differentiation. This role of GILZ is similar to what has been shown with TAZ (transcriptional coactivator with PDZ-binding motif), a transcriptional modulator that fine-tunes the balance of MSC osteoblast and adipocyte differentiation (45). Any potential GILZ and TAZ interactions remain to be defined.

GILZ is a relatively new transcription factor originally identified from dexamethasone-treated thymocytes (10). GILZ is also reported to be induced by estrogen (46), which is important for bone homeostasis, and by sonic hedgehog (Shh) (47), a morphogen that plays a critical role in embryogenesis. Our studies demonstrate that GILZ regulates MSC adipocyte and osteoblast differentiation. It is worth noting that the induction of
FIGURE 6. GILZ does not affect MSC proliferation. MSC-GFP and MSC-GILZ cells were plated in triplicate in 96-well plates at a density of 1 × 10^4 cells/cm² and cell proliferation was assayed using a Cell Growth Determination kit for 11 consecutive days. The experiment was repeated two times (p > 0.05, t test).

GILZ in MSCs in response to dexamethasone treatment seems transient (Fig. 1C), suggesting that the lack or low levels of GILZ expression in MSCs could be a key point linking long term GC therapy and GC-induced osteoporosis.

Acknowledgment—We thank Dr. Rhea-Beth Markowitz for critical reading of the manuscript.

REFERENCES

1. Bruder, S. P., Jaiswal, N., and Haynesworth, S. E. (1997) J. Cell. Biochem. 64, 278–294
2. Engler, A. J., Sen, S., Sweeney, H. L., and Discher, D. E. (2006) Cell 126, 677–689
3. Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S., and Marshak, D. R. (1999) Science 284, 143–147
4. Prockop, D. J. (1997) Science 276, 71–74
5. Abhdjoudj, S., Lasmoles, F., Oyaobi, B. O., Lomri, A., Delannoy, P., and Marie, P. J. (2001) J. Cell. Biochem. 81, 23–38
6. Beresford, J. N., Bennett, J. H., Devlin, C., Leboy, P. S., and Owen, M. E. (1992) J. Cell Sci. 102, 341–351
7. Gori, F., Thomas, T., Hicok, K. C., Spelsberg, T. C., and Riggs, B. L. (1999) J. Bone Miner. Res. 14, 1522–1535
8. Jaiswal, R. K., Jaiswal, N., Bruder, S. P., Mbalaviele, G., Marshak, D. R., and Pittenger, M. F. (2000) J. Biol. Chem. 275, 9645–9652
9. Lecka-Czernik, B., Gubrij, I., Moerman, E. J., Kajkenova, O., Lipschitz, D. A., Manolagas, S. C., and Jilka, R. L. (1999) J. Cell. Biochem. 74, 357–371
10. D’Adamo, F., Zollo, O., Moraca, R., Ayroldi, E., Bruscoli, E., Bartoli, A., Cannarile, L., Migliorati, G., and Riccardi, C. (1997) Immunity 7, 111–120
11. Shi, X., Shi, W., Li, Q., Song, B., Wan, M., Bai, S., and Cao, X. (2003) EMBO Rep. 4, 374–380
12. Ayroldi, E., Zollo, O., Macchiariello, A., Di Marco, B., Marchetti, C., and Riccardi, C. (2002) Mol. Cell. Biol. 22, 7929–7941
13. Shibanuma, M., Kuroki, T., and Nose, K. (1992) J. Biol. Chem. 267, 10219–10224
14. Ayroldi, E., Migliorati, G., Bruscoli, S., Marchetti, C., Zollo, O., Cannarile, L., D’Adamo, F., and Riccardi, C. (2001) Blood 98, 743–753
15. Mittelstadt, P. R., and Ashwell, J. D. (2001) J. Biol. Chem. 276, 29603–29610
16. Yang, N., Zhang, W., and Shi, X. M. (2007) J. Cell. Biochem., in press
17. Gimble, J. M., Robinson, C. E., Wu, X., Kelly, K. A., Rodriguez, B. R., Kliewer, S. A., Lehmann, I. M., and Morris, D. C. (1996) Mol. Pharmacol. 50, 1087–1094
18. Peister, A., Mellad, J. A., Larson, B. L., Hall, B. M., Gibson, L. F., and Prockop, D. J. (2004) Blood 103, 1662–1668
19. Tropel, P., Noel, D., Piatet, N., Legrand, P., Benabid, A. L., and Berger, F. (2004) Exp. Cell Res. 295, 395–406
20. Ory, D. S., Neugeboren, B. A., and Mulligan, R. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11400–11406
21. Stanford, C. M., Jacobson, P. A., Eanes, E. D., Lemble, L. A., and Midura, R. J. (1995) J. Biol. Chem. 270, 9420–9428
22. Tichopad, A., Dilger, M., Schwarz, G., and Pfaff, M. W. (2003) Nucleic Acids Res. 31, e122
23. Pfaff, M. W. (2001) Nucleic Acids Res. 29, e45
24. Robinzon, D. A., Dillon, C. P., Kwiakowski, A. V., Sievers, C., Yang, L., Kopinja, J., Rooney, D. L., Ihrig, M. M., McManus, M. T., Gertler, F. B., Scott, M. L., and Van Parijs, L. (2003) Nat. Genet. 33, 401–406
25. Reichardt, H. M., Tuckermann, J. F., Bauer, A., and Schutz, G. (2000) Z. Rheumatol. 59, Suppl. 2, I/1–I/15
26. Barnes, P. J. (2005) Immunol. Allergy Clin. N. Am. 25, 451–468
27. Harris, E. D., Jr. (1983) Hosp. Pract. (Off Ed.) 18, 137–141
28. Conn, D. L. (2001) Arthritis Rheum. 45, 462–467
29. Kirwan, J. R. (2000) Z. Rheumatol. 59, Suppl. 2, II/85–II/89
30. The Lung Health Study Research Group (2000) N. Engl. J. Med. 343, 1902–1909
31. Bazzy-Asaad, A. (2001) Curr. Opin. Pediatr. 13, 523–527
32. Corren, J., Nelson, H., Greos, L. S., Bensch, G., Goldstein, M., Wu, J., Wang, S., and Newman, K. (2001) Ann. Allergy Asthma Immunol. 87, 405–411
33. Park, S. J., Nguyen, D. Q., Savik, K., Hertz, M. L., and Bolman, R. M., III (2001) J. Heart Lung Transplant. 20, 304–309
34. Walsh, L. J., Wongs, C. A., Oborne, J., Cooper, S., Lewis, S. A., Pringle, M., Hubbard, R., and Tattersfield, A. E. (2001) Thorax 56, 279–284
35. Julian, B. A., Laskow, D. A., Dubovsky, J., Dubovsky, E. V., Curtis, J. J., and Quares, L. D. (1991) N. Engl. J. Med. 325, 544–550
36. Locascio, V., Bonucci, E., Imbimbo, B., Ballanti, P., Adami, S., Milani, S., Tartarotti, D., and DellaRocca, C. (1996) Bone Miner. 8, 39–51
37. Chawes, J. A., Peel, N., and Eastell, R. (2001) Curr. Opin. Rheumatol. 13, 326–332
38. Larkett, B. P., and Raisz, L. G. (1990) Ann. Intern. Med. 112, 352–364
39. Cruess, R. L., Ross, D., and Cawrash, E. (1975) Clin. Orthop. 113, 178–183
40. Cui, Q., Wang, G. J., and Balian, G. (1997) J. Bone Joint Surg. Am. 79, 1054–1063
41. Meunier, P., Aaron, J., Edouard, C., and Vignon, G. (1971) Clin. Orthop. 80, 147–154
42. Weinstein, R. S., Ilka, R. L., Parfitt, A. M., and Manolagas, S. C. (1998) J. Clin. Investig. 102, 274–282
43. Beresford, J. N., Jommer, C. J., Devlin, C., and Triffitt, J. T. (1994) Arch. Oral Biol. 39, 941–947
44. Cheng, S. L., Yang, J. W., Rifas, L., Zhang, S. F., and Avioli, L. V. (1994) Endocrinology 134, 278–286
45. Hong, J. H., Hwang, E. S., McManus, M. T., Amsterdam, A., Tian, Y., Kalmukova, R., Mueller, E., Benjamin, T., Spiegelman, B. M., Sharp, P. A., Hopkins, N., and Yaffe, M. B. (2005) Science 309, 1074–1078
46. Tynan, S. H., Lundeen, S. G., and Allan, G. F. (2004) J. Steroid Biochem. Mol. Biol. 91, 225–239
47. Ingram, W. J., Wicking, C. A., Grimmion, S. M., Forrest, A. R., and Wainwright, B. J. (2002) Oncogene 21, 8196–8205