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VDR-Mediated Inhibition of DKK1 and SFRP2 Suppresses Adipogenic Differentiation of Murine Bone Marrow Stromal Cells

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Abstract Osteoblasts and adipocytes are thought to derive from a common bone marrow stromal cell (BMSC) precursor. Activation of the canonical Wnt signaling pathway plays a pivotal role in the differentiation of BMSCs along either of these two lineages, promoting osteogenesis and inhibiting adipogenesis. Liganded nuclear receptors, including the vitamin D receptor (VDR) and peroxisomal proliferator-activated receptor γ (PPARγ), can also affect BMSCs differentiation. To address whether VDR ablation modulates the differentiation of BMSCs into the osteoblast or adipogenic lineages, BMSCs were isolated from VDR null mice and from their wild-type littermates. VDR ablation did not alter osteoblastic differentiation. However, when cultured under adipogenic conditions, BMSCs from the VDR null mice expressed higher mRNA levels of PPARγ and of markers of adipogenic differentiation. An increase in the size and number of mature adipocyte foci was also observed in cultures isolated from VDR null mice relative to those isolated from wild-type mice. To address whether the increased adipogenesis observed in the VDR null cultures was associated with inhibition of the canonical Wnt signaling pathway, mRNA levels for DKK1 and SFRP2 were examined. Cultures from the VDR null mice expressed higher levels of mRNA encoding DKK1 and SFRP2 than did the wild-type cultures. This difference is, at least in part, due to ligand-dependent actions of the VDR, since 1,25-dihydroxyvitamin D_3 suppressed DKK1 and SFRP2 expression in wild-type cultures. Thus, the VDR inhibits adipogenesis of BMSCs at least in part by suppressing the expression of inhibitors of the canonical Wnt signaling pathway. J. Cell. Biochem. 101: 80–88, 2007.

Key words: $1,25(OH)_2D_3$; PPARγ; osteoblast differentiation; adipocyte differentiation; Wnt signaling

Common precursors found in the bone marrow give rise to osteoblasts and adipocytes [Aubin, 1998]. In vivo studies showing an inverse relationship between trabecular bone volume and adipose content of bone marrow suggest that differentiation into these two lineages is reciprocally regulated [Beresford et al., 1992]. Nuclear receptor ligands such as 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] modulate the early differentiation phase of bone marrow stromal cells (BMSCs) [Beresford et al., 1992; Dorheim et al., 1993]. The main biological effects of $1,25(OH)_2D_3$ are mediated by the vitamin D receptor (VDR) [Haussler et al., 1998; Erben et al., 2002] that is expressed in numerous cell types including BMSCs, osteoblasts and adipocytes [Gruber et al., 1999; Langub et al., 2000; Schneider et al., 2005; Kong and Li, 2006]. In vitro studies in osteoblast and adipocyte cell models demonstrate that $1,25(OH)_2D_3$ regulates differentiation and gene transcription, but the results of these studies differ, depending on the species, the maturational stage of the cells and the time and duration of hormone treatment [Beresford et al., 1986, 1992; Bellows et al., 1994, 1999; Chen and Fry, 1999].

The role of the VDR in vivo also remains unclear. An increase in bone volume due to an increase in cortical bone formation and a decrease in trabecular resorption is observed in mice overexpressing the VDR in mature osteoblasts [Gardiner et al., 2000], suggesting

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a critical role for the VDR in endochondral bone formation. Paradoxically, mice lacking the VDR in pre-osteoblasts also have an increase in bone volume [Yamamoto et al., 2004]. However, studies in mice with global ablation of the VDR demonstrate that prevention of abnormalities in mineral ion homeostasis leads to a normal skeletal phenotype, suggesting that the VDR is not essential for skeletal homeostasis [Li et al., 1998; Amling et al., 1999]. In vitro studies using cells from VDR knockout mice demonstrate that, in the absence of systemic or paracrine factors compensating for the absence of the VDR, osteoblasts lacking the VDR cannot support osteoclastogenesis in response to $1,25(OH)_2D_3$ but do so in response to PTH [Takeda et al., 1999]. These latter data suggest that systemic or paracrine factors can compensate for the absence of the VDR to maintain skeletal homeostasis in vivo.

Similarly, the role of $1,25(OH)_2D_3$ in adipogenesis remains unclear. $1,25(OH)_2D_3$ inhibits pre-adipocyte differentiation [Sato and Hiragun, 1988], however promotes adipocyte differentiation of 3T3-L1 cells [Vu et al., 1996] and synergizes with glucocorticoids, stimulating adipogenesis in rat calvaria cells [Bellows et al., 1994] and in rat bone marrow stromal cells [Atmani et al., 2003]. However other studies in murine BMSCs demonstrate that $1,25(OH)_2D_3$ antagonizes the adipogenic effect of glucocorticoids, decreasing the expression of markers of late adipocytes including adipsin and aP2 [Kelly and Gimble, 1998].

The mechanism by which $1,25(OH)_2D_3$ inhibits adipogenesis has not yet been clarified. Overexpression of the VDR inhibits the expression of a key regulator of adipocyte differentiation, peroxisomal proliferator-activated receptor γ (PPARγ) [Kong and Li, 2006], thereby inhibiting adipogenic differentiation of 3T3-L1 pre-adipocytes. The key role of PPARγ in adipogenic differentiation is clearly demonstrated by the fact that embryonic stem cells lacking PPARγ fail to differentiate into adipocytes [Rosen et al., 1999], but spontaneously differentiate into osteoblasts. Interestingly, PPARγ haploinsufficiency enhances osteogenesis in vitro and in vivo, suggesting a role for this receptor in the lineage progression of BMSCs [Akune et al., 2004]. Thus, inhibition of PPARγ expression by the VDR implicates the VDR as having an important role in the reciprocal regulation of adipogenesis and osteogenesis in the bone marrow. Therefore, to evaluate the consequences of VDR ablation on the differentiation of BMSCs, the osteogenic and adipogenic potential of BMSCs isolated from the VDR null mice was examined.

MATERIALS AND METHODS

Animals

Wild-type mice and their VDR null littermates [Li et al., 1997] were maintained in a virus and parasite-free barrier facility. They were exposed to a 12-h light, 12-h dark cycle and fed a standard chow containing 1% calcium and 0.44% phosphorus. All studies performed were approved by the institutional animal care committee.

Primary Cultures of Murine BMSC

BMSC were isolated from 18-day-old wild-type and VDR knockout littermates, prior to the development of abnormal mineral ion homeostasis. Femora and tibiae were removed under aseptic condition and dissected free of muscle, connective tissue and epiphyses. To evaluate osteogenic differentiation, cells were isolated by flushing bones with α-MEM/10% heat-inactivated FBS, seeded at a cell density of 4×10^5 cells/cm^2 and maintained at 37°C in 5% CO_2. After 4 days in culture, the medium was supplemented with 50 μM ascorbic acid (Sigma, St. Louis, MO). The medium was replaced after 1 week and twice weekly thereafter. For studies evaluating mineralization, 10 mM β-glycerol-phosphate (Sigma) was added 3 days prior to harvest. To evaluate adipogenic differentiation, cells were isolated by flushing bones with supplemented DMEM [Kelly and Gimble, 1998], plated at a cell density of 4×10^5 cells/cm^2 and maintained at 37°C in 7% CO_2. Media was replaced 2 h post plating and weekly thereafter. At 80% confluence, the cells were treated with MHI (0.5 mM methylisobutyl xanthine, 0.5 μM hydrocortisone, and 60 μM indomethacin in DMSO; Sigma), in the presence or absence of 10^{-8} M $1,25(OH)_2D_3$ for 3 days. Cells were then maintained in supplemented DMEM until harvesting.

Alkaline Phosphatase Activity

Cells were fixed with 10% phosphate-buffered formalin for 30 min [Kostenuik et al., 1997], rinsed with distilled water, then incubated for 30 min at 37°C in alkaline buffer solution

containing 1.5 M 2-amino-2-methyl-1-propanol (Sigma), using *p*-nitrophenyl phosphate (Sigma) as a substrate. *P*-nitrophenol release was quantitated by measuring absorbance at 405 nm. Alkaline phosphatase activity was reported as nmol PNPP/min/35 mm well.

Mineralization Assay

To evaluate calcium deposition into the matrix, cells were washed with PBS, calcium was solubilized with 0.6 N HCl for 6 h at room temperature, the samples were reacted with methylthymol blue and measured spectrophotometrically at 620 nm [Gindler and King, 1972] against a standard curve of calcium carbonate.

Oil-Red O Staining

Ten days post MHI or vehicle treatment cells were washed with PBS, fixed in 10% buffered formalin for 15 min, stained with 0.5% Oil red-O in isopropyl alcohol: distilled water (6:4) for 30 min at 37°C, washed and air dried [Sordella et al., 2003]. Oil red-O staining was quantified with a light microscope by counting the number of stained foci containing more than 5 mature adipocytes.

Real-Time Quantitative RT-PCR

RNA was extracted (RNeasy-kit protocol, Qiagen, Valencia, CA), DNase digested (Message Clean kit, Genhunter Corporation, Brookline, MA) and cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). mRNA levels were evaluated by quantitative real time PCR (DNA Engine Opticon System, MJ Research, Waltham, MA) and normalized for actin mRNA [Livak and Schmittgen, 2001]. Primer sequences are in Table I.

TABLE I. Oligonucletide Primers Used for RT-PCR Analyses

Mouse osteopontin
Sense: 5′-TGC ACC CAG ATC CTA TAG CC
Antisense: 5′-CTC CAT CGT CAT CAT CAT CG
Mouse BSP
Sense: 5′-AGG GAA CTG ACC AGT GTT GG
Antisense: 5′-ACT CAA CGG TGC TGC TTT TT
Mouse osteocalcin
Sense: 5′-AAG CAG GAG GGC AAT AAG GT
Antisense: 5′-GCG GTC TTC AAG CCA TAC TG
Mouse PPARγ
Sense: 5′-AGG CCG AGA AGG AGA AGC TGT TG
Antisense: 5′-TGG CCA CCT CTT TGC TCT GCT C
Mouse lipoprotein lipase
Sense: 5′-GTG GCC GAG AGC GAG AAC
Antisense: 5′-AAG AAG GAG TAG GTT TTA TTT GTG GAA
Mouse adipsin
Sense: 5′-CCT GAA CCC TAC AAG CGA TG
Antisense: 5′-GGT TCC ACT TCT TTG TCC TCG
Mouse aP2
Sense: 5′-TCT CAC CTG GAA GAC AGC TCC TCC TCG
Antisense: 5′-TTC CAT CCA GGC CTC TTC CTT TGG CTC
Mouse Dkk1
Sense: 5′-GCT GCA TGA CGC ACG CTA T
Antisense: 5′-AGA GGG CAT GCA TAT TCC ATT
Mouse Sfrp2
Sense: 5′-CCCCTGTCTGTCTCGACGA
Antisense: 5′-CTTCACACACCTTGGGAGCTT
Mouse βactin
Sense: 5′-CCT CTA TGC CAA CAC AGT GC
Antisense: 5′-ACA TCT GCT GGA AGG TGG AC

Statistical Analysis

Experimental data are expressed as the mean ± SEM of three independent experiments. Student's *t*-test values of $P < 0.05$ were considered statistically significant.

RESULTS

To determine if absence of the VDR alters the program of osteoblastic differentiation of primary murine BMSCs, the time of onset and the degree of expression of markers of osteoblast differentiation were examined in cells isolated from 18-day-old VDR null mice and their wild-type littermates. Alkaline phosphatase is an early marker of osteoblast differentiation and an increase in alkaline phosphatase activity correlates with the acquisition of a more differentiated osteoblastic phenotype [Stein and Lian, 1993]. No significant difference in ALP activity was found between the cultures isolated from VDR-null mice and those isolated from their wild-type littermates (Fig. 1A), nor did the number of ALP positive CFU-OB colonies differ (data not shown). The expression of other markers of osteoblast differentiation including osteopontin, bone sialoprotein and osteocalcin [Bellows et al., 1999] was assessed by real-time PCR. The mRNA levels of these genes did not differ between the cultures isolated from VDR-knockout mice and those derived from their wild-type littermates (Fig. 1B). Terminal osteoblast differentiation is characterized by mineralized matrix formation. Calcium deposition into the matrix was not altered in the VDR-null cultures (Fig. 1C).

To address whether differentiation of BMSCs into the adipocyte lineage was affected by VDR status, expression of markers of adipocyte differentiation was examined in BMSCs isolated from 18-day-old VDR null mice and their wild-type littermates. In the absence of MHI, the expression of PPARγ, a key regulator

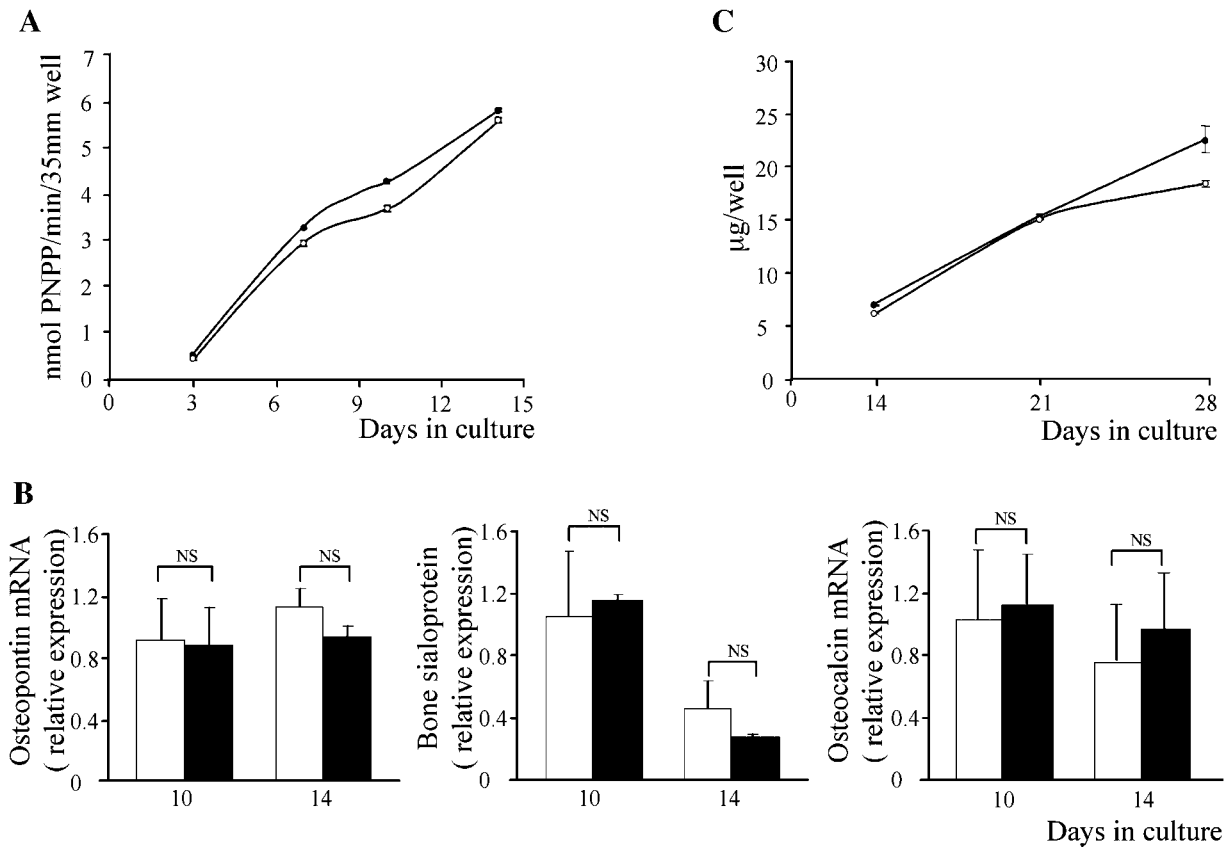

Fig. 1. Acquisition of alkaline phosphatase activity (**A**), expression of osteoblast differentiation markers (**B**) and time course in increase in calcium content (**C**) in BMSCs isolated from vitamin D receptor (VDR) null mice (closed symbol) and wild-type littermates (open symbol), was examined under osteoblastic differentiation conditions. Data represent the mean ± SEM of four independent cultures isolated from four mice of each genotype. NS = not significant.

of adipogenic differentiation [Tontonoz et al., 1994], and lipoprotein lipase (LPL), an early marker of adipogenic differentiation, was unaffected by VDR status. Addition of MHI increased the expression of both PPARγ and LPL to a greater extent in the VDR null cultures than in the wild type cultures (Fig. 2A,B). To examine whether this enhancement of adipogenic differentiation persisted, mRNA levels of aP2 and adipsin, two markers of late adipogenic differentiation, were evaluated (Fig. 2C,D). Although higher levels of aP2 and adipsin mRNA were observed in the VDR null BMSC relative to the wild-type cultures in the absence of MHI, a dramatic enhancement of adipocyte differentiation was seen in the MHI treated VDR null cultures. Oil red-O staining was performed to evaluate the number of mature adipocyte foci. In the absence of MHI, rare Oil red-O stained foci were observed in both the cultures lacking the VDR and those isolated from wild-type mice (Fig. 3A,B). MHI enhanced adipogenesis in cultures isolated from mice of both genotypes, increasing the number and the size of adipocyte foci (Fig. 3A,B and data not shown). However, in the presence of MHI, large adipocyte foci, containing more than 50 adipocytes, represented 37% of the foci in VDR null cultures, and were absent in the wild-type cultures. Medium adipocyte foci, containing between 25 and 50 mature adipocytes, were equally represented in the cultures (36% of in the VDR null cultures and 40% in the wild-type cultures), whereas foci containing 5–25 adipocytes made up 60% of the foci in the wild-type cultures and only 27% of the foci in the VDR null cultures.

The canonical Wnt signaling pathway has been shown to promote osteogenesis and inhibit adipogenesis [Ross et al., 2000]. To address whether increased expression of Wnt inhibitors could, in part, account for the enhanced

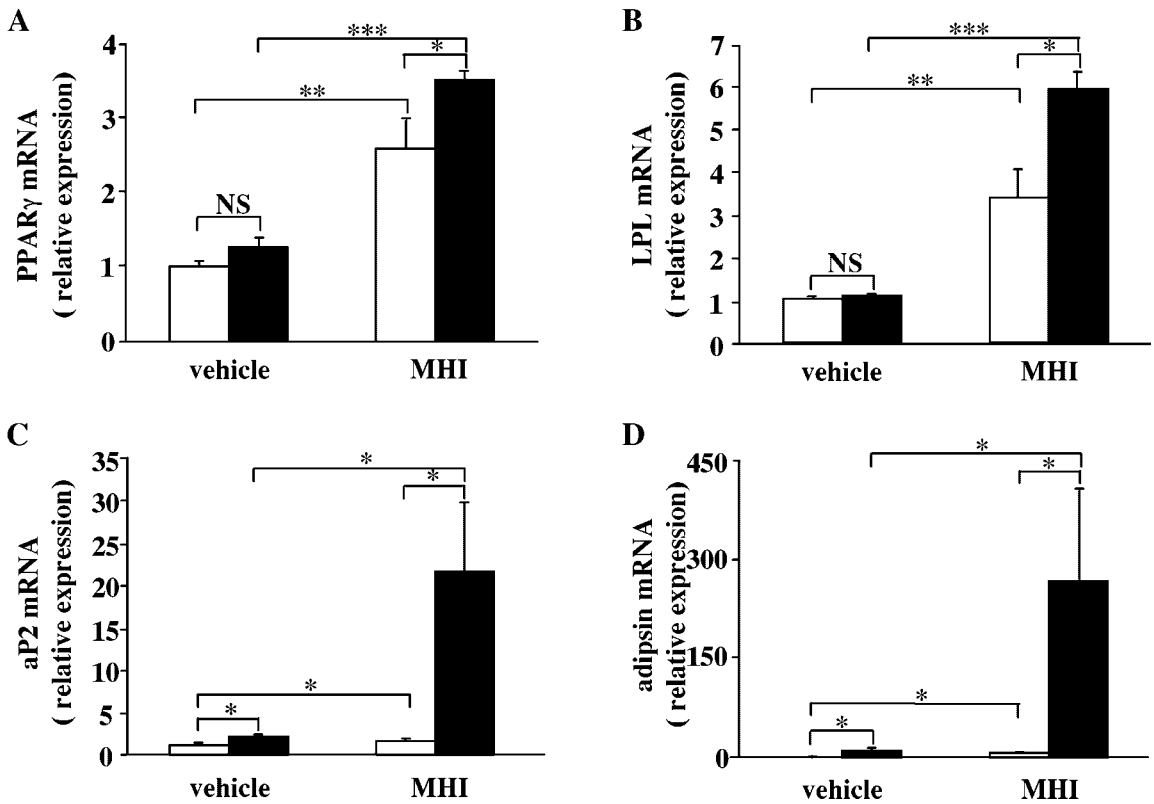

Fig. 2. Early and late markers of adipogenic differentiation were examined 48 h (**A** and **B**), and 10 days (**C** and **D**) after vehicle or MHI treatment of BMSCs isolated from VDR null mice (closed bars) and their wild-type littermates (open bars). Data represent the mean ± SEM of four independent cultures isolated from four mice of each genotype. $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$, NS = not significant.

adipogenesis observed in the VDR null cultures, Dickkopf1 (DKK1) and secreted frizzled-related protein 2 (SFRP2) mRNA levels were evaluated. The expression of DKK1 and SFRP2 was markedly increased in the cultures isolated from the VDR-null mice, both in the absence (Fig. 4A) and presence of MHI (data not shown).

To address whether the VDR suppressed the expression of DKK1 and SFRP2 in a ligand-dependent fashion, primary wild-type BMSCs, cultured under adipogenic conditions, were treated with $1,25(OH)_2D_3$. The expression of DKK1 and SFRP2 mRNA was repressed 13.4 ± 0.9 and 14.5 ± 0.2 fold respectively 48 h after $1,25(OH)_2D_3$ treatment (Fig. 4B,D) in the absence of MHI. $1,25(OH)_2D_3$-mediated repression of Dkk1 (2 ± 0.1) and Sfrp2 (3.7 ± 0.2) was maintained in the presence of MHI (Fig. 4C,E).

DISCUSSION

Canonical Wnt signaling has been shown to regulate the early commitment of pluripotential stem cells to the osteoblast lineage [Gaur et al., 2005]. Further, transgenic overexpression of Wnt10b in adipogenic cells in the bone marrow, under the control of the fatty acid binding protein 4 promoter, leads to an increase in bone mass [Bennett et al., 2005], confirming a key role for Wnt signaling in promoting the differentiation of BMSCs into the osteoblast lineage. Conversely, inhibition of the canonical Wnt signaling pathway promotes adipogenic differentiation, at least in part, by modulating the expression of adipogenic transcription factors including PPARγ [Ross et al., 2000; Kawaguchi et al., 2005]. Canonical Wnt-signaling is negatively regulated by DKKs and SFRPs both of which inhibit Wnt-LRP receptor interactions [Kawano and Kypta, 2003; Bodine et al., 2004]. Haploinsufficiency of DKK1 increases bone formation [Glinka et al., 1998], whereas overexpression of DKK1 in rat calvarial osteoblasts enhances adipocyte differentiation [Morvan et al., 2006].

Our investigations in BMSCs lacking the VDR demonstrate an increase in adipogenic

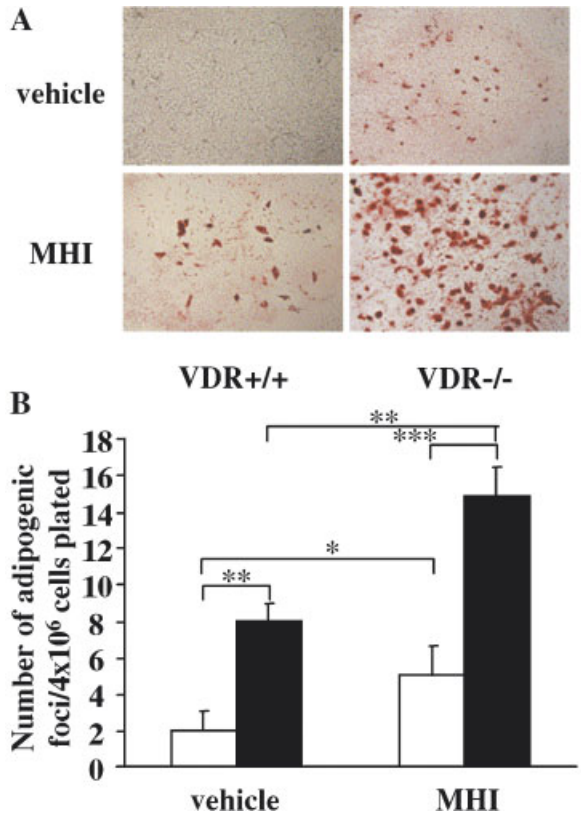

Fig. 3. Oil red-O staining was performed 10 days after vehicle or MHI treatment of BMSCs isolated from VDR null mice (VDR-/- and closed bars) and their wild-type littermates (VDR+/+ and open bars). Photographs (**A**) are representative of the adipocyte foci observed under the culture conditions indicated. The quantitation of adipocyte foci is shown in (**B**). Data represent the mean ± SEM of preparations of BMSCs from three mice of each genotype. $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

potential associated with increased expression of DKK1 mRNA. DKK1 is upregulated in response to activation of the canonical Wnt signaling pathway [Gonzalez-Sancho et al., 2005], thus the observed increase in DKK1 expression could be a primary event leading to inhibition of this pathway or could reflect an upregulation of canonical Wnt signaling, the latter of which would not be expected to lead to enhanced adipogenesis. Investigations were, therefore, performed to examine the expression of SFRP2, a Wnt inhibitor that, unlike DKK1, is repressed rather than induced, by canonical Wnt signaling [Jackson et al., 2005]. The observation that SFRP2 mRNA levels are also increased in the absence of the VDR, suggests that inhibition of the canonical Wnt signaling pathway via increased expression of these Wnt antagonists is, at least in part, responsible for the increased adipogenic differentiation observed in BMSCs lacking the VDR.

Interestingly, although we observed an increase in adipogenic potential in BMSC isolated from VDR null mice, no difference in osteogenic potential was detected. These findings are somewhat at odds with our previous investigations in calvarial osteoblasts lacking the VDR [Sooy et al., 2005]. These differences may reflect the disparate effects of $1,25(OH)_2D_3$ on osteoblast differentiation previously reported. While $1,25(OH)_2D_3$ promotes terminal osteoblast differentiation [Bellows et al., 1999; Chen and Fry, 1999], the effects of $1,25(OH)_2D_3$ on the early stages of differentiation depend on the species, the stage of hormone treatment (proliferative vs. post-proliferative cultures), the duration of treatment and the cell model examined. Calvarial osteoblasts represent a relatively homogeneous population of committed cells, whereas early osteoblast precursors represent approximately 1×10^{-5} of the nucleated bone marrow cells [Falla et al., 1993]. Thus, the paracrine actions of other cells in the bone marrow microenvironment may modulate the consequences of VDR ablation. Notable in this respect is the fact that DKK1 expression is unaltered in calvarial osteoblasts lacking the VDR (data not shown). It is, therefore, likely that the paracrine effects of other cells in the bone marrow increase expression of inhibitors of the canonical Wnt signaling pathway in the VDR null background. Thus, in the absence of increased expression of DKK1 and SFRP2, we would anticipate that BMSCs from the VDR null mice would exhibit the same enhanced osteogenic potential observed in vitro in calvarial osteoblasts lacking the VDR and in vivo in mice with osteoblast—specific ablation of the VDR [Yamamoto et al., 2004]. Interestingly, in addition to our investigations demonstrating that the VDR represses expression of inhibitors of canonical Wnt signaling in a ligand-dependent fashion, $1,25(OH)_2D_3$ has also been shown to increase expression of the Wnt receptor, LDL receptor-related protein 5 (LRP5) in ST2 stromal cells [Fretz et al., 2006].

Although investigations in 70-day-old VDR null mice with normal mineral ion homeostasis, failed to reveal a demonstrable skeletal phenotype [Amling et al., 1999], by 4 months of age, the VDR null mice have a reduction in CFU-OB, accompanied by a decrease in trabecular bone

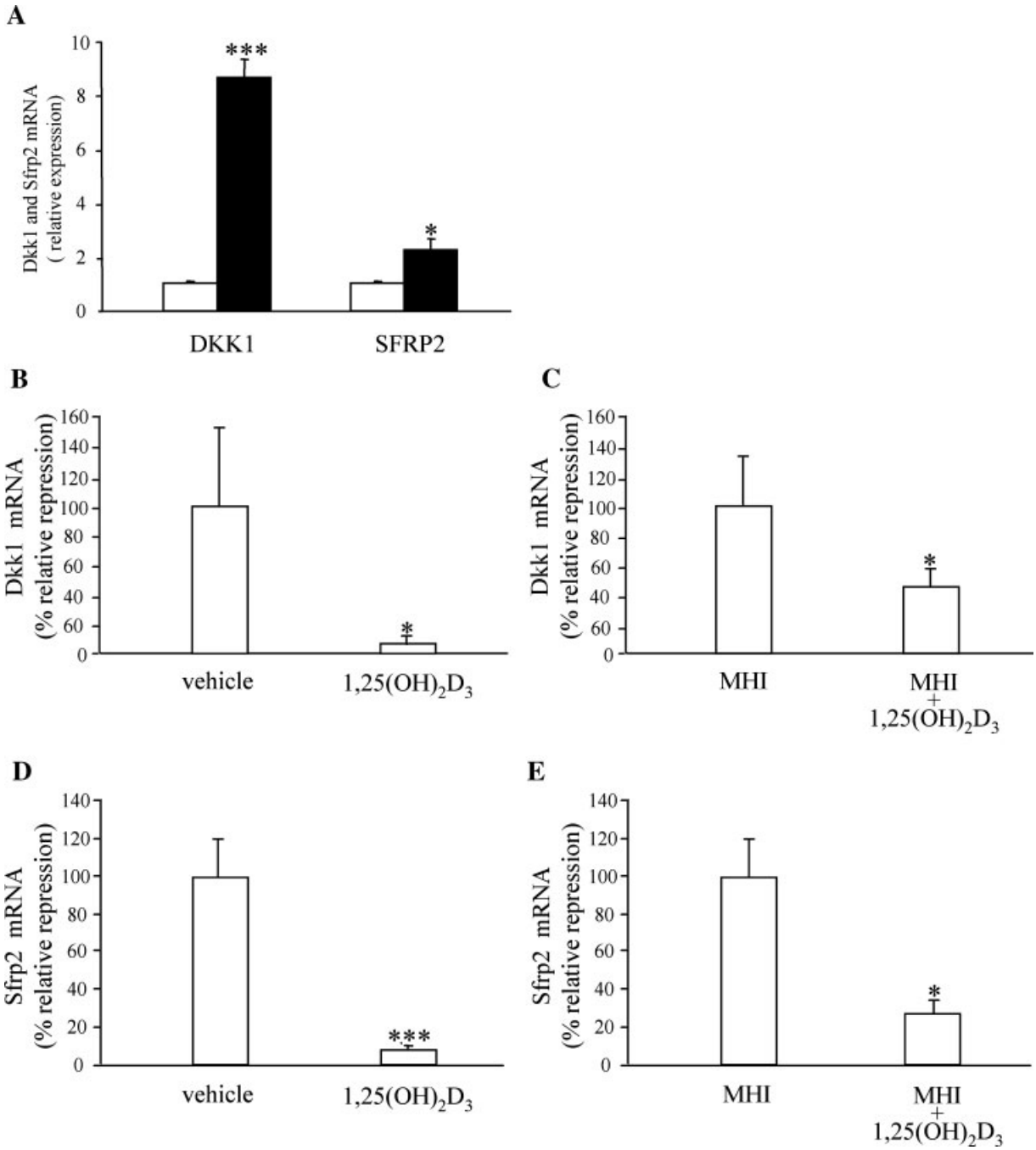

Fig. 4. DKK1 and SFRP2 expression was evaluated 48 h after vehicle treatment of BMSCs from VDR null mice (closed bars) and wild-type littermates (open bars) (A). Data represent the mean ± SE of preparations of BMSCs from three mice of each genotype. $*P < 0.05$, $***P < 0.0005$ compared with the wild-type bone marrow stromal cells. Expression of DKK1 and SFRP2 was evaluated 48 h after a single dose of 10^{-8} M $1,25(OH)_2D_3$ treatment of BMSCs isolated from wild-type mice, in the absence (**B** and **D**) or presence of MHI (**C** and **E**). Data represent the mean ± SEM of cells from three mice. $*P < 0.05$, $***P < 0.0005$ compared with vehicle- or MHI-treated wild-type bone marrow stromal cells.

volume [Panda et al., 2004], suggesting that the VDR may play a critical role in skeletal homeostasis in ageing mice.

Thus, we propose that ligand-dependent actions of the VDR in BMSCs promote canonical Wnt signaling by inhibiting the expression of DKK1 and SFRP2 and inducing the expression of LRP5. Additional investigations will be required to address which cell type and at what stage of differentiation the VDR acts to repress

the expression of these inhibitors of the canonical Wnt signaling pathway.

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