

Differential Signalling Through ALK-1 and ALK-5 Regulates Leptin Expression in Mesenchymal Stem Cells

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Leptin plays a central role in maintaining energy balance, with multiple other systemic effects. Despite leptin importance in peripheral regulation of mesenchymal stem cells (MSC) differentiation, little is known about its expression mechanism. Leptin is often described as adipokine, while it is expressed by other cell types. We have recently shown an *in vitro* leptin expression, enhanced by glucocorticoids in synovial fibroblasts (SVF). Here, we investigated leptin expression in MSC from bone marrow (BM-MSC) and umbilical cord matrix (UMSC). Results showed that BM-MSC, but not UMSC, expressed leptin that was strongly enhanced by glucocorticoids. Transforming growth factor β 1 (TGF- β 1) markedly inhibited the endogenous- and glucocorticoid-induced leptin expression in BM-MSC. Since TGF- β 1 was shown to signal via ALK-5-Smad2/3 and/or ALK-1-Smad1/5 pathways, we analyzed the expression of proteins from both pathways. In BM-MSC, TGF- β 1 increased phosphorylated Smad2 (p-Smad2) expression, while ALK-5 inhibitor (SB431542) induced leptin expression and significantly restored TGF- β 1-induced leptin inhibition. In addition, both prednisolone and SB431542 increased p-Smad1/5 expression. These results suggested the ALK-5-Smad2 pathway as an inhibitor of leptin expression, while ALK-1-Smad1/5 as an activator. Indeed, Smad1 expression silencing induced leptin expression inhibition. Furthermore, prednisolone enhanced the expression of TGF- β RII while decreasing p-Smad2 in BM-MSC and SVF but not in UMSC. *In vitro* differentiation revealed differential osteogenic potential in SVF, BM-MSC, and UMSC that was correlated to their leptin expression potential. Our results suggest that ALK-1/ALK-5 balance regulates leptin expression in MSC. It also underlines UMSC as leptin nonproducer MSC for cell therapy protocols where leptin expression is not suitable.

Introduction

LEPTIN IS A NONGLYCOSYLATED peptide hormone that plays a crucial role in regulating central energy balance, and controlling appetite in humans. In addition, it has been shown that leptin plays a role in regulating bone mass through osteogenic differentiation of mesenchymal progenitor cells [1,2]. Despite the fact that it was discovered in 1994, progress in understanding mechanisms of leptin expression has been slowed by the lack of cell lines that express leptin and respond robustly to hormonal signals [3]. Leptin was widely studied in 3T3-L1 mouse cell line described as having the potential to differentiate into adipocytes, but with a low level of leptin mRNA expression [4,5]. The transcriptional regulation of the leptin gene was described as being controlled by Peroxisome proliferator-activated receptor- γ (PPAR- γ) [6–8], and

down-regulated by adrenergic stimulation [9]. Although described as an adipocyte-derived hormone, leptin is also secreted by several other cell types [7,10]. We have recently shown an *in vitro* leptin expression that was enhanced by glucocorticoids in mesenchymal synovial fibroblasts (SVF) [11]. Single or repeated injections of glucocorticoids were also shown to elevate rat plasma leptin in a dose-dependent manner [12]. On the other hand, leptin receptor expression was decreased *in vitro* in human bronchial epithelial cells by transforming growth factor β 1 (TGF- β 1) [13]. Despite these results, no mechanism of leptin expression has been proposed. In this study, we investigated leptin expression in bone marrow-derived mesenchymal stem cells (BM-MSC) that are widely used in various cell therapy protocols, and in umbilical cord matrix-derived MSCs (UMSC) that are considered as an emerging source of MSC for cell therapy [14]. Results

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demonstrated that BM-MSC but not UMSC produced leptin. We also showed that glucocorticoids and TGF- β 1 can regulate leptin expression through ALK-1-Smad1/5 and ALK-5-Smad2/pathways.

Materials and Methods

Isolation and culture of MSC

Processing of umbilical cord samples and subsequent isolation of UMSC ($n=9$) from human umbilical cord were achieved as described in Ref. [15]. BM was obtained from iliac crest marrow aspiration of healthy donors after informed consent. Isolation of BM MSCs ($n=7$) was performed as described in Ref. [14]. The synovial tissue were obtained from patients during joint replacement. Informed consents were obtained, and experiments were approved by the ethics committee of our academic hospital (CHU, Liège, Belgium). SVF ($n=10$) were isolated as previously explained [16,17]. MSC were cultured in a complete medium consisting of Dulbecco's modified Eagle's medium (DMEM) medium (Cambrex Bio Science), with L-glutamine (2 mM), streptomycin (100 mg/mL), and penicillin (100 U/mL) (BioWhittaker), and supplemented with 10% fetal bovine serum (FBS) (Lonza). Cells (5×10^4 cells/0.5 mL of medium) were seeded in 24-well plates (BD Biosciences) in triplicate. On the second day of culture, cells were stimulated with prednisolone, TGF- β (GIBCO-BRL), or SB 431542 (Sigma-Aldrich). After 1 week, the medium was collected for enzyme-linked immunosorbent assay (ELISA) tests, and cells were harvested for protein extraction.

Enzyme-linked immunosorbent assay

Leptin concentrations were determined in the cell supernatant using a commercial kit (R&D Systems) according to the manufacturer's instructions.

Transfection with lentivirus expressing Smad1 shRNA

SVF were plated at a density of 5×10^3 cells/cm² in 6-well plates and cultured overnight in complete medium (day 0). At day 1, the medium was replaced by a mixture of complete medium with polybrene (6 μ g/mL) (sc-134220). Infection was performed by adding 15 μ L of the pool of Smad1 shRNA (sc-29483) or control shRNA (sc-108080) infecting lentivirus (Santa Cruz Biotechnology). After over-night incubation with lentivirus, the medium was replaced, and the cells were incubated for 24 h. Selection of the cells was performed with 2 μ g/mL of puromycin (P7255) (Sigma-Aldrich) for 48 h.

Western blot

Cells were collected and lysed, and total were proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as explained earlier in Ref. [16]. Membranes were incubated with TGF β -RI (#3712), Smad1 (#9743), phosphorylated Smad1 (p-Smad1)1/5 (#9516), p-Smad2 (S465/467) (#3101), Smad2 (L16D3) (#3103), (Cell Signaling), ALK-1 (H-150) (sc-28976), TGF β -RII (C-16) (sc-220), OB-R (B-3) (sc-8391), ERK1/2 (K-23) (sc-94), Runx-2 (27-K) (Sc-101145) (Santa Cruz Biotechnology), and α -Tubulin (T6074) (Sigma-Aldrich) anti-

bodies for 1–3 h at room temperature. Western blots were shown with 1:2,000 diluted anti-mouse or anti-rabbit (DAKO A/S) antibodies and ECL chemiluminescent reagents (Amersham Biosciences). Bands intensities were quantified using Quantity one 4.6.5 software (Bio-Rad Laboratories, Inc.). Relative quantifications represent the mean \pm standard deviation of 3 different experiments performed on 3 different donors. The Mann-Whitney test was used to calculate significant differences between conditions.

Osteogenic differentiation

To induce osteogenic differentiation, 3×10^3 cells/cm² were plated in DMEM supplemented with 10% FBS, 10 mM β -glycerophosphate, 60 μ M ascorbic acid, and 10^{-7} M dexamethasone (Sigma Aldrich), and cultured for 3–4 weeks. Medium was replaced every 3–4 days. The induction of alkaline phosphatase (ALP) expression was detected after 3 weeks using histochemical staining assays. Briefly, cells were fixed with 70% (v/v) ethanol (Sigma-Aldrich) at room temperature for 10 min. After being washed with phosphate-buffered saline solution, cells were stained using a mixture of 0.3 mg/mL naphthol AS-MX phosphate, 0.005 mg/mL of NN dimethylformamide in Tris buffer (0.2 M, pH 9.1), and 1 mg/mL fast blue BB salt (Sigma-Aldrich).

Microscopy

Photos were captured using a digital sight camera (2 MV) under the inverted microscope Eclipse TS100 (Nikon Instruments, Inc.), using NIS-Elements Basic Research software (Nikon Instruments, Inc.) and objectives with phase contrast.

Statistics

P values were obtained using the Mann-Whitney *U*-test and were considered significant when <0.05 .

Results

BM-MSC are robust leptin producers, while UMSC do not express leptin

BM-MSC and UMSC were cultured for 7 days in the presence or absence of the glucocorticoid prednisolone. SVF, which we have recently shown to be leptin producers [11], were used as a positive control. ELISA analysis showed that BM-MSC expressed leptin, and that this expression was markedly up regulated by prednisolone (10^{-6} M) treatment (Fig. 1). In contrast, no leptin production was detected in UMSC cultures in the absence or presence of prednisolone (Fig. 1). The effect of glucocorticoids (prednisolone or dexamethasone) on BM-MSC and SVF was clearly dose dependent (10^{-9} to 10^{-6} M), with leptin expression reaching a plateau for a concentration of prednisolone around 10^{-6} M (data not shown).

SB 431542, a specific ALK-5 inhibitor, increased leptin expression and restored TGF- β -inhibited leptin expression in BM-MSC

Searching for the signalling pathways involved in MSC leptin expression, we observed that TGF β 1 is an inhibitor of leptin expression in both SVF (data not shown) and BM-MSC

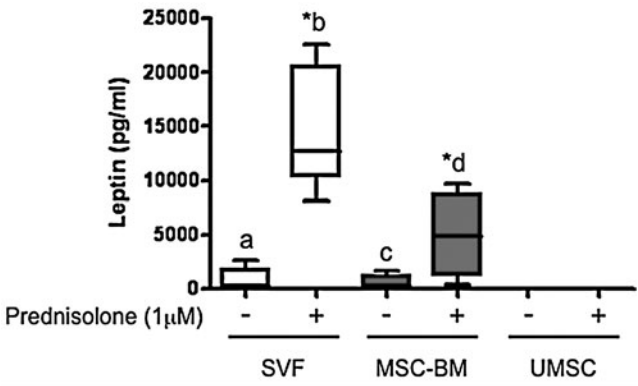


FIG. 1. Bone marrow-derived stem cells BM-MSC and synovial fibroblasts (SVF), but not umbilical cord matrix-derived stem cells (UMSC), produce leptin. BM-MSC ($n=7$), UMSC ($n=9$) and SVF ($n>10$) were cultured in the presence or absence of prednisolone for 7 days. Leptin production was measured in the culture supernatant by enzyme-linked immunosorbent assay (ELISA). *b, statistically different from a and d. *d, statistically different from c. Results are shown as the average of 3 representative experiments.

(Fig. 2A). TGF- β 1 was shown to signal via ALK-1 (Smad1/5 pathway) and/or ALK-5 (Smad2/3 pathway) [18]. To investigate these pathways, we treated BM-MSC with an ALK-5 inhibitor (SB 431542) in the presence or absence of prednisolone and TGF- β 1 (Fig. 2A). ELISA analysis showed that TGF- β 1 significantly inhibited both endogenous and glucocorticoid-induced leptin expression. The inhibition of leptin expression observed by TGF- β 1 was partially blocked in the presence of SB 431542 (Fig. 2A). This effect was associated with increased p-Smad-1/5 expression (Fig. 2A). SB 431542 alone induced leptin expression in BM-MSC (Fig. 2A) but not in UMSC (data not shown), suggesting that the ALK-5/Smad2 pathway may not be the only leptin repressing pathway in these cells. The effect of SB 431542 was dose dependent (0.5–10 μ M), with leptin expression reaching a plateau for a concentration of SB 431542 around 10 μ M (data not shown).

We also tested the effect of glucocorticoids, TGF- β 1 and SB 431542 on leptin receptor (OB-R) expression in BM-MSC and

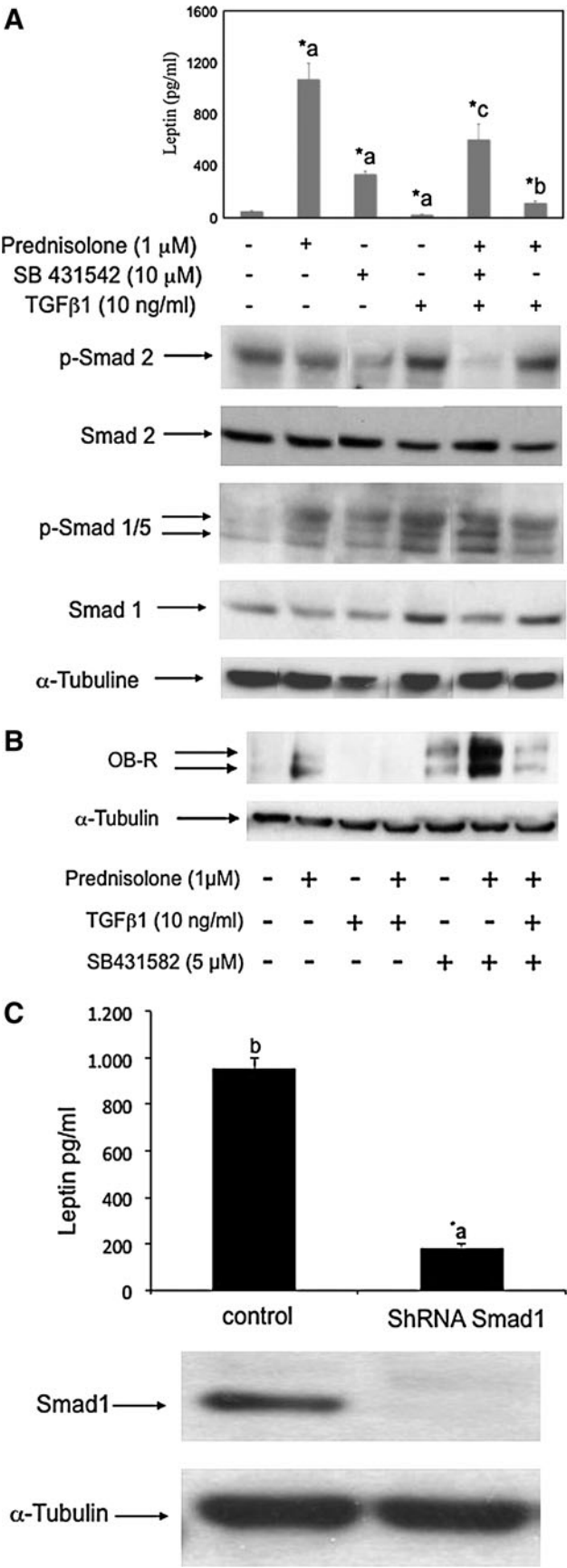


FIG. 2. SB 431542, an ALK-5 inhibitor, induces leptin expression and restores transforming growth factor β (TGF- β)-inhibited leptin expression in BM-MSC. BM-MSC ($n=4$) were cultured for 7 days in the presence or absence of prednisolone, TGF- β 1, and/or SB431542 at the indicated concentrations. (A) Leptin was determined by ELISA in the culture supernatants, and phosphorylated Smad2 (p-Smad2), Smad2, p-Smad1/5, Smad1, and α -tubulin expressions were detected by western blot. *a, statistically different from control. *b, statistically different from prednisolone-treated cells. *c, statistically different from prednisolone and TGF- β -treated cells. (B) Western blot showing leptin receptor expression (OB-R) in BM-MSC cultured for 7 days in the presence of prednisolone, TGF- β 1, and/or SB431542 at the indicated concentrations. (C) Leptin (ELISA) and Smad1 expression (western blot) in SVF transduced or not with lentiviral particles for ShRNA of Smad1. *a, statistically different from b.

found that OB-R expression was enhanced by prednisolone (Fig. 2B). TGF- β 1 inhibited endogenous and prednisolone induced OB-R expression. Of interest, SB 431542 also induced up-regulation of OB-R expression and partially restored TGF- β 1-induced OB-R expression inhibition (Fig. 2B).

These results suggested that the TGF- β pathway, through ALK-5-Smad2/3 and ALK-1-Smad1/5 routes, may be involved in negative and positive leptin expression regulation, respectively.

Smad1 silencing inhibits leptin expression in MSCs

To further test the involvement of Smad1 in MSCs leptin expression, we used lentiviral particles expressing Smad1 shRNA to infect SVF cells. Western blot analysis demonstrated a significant decrease of Smad1 expression in infected cells (Fig. 2C). ELISA analysis showed that Smad1 silencing resulted in significant decrease in leptin expression (Fig. 2C). No significant difference in leptin expression was observed when we used lentiviral particles for the expression of control shRNA. These results further suggested that Smad1 is involved in positive leptin expression regulation.

TGF- β RII and p-Smad-2 expression are modulated by prednisolone in BM-MSC and SVF but not in UMSC

We demonstrated that prednisolone significantly increased leptin expression in SVF and BM-MSC but not in UMSC. Thus, the effect of prednisolone on TGF- β RII and p-Smad-2 expression was determined in BM-MSC, UMSC, and SVF protein extracts (Fig. 3). Western blotting analysis showed that prednisolone increased TGF- β RII and decreased

p-Smad2 expression in BMSC and SVF but not in UMSC. Of interest, SVF, which are the most robust leptin producers, had the lowest p-Smad2 expression level and showed the highest TGF- β RII expression increase in the presence of prednisolone. These results suggested that down-regulation of Smad2 phosphorylation may be associated to leptin expression in BM-MSC as well as in SVF.

Differential osteogenic potential in SVF, BM-MSC, and UMSC

Leptin was shown to be implicated in osteogenic differentiation [1,2]. We, therefore, tested SVF, BM-MSC, and UMSC for their osteogenic differentiation potential. SVF, BM-MSC, and UMSC, cultured in DMEM+10% FBS (control) or in osteogenic medium for 3 weeks, were tested for ALP activity and Runx-2 expression (Fig. 4A, B, respectively). In parallel, leptin expression was measured in culture supernatants (Fig. 4C). ALP activity analysis revealed an important staining with SVF that was increased in osteogenic conditions. A less important ALP activity was detected with BM-MSC, whereas no detectable ALP activity was observed with UMSC (Fig. 4A). UMSC osteogenic differentiation for longer than 3 weeks led to barely detectable ALP activity (data not shown). Western blot analysis showed an obvious up-regulation of Runx-2 expression in SVF in the presence of osteogenic medium (Fig. 4B). Less Runx-2 expression was detected in BM-MSC, whereas barely detectable Runx-2 expression was detected in UMSC in the presence or absence of osteogenic medium (Fig. 4B). A higher level of leptin was detected in osteogenic medium with SVF than with BM-MSC, whereas no leptin expression was detected in osteogenic medium with UMSC (Fig. 4C). BM-MSC cultured in osteogenic medium, in the absence of dexamethasone, showed a significantly lower leptin expression level, and decreased ALP activity (Fig. 4D). This suggests that one of the roles of glucocorticoids (dexamethasone) in osteogenic mixture may be to enhance leptin expression.

Discussion

Although it is known that leptin expression is modulated by glucocorticoids in vitro and in vivo [12,19], very little is known about the mechanisms involved. Leptin was widely studied in 3T3-L1 mouse cell line described to have the potential to differentiate into adipocytes but with a low level of leptin mRNA expression [4]. Thus, recently, the need for a cell model that expresses leptin and robustly responds to hormonal signals for the study of leptin expression has emerged [3]. Our earlier study showed that SVF, belonging to mesenchymal lineages, expresses leptin, which is strongly enhanced by glucocorticoids [11]. Here, we have shown that BM-MSC, another MSCs, also expressed a basal level of leptin that was markedly enhanced by glucocorticoids. Surprisingly, UMSC did not express leptin. In an attempt to understand the pathways involved in leptin expression, we investigated TGF β 1 pathways, as we observed that TGF β 1 had an inhibitory effect on leptin expression in SVF (data not shown) and BM-MSC (this work). We showed that leptin expression parallels ALK-5 and p-Smad2 down-regulation as well as the gain of ALK-1 and p-Smad1/5 expression. It was previously reported that in addition to the well-characterized ALK-5-Smad2/3 route, TGF- β 1 can also signal via the

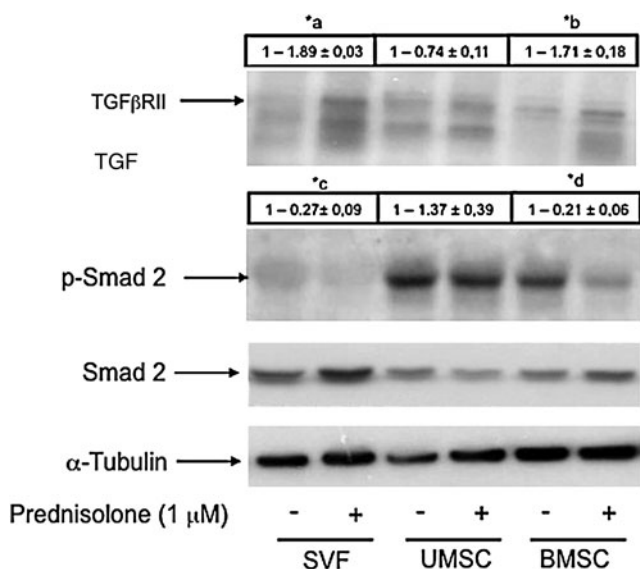


FIG. 3. Prednisolone up-regulates TGF- β RII and down-regulates p-Smad2 expression in SVF and BM-MSC but not in UMSC. BM-MSC ($n=4$), UMSC ($n=4$), and SVF ($n=4$) were cultured in the presence or absence of prednisolone for 7 days. Western blot shows TGF- β RII, p-Smad2, Smad2, and α -tubulin expression in protein extracts from each culture. *a, *b, *c, and *d: prednisolone stimulated statistically different from control.

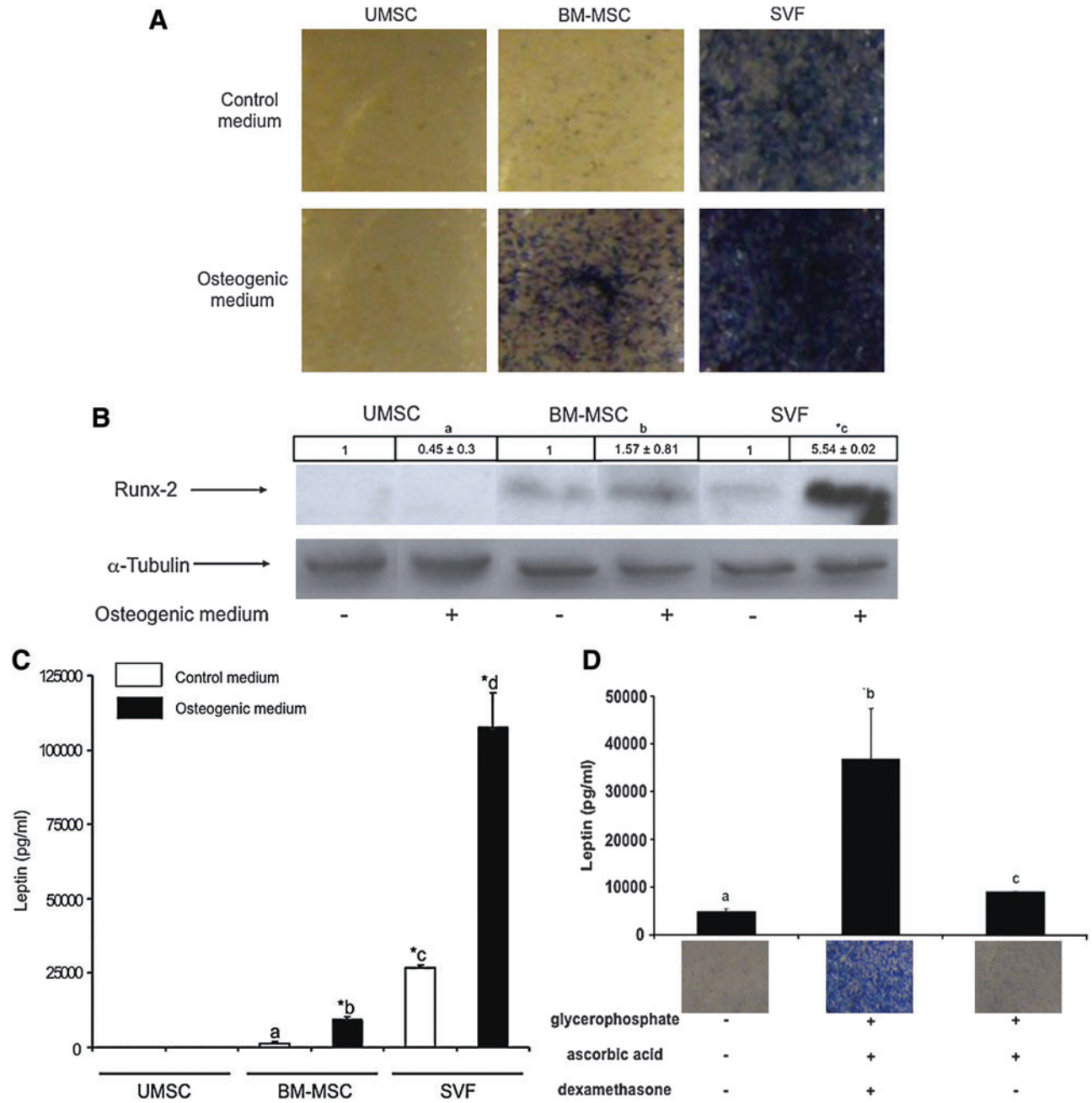


FIG. 4. Osteogenic differentiation capacity of UMSC, BM-MSC, and SVF. UMSC, BM-MSC, and SVF ($n=3$) were exposed in vitro to control [Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS)] or osteogenic (DMEM supplemented with 10% FBS + 10 mM β -glycerophosphate, 60 μ M ascorbic acid, and 10^{-7} M dexamethasone) medium. Osteogenic differentiation is shown by (A) alkaline phosphatase (ALP) activity and (B) Runx-2 expression in control and differentiated cells (western blot). Relative quantification is shown as mean \pm standard deviation of 3 different experiments performed on 3 different donors. *c, statistically different from b and a. (C) Leptin expression was measured by ELISA in supernatant of control and differentiated MSC. *b, statistically different from a. *d, statistically different from c and b. *c, statistically different from a. (D) ALP and leptin expression in BM-MSC cultured for 2 weeks in the presence of osteogenic medium supplemented or not with dexamethasone. *b, statistically different from a and c. Color images available online at www.liebertonline.com/scd

alternative receptor ALK-1, resulting in Smad1/5 pathway activation [18]. According to our results, we postulated that ALK-5-Smad2 pathway inhibits leptin expression, whereas ALK-1-Smad1/5 may function as an activator. Indeed, when we compared p-Smad2 expression between different MSC

types, we found the highest level of p-Smad2 expression in UMSC that do not express leptin, and the lowest p-Smad2 level in SVF that are robust leptin producers. In BM-MSC, TGF- β 1 induced p-Smad2 expression and markedly inhibited leptin expression. These results supported the hypothesis

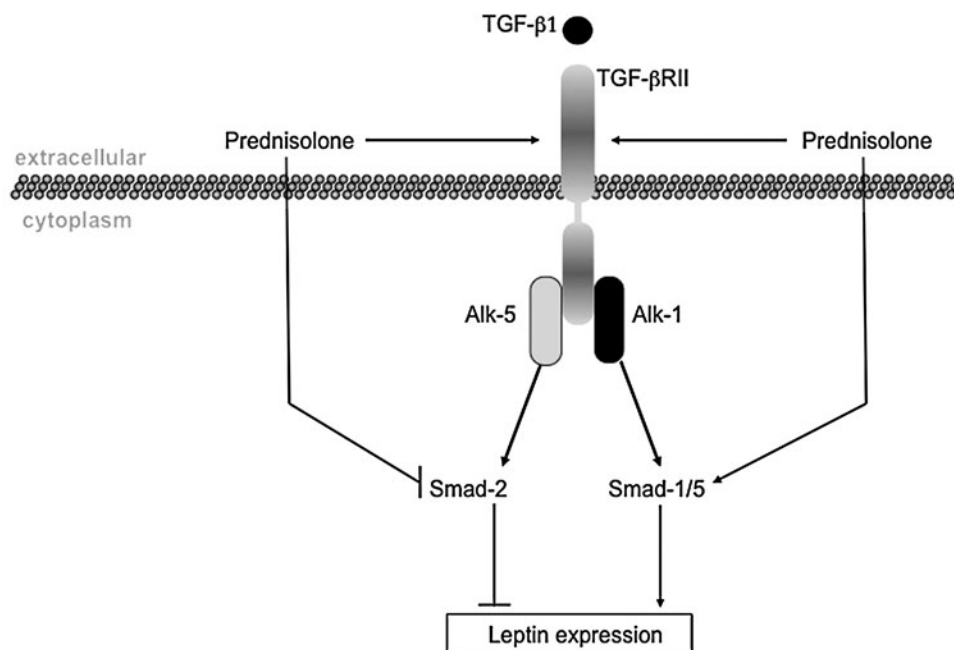


FIG. 5. The balance in signalling through either ALK-1 or ALK-5 regulates leptin expression in MSC. TGF β 1 was shown to signal by the ALK-5-Smad2/3 pathway and/or the ALK-1-Smad1/5 pathway [18]. When TGF β 1 binds to TGF β RII, it induces the activation of both ALK-1 and ALK-5 pathways, with a predominance of the inhibitory ALK-5-Smad-2 route. However, the inhibition of ALK-5 favors the activator ALK-1 route, leading to increased leptin expression. In the presence of prednisolone, TGF β RII expression is up-regulated, but the ALK-5-p-Smad2 pathway is down-regulated and the ALK-1-Smad1/5 pathway is favored, thereby leading to amplified leptin expression.

that the ALK-5 pathway is an inhibitor of leptin expression. Furthermore, when we inhibited the ALK-5 pathway using SB431542, leptin expression was significantly increased. Interestingly, this increased leptin expression was associated with increased p-Smad1/5 expression. Finally, silencing Smad1 expression resulted in leptin expression inhibition, suggesting that the ALK-1 pathway is a leptin expression activator. We also showed that prednisolone-induced leptin expression in BM-MSC, and SVF was accompanied by TGF- β RII expression up-regulation and p-Smad2 expression down-regulation. Taken collectively, these results suggest a model where the signalling balance between both ALK-5 and ALK-1 can determine leptin expression in response to the TGF- β pathway (Fig. 5). In this model, glucocorticoid up-regulates TGF- β RII expression, but at the same time, directs the TGF- β pathway to signal through ALK-1.

TGF- β 1 was also able to inhibit OB-R expression in BM-MSC, whereas prednisolone up-regulated its expression. Interestingly, SB431542 induced OB-R expression and blocked the inhibitory effect of TGF- β 1, suggesting a similar mechanism for OB-R expression as for leptin. The effect of prednisolone on leptin expression in BM-MSC was not observed in UMSC. UMSC have functional glucocorticoid receptors, as demonstrated by decreased interleukin-6 production after prednisolone treatment (data not shown). Contrary to BM-MSC, UMSC have a constitutive increased p-Smad2 expression that is not modulated by glucocorticoids. In addition, despite having the same ALK-1 and ALK-5 expression pattern as BM-MSC, UMSC were not able to show any leptin expression after SB 431542 treatment (data not shown). The constitutive p-Smad2 expression in UMSC seems to be independent of TGF- β receptor stimulation. Such constitutive activation was described in PPAR- γ null mouse embryonic fibroblasts (MEFs) [20]. However, SB 431542 was able to abrogate Smad2 phosphorylation in MEFs, whereas SB 431542 treatment did not induce leptin expression in UMSC (data not shown), suggesting a different mechanism for these

cells. Furthermore, unlike BM-MSC and SVF, UMSC did not show any increased TGF- β RII expression in the presence of prednisolone.

It was shown that the gene expression pattern of BM-MSC is more similar to osteoblasts, suggesting a better osteogenic potential. In contrast, UMSCs are more primitive, because they share more common genes with embryonic stem cells [21]. In the present study, we showed a better osteogenic potential with SVF and BM-MSC than with UMSC. This differential osteogenic potential was correlated to leptin expression in these cells. Leptin is now admitted to be implicated in osteogenic differentiation [1,2]. This suggests that the difference in osteogenic potential between SVF, BM-MSC, and UMSC could be attributed to their different leptin expression potential.

Multiple myeloma (MM) expands almost exclusively in the BM and generates devastating bone lesions, in which bone formation is impaired. Takeuchi k et al. attributed this terminal osteoblastic differentiation impairment in MM to TGF- β that is abundantly deposited in the bone matrix. Using SB431542 to inhibit TGF- β action, they potently enhanced osteoblast differentiation from BM stromal cells [22]. Since leptin is implicated in osteogenic differentiation [23], the effect attributed by Takeuchi k et al. to TGF- β signalling inhibition in MM could be mediated through leptin expression.

Leptin is considered a pro-inflammatory peptide in rheumatic diseases, whereas it is not expressed in healthy cartilage [24,25]. Indeed, recent works have shown that leptin inhibits chondrogenesis [26]. Blaney Davidson et al. showed an increased ALK-1/ALK-5 ratio as a cause of deviant chondrocyte behavior (elevated MMP-13 expression), which contributes to age-related cartilage destruction and osteoarthritis in humans [27,28]. Our results suggest that leptin may be one of the aspects of aberrant chondrocytes differentiation that results from changes in TGF- β signalling related to age or osteoarthritis.

In this work, we showed the involvement of ALK-1/ALK-5 pathways for leptin expression in MSC. The field of cell

therapy is experiencing more and more cell types for different protocols that do not necessarily share the same requirement in term of protein expression. Leptin is now admitted to be strongly implicated in osteogenic differentiation [1]. Thus, in osteogenic protocols, the use of naturally leptin MSC producer, such as SVF or BM-MSC, could be more appropriated than UMSC. On the contrary, since healthy chondrocytes do not express leptin, the use of MSC in protocols of cartilage disorders repair would better require a leptin nonproducer MSC such as UMSC.

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Author Disclosure Statement

None to declare.

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