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ABSTRACT

In osteoporosis, bone loss is accompanied by greater adiposity in the marrow. Given the cellular proximity within the bone marrow, we wondered whether adipocytes might have a paracrine impact on osteoblast differentiation. To test this hypothesis, we cocultured adipocytes with osteoblasts derived from mesenchymal stem cells (MSCs) in the absence of direct cell contact and then analyzed gene expression changes in the osteoblastic population by using real-time reverse transcription polymerase chain reaction. We found that, upon coculture, MSC-derived osteoblasts showed appearance of adipogenic (lipoprotein lipase, leptin) and decrease of osteogenic (osteocalcin) mRNA markers. Our results indicate that *in vitro*, MSC-derived adipocytes are capable of inducing MSC-derived osteoblasts to differentiate towards an adipocyte phenotype. These new data suggest that (i) transdifferentiation of committed osteoblasts into adipocytes may contribute to the increase in marrow fat content at the expense of bone-forming cells and (ii) this switch might be initiated by the adipocytes themselves.

Keywords: Human MSC, Osteoblast, Adipocyte, Coculture, Transdifferentiation, Osteoporosis

INTRODUCTION

Mesenchymal stem cells (MSCs) in the bone marrow are able to differentiate into the cell lineages responsible for bone and fat formation, osteoblasts and adipocytes respectively [1-4]. In humans, it has been demonstrated that osteoporosis, resulting from aging, menopause or even anorexia nervosa, is accompanied by an increase in the number of medullary adipocytes at the expense of osteoblasts [5-7]. Although it has been suggested that this accumulation of fat in the marrow cavity is probably the result of preferential MSCs differentiation into the adipocyte cell lineage, the underlying mechanisms have yet to be revealed [8, 9]. It is conceivable that the osteoporosis-associated increase in marrow adipogenesis may contribute to limit osteoblast commitment by acting on MSCs or even directly on osteoblasts [10]. This hypothesis is supported by the proximity of adipocytes to osteoblasts in the bone marrow and the knowledge that adipocytes are secretory cells. *In vitro* studies have demonstrated that adipocyte-secreted factors, including hormones, leptin, adiponectin and fatty acids, may modify the proliferation, function and apoptosis of neighbouring cells [11-13]. We wondered whether adipocytes might also have a strong impact on osteoblast differentiation in the marrow cavity. In an attempt to reproduce cellular interactions within the bone marrow, we used a cell coculture system to expose either MSCs or MSC-derived osteoblasts to the products secreted by MSC-derived adipocytes.

MATERIALS AND METHODS

Cell culture and induction of osteoblastogenic and adipogenic differentiation

Purified MSCs obtained from three different donors (two men and one woman; mean age +/- SD: 25.6 +/- 7.4 years) were purchased from Lonza (Verviers, Belgium). Cells were plated in

expansion medium composed of Dulbecco's Modified Eagle Medium (DMEM) (Dutscher, Brumath, France) containing 10% fetal calf serum (FCS), 1% penicillin/streptomycin and 1% glutamine (Dutscher). Cell cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2, and media were changed twice weekly.

Differentiation experiments were started when MSCs had reached confluence (day 0). To induce osteogenesis, MSCs were cultured in DMEM with 10% FCS supplemented with osteogenic medium (50 µM ascorbic acid, 10 mM β-glycerophosphate and 10^{-8} M vitamin D3 (Sigma-Aldrich Corporation, St Quentin Fallavier, France)) for 7 or 21 days.

For adipogenic differentiation, MSCs were cultured in DMEM with 10% FCS supplemented with adipogenic medium (0.5 µM dexamethasone, 0.5 µM isobutyl-1-methylxanthine and 50 µM indomethacin (Sigma-Aldrich Corporation)) for 14 days.

Coculture of adipocytes and osteoblasts cells

MSCs from the same donor were either seeded on cell culture inserts (pore size 0.4 µm, Millicell) at a density of 9×10^3 cells per cm^2 and cultured after confluence in adipogenic medium (A) or plated in the basal compartment of 6-well plates at a density of 9×10^3 cells per cm^2 and cultured after confluence in osteogenic medium (O). Inserts were then transferred to plates and cocultures were maintained in serum-free DMEM for 48h. Cocultures were performed with MSCs cultured in osteogenic medium for 7 days (O7/A14) or for 21 days (O21/A14) and with undifferentiated MSCs cultured in expansion medium until confluence (MSCs/A14). In each case, cells were cultured under the same conditions but in the absence of adipocytes and were considered as controls (O7, O21, MSCs). In another control experiment, inserts were seeded with MSCs cultured in expansion medium for 21 days, rather than MSC-derived adipocytes (O21/MSCs21). Cells plated in the basal compartment of 6-well plates were collected for the determination of changes in gene expression and for

alkaline phosphatase (ALP) activity assay. The determination of adipocyte differentiation for cells seeded on inserts was performed by using Oil Red O staining and reverse transcription polymerase chain reaction (RT-PCR).

Oil Red O staining

Cells were fixed in 2% paraformaldehyde for 15 min, washed in water, incubated with 60% isopropanol for 5 min and stained with newly filtered Oil Red O solution for 10 min at room temperature. After staining, the cells were rinsed with water before counterstaining with Gill-3 Hematoxylin for 5 min at room temperature.

Alkaline phosphatase (ALP) activity

The cells were washed three times with 1X phosphate-buffered saline (PBS) and scraped immediately after adding 500 µl of Nonidet P40 0.2%. The collected lysates were then sonicated for 30 s. Enzyme activity assay was performed in assay buffer (100 mM $MgCl_2$ and 0.7 mM 2-amino-2-methyl-1-propanol pH 10.3) containing 100 mM p-nitrophenylphosphate as substrate and incubated at 37°C. The reaction was stopped by adding 1N NaOH and absorbance was read at a wavelength of 405 nm wavelength. ALP activity was defined as nmol of p-nitrophenylphosphate hydrolyzed per min per mg of total protein. Experiments were performed in triplicate.

RNA extraction

Total RNA was extracted using Extract-All reagent (Eurobio, Les Ulis, France), according to the manufacturer's instructions. Total RNA was quantified by spectrophotometer at 260 nm wavelength and the integrity of RNA was controlled by the 28S/18S rRNA ratio after agarose

gel electrophoresis. Contaminating DNA was removed from RNA samples in a 30 min digestion at 37°C with DNase I (Roche Diagnostics, Meylan, France).

Reverse transcription

1 µg of each RNA sample was used for reverse transcription (RT) performed under standard conditions with Superscript II reverse transcriptase (Life Technologies, Cergy Pontoise, France) and random hexamer primers (Amersham Pharmacia Biotech, Saclay, France) in a 20 µl final volume. The reaction was carried out at 42°C for 30 min and stopped by incubation at 99°C for 5 min. The RT reaction products were then diluted to 100 µl in water. 1 µl of stock cDNA template was used in subsequent PCR reactions.

PCR

cDNA was subjected to PCR in a 25 µl mix in 1X reaction buffer with 0.2 mM of each dNTP, 0.3 µM of each primer and 1U *Taq* Polymerase (Roche Diagnostics). After a 5 min denaturation at 94°C, amplification cycles were carried out as follows: 40s denaturation at 94°C followed by 30s annealing at the temperature indicated in Table 1 and 30s elongation at 72°C, for 30 cycles. The amplification products were visualized on 2% agarose gel.

Real-time PCR experiments

Real-time PCR was performed using a LightCycler system (Roche Diagnostics), according to the manufacturer's instructions. Primers were obtained from TibMolBiol (Berlin, Germany), the sequences are listed in Table 1. Reactions were performed in 10 µl volumes with 0.5 µM primers, 4 mM $MgCl_2$ and 1 µl of LightCycler-FastStartDNA Master SYBR Green I mix (Roche Diagnostics). The protocol consisted of a hot start step (8 min at 95°C), followed by 40 cycles including a 10 s denaturation step (95°C), a 10 s annealing step and an elongation

step at 72°C varying from 8 s to 16 s. Efficiencies of PCR were optimized according to Roche Diagnostic's recommendations on a standard sample expressing all studied genes. To confirm the amplification specificity, PCR products were subjected to a melting curve analysis and a subsequent gel electrophoresis. Two housekeeping genes, YWHAZ (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein) and PPIA (peptidylprolyl isomerase A), identified as the most stable under all conditions, were used to normalize transcription levels [14]. Quantification data represented the mean of at least four experiments performed in duplicate. Relative quantification analyses were performed by RelQuant 1.01 Software (Roche Diagnostics).

Statistical analysis

Statistical significance of mRNA level differences between cocultured cells and controls was determined for each gene by a uni-variate analysis (Mann-Withney *U*-test). Results were considered to be significantly different at $P < 0.05$.

RESULTS

Differentiation of MSCs into osteoblasts and adipocytes

The present study was performed on human MSCs from three different donors. Prior to coculture, the cells were incubated in either osteogenic or adipogenic differentiation medium. Two time points (namely 7 and 21 days) were chosen during osteogenic differentiation, whereas cells on inserts were incubated in adipogenic medium for 14 days. Late osteoblastic differentiation was evidenced by the expression of osteocalcin (OC). Adipocyte differentiation was corroborated by (i) expression of the specific marker lipoprotein lipase (LPL) and (ii) the formation of cytoplasmic lipid droplets stained with Oil Red O (Figure 1).

Effects of coculture on the differentiation of MSC-derived osteoblasts

Cell phenotype of MSC-derived osteoblasts, cocultured or not with MCS-derived adipocytes, was determined using the technique of RT-PCR. As shown in Figure 2-A, expression of LPL was observed in MSC-derived osteoblasts after coculture but was undetectable in all the controls. This surprising result was corroborated by quantitative analysis of two other markers of adipogenic differentiation, PPARγ and leptin. Indeed, there was a consistent increase in PPARγ mRNA levels (versus control experiments) in MSC-derived osteoblasts cocultured with MSC-derived adipocytes (Figure 2-B). Likewise, leptin mRNA was expressed at either very low or undetectable levels in MSCs incubated in osteogenic medium but was strongly upregulated upon coculture (Figure 2-B). It is noteworthy that GLUT4 expression was not detected in any of the samples (data not shown). These results were observed for all three human MSCs, although the relative difference in mRNA levels varied from one MSC to another. For example, coculture with cells in osteogenic medium for 21 days (O21/A14) led to a 130-fold, 80-fold and 260-fold increase in mean leptin expression normalized with YWHAZ by the MSC1, MSC2 and MSC3, respectively.

The mRNA changes were less consistent for markers of osteogenic differentiation. There was no change in COL1 or RUNX2 mRNA levels upon coculture (Figure 2-B). In contrast, coculture led to a decrease in OC mRNA levels and an increase in ALP mRNA levels (Figure 2-B).

These effects were specific, as demonstrated by our observation that coculturing MSC-derived osteoblasts with undifferentiated MSCs (i.e. grown in expansion medium) did not change the osteoblasts' differentiation state (data not shown).

Effects of coculture on MSC differentiation

Coculture was also performed with undifferentiated MSCs that had previously been cultured in expansion medium until confluence (MSCs/A14). As for MSC-derived osteoblasts (although to a lesser extent), coculture increased PPAR mRNA expression levels and led to the expression of leptin. There was no significant change in ALP, COL1 or RUNX2 mRNA levels upon coculture. Lipoprotein lipase and OC were not detected in undifferentiated MSCs, regardless of whether the cells were cocultured or grown alone (Figure 2-A and 2-B).

Effects of coculture on MSC-derived osteoblast function: ALP activity

The ALP activity of MSCs cultured in osteogenic medium for 21 days was significantly higher after coculture (870 ± 53 per mg of protein, versus a value of 491 ± 123 in control experiments) (Figure 3).

DISCUSSION

There is considerable evidence in support of a causative role for bone marrow adipogenesis in the bone loss observed in osteoporosis. Given the proximity of adipocytes to osteoblasts in the bone marrow and in view of the fact that adipocytes are secretory cells, we wondered whether adipocytes might have a paracrine impact on osteoblast differentiation. To test this hypothesis and in an attempt to reproduce at least the indirect cell-cell interactions within the marrow cavity, we studied the differentiation of bone marrow MSCs and MSC-derived osteoblasts in a cell coculture system. We found that MSC-derived osteoblasts differentiated into adipocyte-like cells upon coculture, as evidenced by the expression of adipogenic mRNA markers (LPL and leptin) and a decrease in levels of osteogenic mRNA marker (OC). We also observed that ALP mRNA levels increased upon coculture. Although alkaline phosphatase is

a well-known osteogenic marker, it was recently reported that ALP activity also increases during adipogenesis [15]. We have confirmed this increase at the mRNA level during MSC-derived adipocyte differentiation (data not shown). Lastly, the lack of change in COL1 and RUNX2 mRNA levels upon coculture was not surprising, considering that these markers are expressed throughout the osteoblastic differentiation process.

Given that MSCs constitute a heterogeneous cell population, it could be argued that the observed effects of coculture were due to contaminating progenitor cells present in the starting population. Coculture led to a number of modifications in the MSCs' phenotype. However, the lack of expression of OC and LPL (both with and without coculture) ruled out a significant contribution to the observed adipogenic phenotype by these cells.

In addition to the consistent appearance of adipogenic markers (LPL and leptin), the decrease in OC mRNA expression upon coculture suggested that the cells might partially de-differentiate before committing to another cell type. This type of phenotype switch between committed or differentiated osteoblasts and adipocytes has already been reported in various cell culture systems [16-19]. Some authors have shown that in response to inductive media, fully differentiated MSC-derived cells are capable of transdifferentiation (i.e. de-differentiation prior to re-differentiation) [16]. In addition to the enhanced adipogenic differentiation of MSCs, transdifferentiation of committed osteoblasts into adipocytes might contribute to the increase in bone marrow fat and the concomitant decrease in the number of bone-forming cells. Our data suggest that this conversion might be caused by adipocytes themselves and supports the controversial hypothesis whereby fat cells have a detrimental effect on bone-forming cells [20].

In the present study, there was no physical contact between the cocultured cell types. This suggests that MSC-derived osteoblasts responded to one or more soluble factors secreted by MSC-derived adipocytes. Several studies have shown that PPARγ (one of the key

transcription factors in adipogenesis) plays a crucial role in driving committed osteoblasts to differentiate into adipocytes [17, 21, 22]. Moreover, the inhibition of MSC-to-osteoblast differentiation by PPARγ–ligands has been reported [23-24]. Fatty acids and their metabolites are secreted by adipocytes and are potential PPARγ–ligands [25-26] - making them good candidates for a paracrine effect of this type. This supposition is further supported by *in vitro* studies showing that polyunsaturated fatty acids have a negative impact on osteoblast proliferation, function and apoptosis [11-13]. Among the numerous factors released by adipocytes, chemerin seems to be another good candidate for the paracrine effect observed here. Indeed, a recent study has shown that this adipokine is involved (via its receptor, CMKLR1) in the positive regulation of adipogenesis and the negative regulation of osteoblastogenesis in bone-marrow-derived cells and is thus a potential modulator of bone mass [27].

Under either hypothesis, one explanation for the observed phenotypic differences between MSCs and MSC-derived osteoblasts upon coculture may be the cellular differentiation state. Since PPARγ activation regulates a variety of pathways, one can legitimately presume that there are differentiation-dependent changes in the signalling pathways involved, leading to variable expression of the adipocyte phenotype. For chemerin, differences between MSCs and MSC-derived osteoblasts in terms of differentiation-dependent expression of its receptor may explain why the expression of adipocyte-specific genes is less readily detected in MSCs than in MSC-derived osteoblasts upon coculture. Further investigations are needed to confirm these hypotheses and characterize the underlying mechanisms.

In summary, our data suggest that (i) transdifferentiation of committed osteoblasts into adipocytes contributes to the increase in bone marrow fat and the concomitant decrease in bone-forming cells and (ii) this switch might be initiated by the adipocytes themselves.

Targeting marrow adipocytes, as it has been suggested [8, 28], may be of relevance in the development of therapeutic methods for limiting bone loss in osteoporosis.

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REFERENCES

[1] M. Owen, Marrow stromal stem cells, J. Cell Sci. Suppl. 10 (1988) 63-76.

[2] J.M. Gimble, The function of adipocytes in the bone marrow stroma, New Biol. 2 (1990) 304-312.

[3] J.N. Beresford, J.H. Bennett, C. Devlin, P.S. Leboy, M.E. Owen, Evidence for an inverse relationship between the differentiation of adipocytic and osteogenic cells in rat marrow stromal cell cultures, J. Cell Sci. 102 (Pt 2) (1992) 341-351.

[4] M.F. Pittenger, A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, D.R. Marshak, Multilineage potential of adult human mesenchymal stem cells, Science 284 (1999) 143-147.

[5] P. Meunier, J. Aaron, C. Edouard, G. Vignon, Osteoporosis and the replacement of cell populations of the marrow by adipose tissue. A quantitative study of 84 iliac bone biopsies, Clin. Orthop. Relat. Res. 80 (1971) 147-154.

[6] J. Justesen, K. Stenderup, E.N. Ebbesen, L. Mosekilde, T. Steiniche, M. Kassem, Adipocyte tissue volume in bone marrow is increased with aging and in patients with osteoporosis, Biogerontology 2 (2001) 165-171.

[7] K. Ecklund, S. Vajapeyam, H.A. Feldman, C.D. Buzney, R.V. Mulkern, P.K. Kleinman, C.J. Rosen, C.M. Gordon, Bone Marrow Changes in Adolescent Girls with Anorexia Nervosa, J. Bone Miner. Res. (2009).

[8] G. Duque, Bone and fat connection in aging bone, Curr. Opin. Rheumatol. 20 (2008) 429-434.

[9] J.M. Gimble, S. Zvonic, Z.E. Floyd, M. Kassem, M.E. Nuttall, Playing with bone and fat, J. Cell Biochem. 98 (2006) 251-266.

[10] S. Muruganandan, A.A. Roman, C.J. Sinal, Adipocyte differentiation of bone marrow-derived mesenchymal stem cells: cross talk with the osteoblastogenic program, Cell Mol. Life Sci. 66 (2009) 236-253.

[11] A.C. Maurin, P.M. Chavassieux, L. Frappart, P.D. Delmas, C.M. Serre, P.J. Meunier, Influence of mature adipocytes on osteoblast proliferation in human primary cocultures, Bone 26 (2000) 485-489.

[12] A.C. Maurin, P.M. Chavassieux, E. Vericel, P.J. Meunier, Role of polyunsaturated fatty acids in the inhibitory effect of human adipocytes on osteoblastic proliferation, Bone 31 (2002) 260-266.

[13] A. Elbaz, X. Wu, D. Rivas, J.M. Gimble, G. Duque, Inhibition of Fatty Acid Biosynthesis Prevents Adipocyte Lipotoxicity on Human Osteoblasts In Vitro, J. Cell Mol. Med. (2009).

[14] T. Fink, P. Lund, L. Pilgaard, J.G. Rasmussen, M. Duroux, V. Zachar, Instability of standard PCR reference genes in adipose-derived stem cells during propagation, differentiation and hypoxic exposure, BMC Mol. Biol. 9 (2008) 98.

[15] A.T. Ali, C.B. Penny, J.E. Paiker, G. Psaras, F. Ikram, N.J. Crowther, The relationship between alkaline phosphatase activity and intracellular lipid accumulation in murine 3T3-L1 cells and human preadipocytes, Anal. Biochem. 354 (2006) 247-254.

[16] L. Song, R.S. Tuan, Transdifferentiation potential of human mesenchymal stem cells derived from bone marrow, Faseb J. 18 (2004) 980-982.

[17] S.W. Kim, S.J. Her, S.Y. Kim, C.S. Shin, Ectopic overexpression of adipogenic transcription factors induces transdifferentiation of MC3T3-E1 osteoblasts, Biochem. Biophys. Res. Commun. 327 (2005) 811-819.

[18] T. Schilling, U. Noth, L. Klein-Hitpass, F. Jakob, N. Schutze, Plasticity in adipogenesis and osteogenesis of human mesenchymal stem cells, Mol. Cell Endocrinol. 271 (2007) 1-17.

[19] T. Schilling, R. Kuffner, L. Klein-Hitpass, R. Zimmer, F. Jakob, N. Schutze, Microarray analyses of transdifferentiated mesenchymal stem cells, J. Cell Biochem. 103 (2008) 413-433.

[20] C.J. Rosen, M.L. Bouxsein, Mechanisms of disease: is osteoporosis the obesity of bone?, Nat. Clin. Pract. Rheumatol. 2 (2006) 35-43.

[21] B. Lecka-Czernik, I. Gubrij, E.J. Moerman, O. Kajkenova, D.A. Lipschitz, S.C. Manolagas, R.L. Jilka, Inhibition of Osf2/Cbfa1 expression and terminal osteoblast differentiation by PPARgamma2, J. Cell Biochem. 74 (1999) 357-371.

[22] O.P. Lazarenko, S.O. Rzonca, L.J. Suva, B. Lecka-Czernik, Netoglitazone is a PPAR-gamma ligand with selective effects on bone and fat, Bone 38 (2006) 74-84.

[23] B. Lecka-Czernik, E.J. Moerman, D.F. Grant, J.M. Lehmann, S.C. Manolagas, R.L. Jilka, Divergent effects of selective peroxisome proliferator-activated receptor-gamma 2 ligands on adipocyte versus osteoblast differentiation, Endocrinology 143 (2002) 2376-2384.

[24] O.P. Lazarenko, S.O. Rzonca, W.R. Hogue, F.L. Swain, L.J. Suva, B. Lecka-Czernik, Rosiglitazone induces decreases in bone mass and strength that are reminiscent of aged bone, Endocrinology 148 (2007) 2669-2680.

[25] S.L. Marcus, K.S. Miyata, R.A. Rachubinski, J.P. Capone, Transactivation by PPAR/RXR heterodimers in yeast is potentiated by exogenous fatty acid via a pathway requiring intact peroxisomes, Gene Expr. 4 (1995) 227-239.

[26] G. Krey, O. Braissant, F. L'Horset, E. Kalkhoven, M. Perroud, M.G. Parker, W. Wahli, Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay, Mol. Endocrinol. 11 (1997) 779-791.

[27] S. Muruganandan, A. Roman, C.J. Sinal, Role of Chemerin/CMKLR1 Signaling in Adipogenesis and Osteoblastogenesis of Bone Marrow Stem Cells, J. Bone Miner. Res. 25 (2009) 222-234.

[28] M.E. Nuttall, J.M. Gimble, Is there a therapeutic opportunity to either prevent or treat osteopenic disorders by inhibiting marrow adipogenesis?, Bone 27 (2000) 177-184.

CAPTIONS

Figure 1. Adipocyte differentiation of MSCs seeded on inserts

(A) Oil red O staining of mesenchymal stem cells (MSCs) seeded on inserts and incubated in adipogenic medium for 14 days. The arrow indicates lipid droplets stained with Oil red O. (B) RT-PCR analysis of lipoprotein lipase (LPL). mRNA coding for LPL was detected by PCR amplification of cDNA. T-: negative control with H_2O; T+: positive control

Figure 2. Effects of coculture on the differentiation of MSCs and MSC-derived osteoblasts

(A) RT-PCR analysis of the lipoprotein lipase (LPL) gene from three different mesenchymal stem cell-derived cultured cells (MSC1, MSC2 and MSC3). O7: MSCs cultured in osteogenic medium for 7 days, O7/A14: MSCs cultured in osteogenic medium for 7 days and then cocultured with MSCs previously cultured in adipogenic medium for 14 days, O21: MSCs cultured in osteogenic medium for 21 days, O21/A14: MSCs cultured in osteogenic medium for 21 days and then cocultured with MSCs previously cultured in adipogenic medium for 14 days, MSCs: MSCs cultured in expansion medium until confluence, MSCs/A14: MSCs cultured in expansion medium until confluence and then cocultured with MSCs previously cultured in adipogenic medium for 14 days. (B) Quantitative RT-PCR analysis of adipocyte-specific genes [peroxisome proliferator-activated receptor gamma (PPARγ) and leptin] and osteoblast-specific genes [osteocalcin (OC), alkaline phosphatase (ALP), collagen type 1 (COL1), runt-related transcription factor 2 (RUNX2)]. mRNA expression levels were normalized to O7 expression against the signal from two housekeeping genes for peptidylprolyl isomerase A (PPIA) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (YWHAZ). Graphs are representative of 4 independent experiments for MSC and MSC/A14 and for 5 independent experiments for O7, O7/A14, O21 and O21/A14. Error bars are standard deviations. * $P \leq 0.05$, ** $P \leq 0.01$, cocultured cells compared to cells grown alone.

Figure 3. Effects of coculture on ALP activity

Representative alkaline phosphatase (ALP) activity for mesenchymal stem cell- (MSC-) derived osteoblasts cultured alone (open bars) and after coculture with MSC-derived adipocytes (black bars) at 7 or 21 days after initiation of osteogenesis. Bars represent the mean ± standard error of three independent experiments.

Figure 1

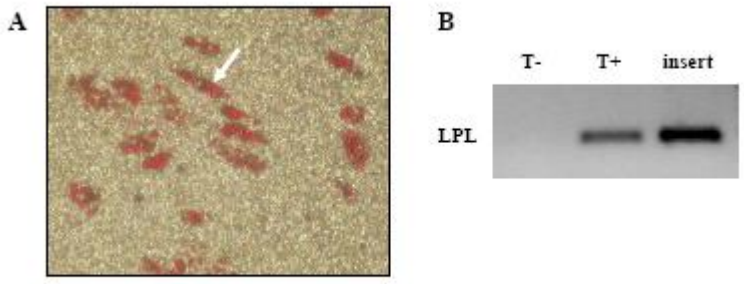
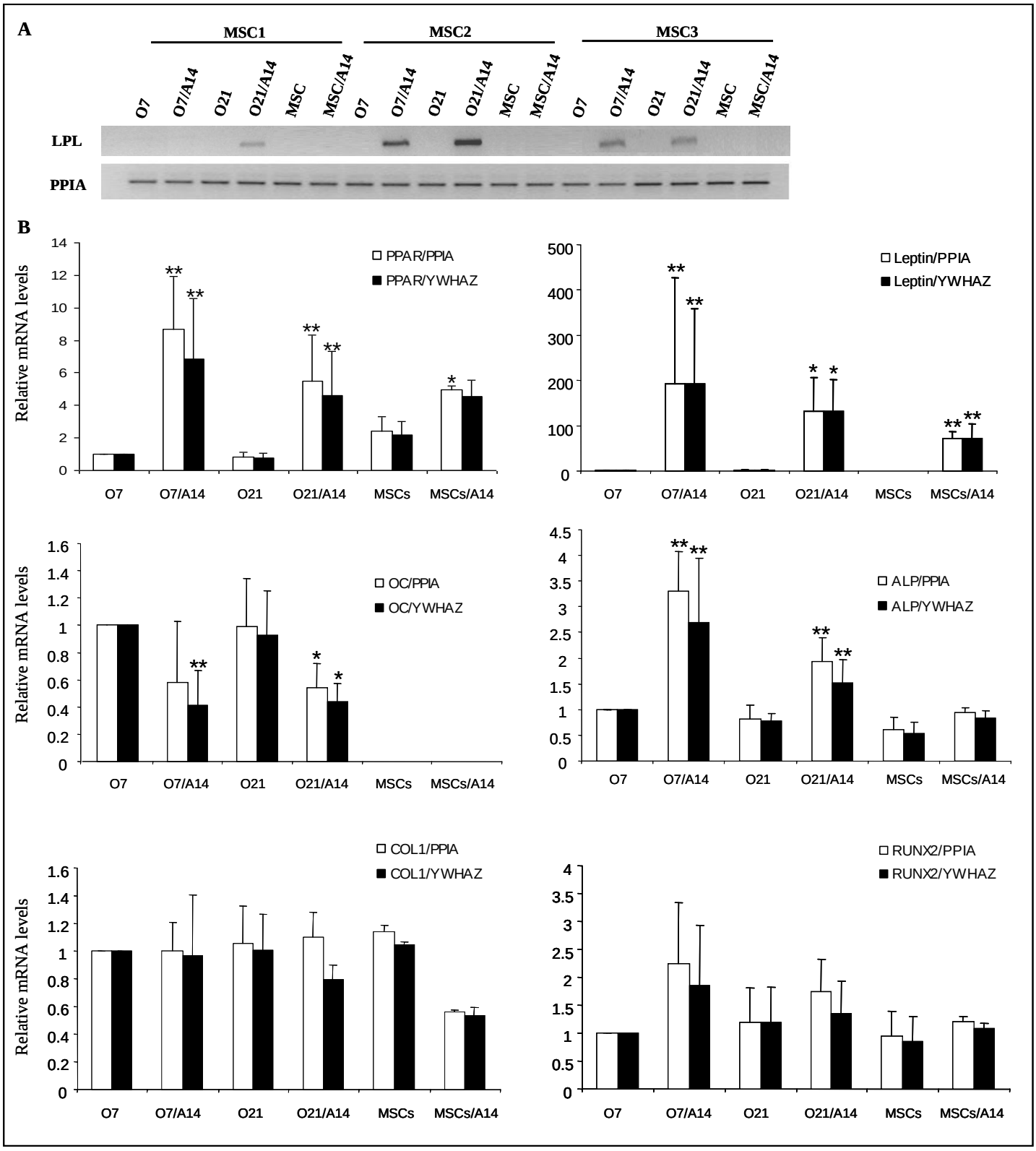


Figure 2


A
MSC1
MSC2
MSC3
O7
O7/A14
O21
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MSC
MSC/A14
LPL
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Relative mRNA levels
PPAR/PPIA
PPAR/YWHAZ
Leptin/PPIA
Leptin/YWHAZ
OC/PPIA
OC/YWHAZ
ALP/PPIA
ALP/YWHAZ
COL1/PPIA
COL1/YWHAZ
RUNX2/PPIA
RUNX2/YWHAZ
O7
O7/A14
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MSCs
MSCs/A14

Figure 3

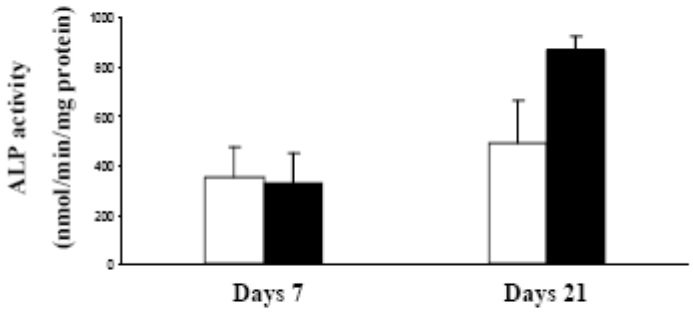
ALP activity
(nmol/min/mg protein)
1000
800
600
400
200
0
Days 7
Days 21

Table 1. Primer sequences and conditions of PCR.

cDNA	Foward and reverse primers	Ta (°C)	Product (bp)	Genbank
adipocyte specific primers				
LPL	F:5'-GACTGAGAGTGAAACCCATAC-3'	54	290	M15856
	R:5'-CCCTAGAACAGAAGATCACC-3'			
PPAR	F:5'-GCTTCTGGATTTCACTATGG-3'	54	195	NM_015869
	R:5'-AAACCTGATGGCATTATGAG-3'			
Leptin	F:5'-TGTGCGGATTCTTGTGGCTT-3'	55	312	BC060830
	R:5'-CAAGTGGCAGCTCTTAGAGA-3'			
GLUT4	R:5'-ATGCTGCTGCCTCCTATGAA-3'	60	146	NM_001042
	R:5'-CAGTTGGTTGAGCGTCCC-3'			
osteoblast specific primers				
COL1	F:5'-GGACACAATGGATTGCAAGG-3'	58	461	NM_000089
	R:5'-TAACCACTGCTCCACTCTGG-3'			
OC	F:5'-ATGAGAGCCCTCACACTCCTC-3'	57	293	NM_199173
	R:5'-GCCGTAGAAGCGCCGATAGGC-3'			
RUNX2	F:5'-AAGTTACAGTAGATGGACCT-3'	69	304	AF001450
	R:5'-GTGGTAGAGTGGATGGAC-3'			
ALP	F:5'-CAAAGGCTTCTTCTTGCTGGT-3'	60	257	AB011406
	R:5'-AAGGGCTTCTTGTCCGTGTC-3'			
reference gene primers				
PPIA	F:5'-ACCGTGTTCTTCGACATTGC-3'	55	274	NM_021130
	R:5'-CAGGACCCGTATGCTTTAGGA-3'			
YWHAZ	F:5'-GGTCATCTTGGAGGGTCGTC-3'	55	245	NM_145690
	R:5'-GTCATCACCAGCGGCAAC-3'			

Shown are the primer sequences, annealing temperatures (Ta), lengths of the corresponding PCR products, and Genbank accession numbers. F: forward; R: reverse. *LPL*, lipoprotein lipase; *PPAR*, peroxisome proliferator-activated receptor gamma; *GLUT4*, insulin-sensitive glucose transporter ; *COL1*, collagen type I; *OC*, osteocalcin; *RUNX2*, runt-related transcription factor 2; *ALP*, alkalin phosphatase; *PPIA*, peptidylprolyl isomerase A; *YWHAZ*, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein.