

# ORIGINAL ARTICLE

## Analysis of fatty acid composition in human bone marrow aspirates

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**Abstract.** In the present study, the fatty acid composition of bone marrow aspirates and serum phospholipids in nine patients with hematologic diseases was investigated, and the effect of fatty acids on osteoblast differentiation in ST2 cells was examined. The concentrations of oleic acid and palmitic acid were significantly higher in bone marrow aspirates than in serum phospholipids, but the concentrations of other fatty acids did not differ. The rate of alkaline phosphatase positive ST2 cells induced by BMP-2 was significantly increased by oleic acid, but was unaffected by the presence or absence of palmitic acid. We conclude that the fatty acid composition of bone marrow aspirates differs from that of serum phospholipids. This difference may affect osteoblast differentiation in the bone marrow microenvironment. (Keio J Med 54 (3): 150–155, September 2005)

**Key words:** fatty acids, osteoporosis, osteoblast, bone marrow

### Introduction

Osteoporosis is a widespread public health problem among postmenopausal women because of the association between low bone mass and the occurrence of vertebral and hip fractures.<sup>1</sup> Bone mass is maintained locally by a balance between bone formation by osteoblasts and bone resorption by osteoclasts. Osteoblasts derived from mesenchymal tissues replace resorbed bone by elaborating matrix, which then becomes mineralized.<sup>2</sup> After menopause, bone resorption exceeds bone formation. Consequent bone loss accompanied by an increased volume of adipose cells in bone marrow may inhibit osteoblast proliferation.<sup>3</sup> Numerous factors, including bone morphogenetic protein-2 (BMP-2),<sup>4</sup> transforming growth factor beta (TGF- $\beta$ ),<sup>5</sup> and retinoic acid,<sup>6</sup> regulate osteoblastic bone formation. The secretion of many of these factors, including fatty acids, is affected during menopause.

Previous studies have documented that dietary fat plays an important role in skeletal biology and bone health.<sup>7</sup> Fatty acids may influence bone metabolism by altering the biosynthesis of prostaglandin (PGE<sub>2</sub>).<sup>8–10</sup> In particular, n-3 polyunsaturated fatty acid (PUFA)

increases bone formation by decreasing PGE<sub>2</sub> biosynthesis.<sup>11</sup> Thus, numerous studies have reported the effects of dietary fat on bone formation *in vitro* or *in vivo*.<sup>7,12,13</sup> However, little is known about the association between fatty acid composition and the microenvironment of human bone marrow, which may influence osteoblastic bone formation. Moreover, the effects of fatty acids in bone marrow on osteoblast differentiation are not fully understood.

In order to elucidate the mechanism of postmenopausal osteoporosis, we analyzed the fatty acid composition of bone marrow aspirates and serum phospholipids in nine patients with hematologic diseases and then examined the effects of fatty acids on osteoblast differentiation, using a mouse mesenchymal cell-derived ST2 cell line.

### Methods

#### Subjects

Nine female patients with hematologic diseases who were referred to the Department of Internal Medicine of Saitama Medical School Hospital between 1999 and

2001, participated in this study. The patients who have received bone marrow transplants or had myelosuppressive chemotherapy within three months were precluded from these subjects. Oral and written informed consent was obtained from each subject before study entry. The study was conducted in accordance with our institutional guidelines. To analyze fatty acid composition, we examined a portion of the serum and the bone marrow aspirates obtained from the patients.

#### *Fatty acid analysis*

The fatty acid composition of the bone marrow aspirates and serum phospholipids were analyzed as described previously.<sup>14–16</sup> The phospholipid fraction was isolated from the extracted lipids with the use of a one-dimensional neutral lipid solvent system of hexane, diethyl ether, and acetic acid (87:2:1, v/v). The phospholipid fraction was scraped into glass tubes, and phospholipid fatty acid methyl esters were prepared by transmethylation with 2 M KOH-ethanol and 6 M HCL-ethanol. Fatty acid methyl ester derivatives formed from the isolated phospholipid fraction were separated by gas-liquid chromatography (Hewlett-Packard 5890). Individual fatty acid methyl esters were identified by comparing sample peak retention times with n-pentadecane (Tokyo Kasei Kogyo). The results were expressed as the relative percentage of total identified fatty acids.

#### *Cell culture and alkaline phosphatase assays*

Mouse mesenchymal cell-derived ST2 cell line was purchased from RIKEN Cell Bank. The cells were maintained at subconfluence in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (GIBCO BRL). To evaluate adipocyte differentiation, cells were grown to confluence in DMEM with 10% FBS. The culture medium was supplemented with  $10^{-7}$  M rosiglitazone (Calbiochem) to allow adipocyte differentiation. Cells were seeded at a density of 4,000 cells per well in 96-well dishes and allowed to grow to confluence for 24 hours. After 7 days of treatment with rosiglitazone, lipid staining was done using oil-red O soluble dye, which stains neutral lipids. To examine osteoblast differentiation, cells were grown to confluence in DMEM with 10% FBS. The culture medium was supplemented with BMP-2 to allow osteoblast differentiation. In the same way as BMP-2, retinoic acid, TGF- $\beta$ , and fatty acids were added to the culture medium for 7 days. Recombinant human BMP-2 was provided by Yamanouchi Pharmaceutical Co., Ltd. Alkaline phosphatase assays were performed using a Sigma–Aldrich diagnostic kit. Cells were seeded at a density of  $2.0 \times 10^4$  cells per well in 24-well dishes and

allowed to grow to confluence for 24 hours. After 7 days of treatment with rh BMP-2, the cells were lysed with 200  $\mu$ l of 0.01% SDS in PBS, and the lysates were scraped and collected. Fifty microliters of lysate was added to 50  $\mu$ l of prewarmed p-nitrophenol disodium phosphate (PNPP) substrate with alkaline phosphatase (ALP) buffer solution (SIGMA 221), and the samples were incubated at 37°C. The reaction was stopped by adding 50  $\mu$ l of 2 N NaOH, and the optical density (OD) was read at 405 nm. The ALP activity was determined by comparison with a PNPP standard curve. All values were normalized against the protein concentration determined with the use of TAKARA BCA Protein Assay Reagent.

#### *Statistical analysis*

The data are expressed as means  $\pm$  standard deviation (SD). Statistical analysis was done by the two-tailed Student's t-test for two unpaired groups. Differences with p values of less than 0.05 were considered statistically significant.

## **Results**

#### *Fatty acid composition of the human bone aspirates and serum phospholipids*

Table 1 shows the clinical characteristics of the patients who underwent bone marrow puncture. All nine female patients had hematologic diseases and despite having different diseases, the fatty acid composition did not differ substantially among these patients. The concentrations of the fatty acids of the bone aspirates and serum phospholipids are shown in Table 2. The concentrations of oleic acid and palmitic acid were significantly higher in bone marrow aspirates than in

**Table 1** Clinical Characteristics of the Subjects

Patient no.	Age (yr)/sex	Pre/post menopause	Disease	TC (mg/dl)	LDL-C (mg/dl)
1	33/female	premenopause	AML	200.1	130.4
2	81/female	postmenopause	CML	243.3	146.4
3	44/female	premenopause	CLL	165.0	112.3
4	61/female	postmenopause	NHL	188.8	128.0
5	49/female	postmenopause	NHL	156.3	115.0
6	68/female	postmenopause	NHL	282.4	186.5
7	65/female	postmenopause	MDS	238.5	152.4
8	40/female	premenopause	myeloma	170.1	111.9
9	71/female	postmenopause	NHL	252.2	159.7

AML: acute myeloid leukemia, CML: chronic myelogenous leukemia, CLL: chronic lymphocytic leukemia, NHL: non-Hodgkin's lymphoma, MDS: myelodysplastic syndromes, TC: total cholesterol, LDL-C: low-density lipoprotein cholesterol.

**Table 2** Fatty Acid Composition of Bone Marrow Aspirates and Serum Phospholipids

Fatty acids	Bone marrow aspirates ( $\times 10^2$ mEq/l)	Serum phospholipids ( $\times 10^2$ mEq/l)	P
palmitic acid	20.75 $\pm$ 3.79	16.90 $\pm$ 3.50	p < 0.05
stearic acid	6.65 $\pm$ 1.53	7.82 $\pm$ 2.03	NS
oleic acid	16.99 $\pm$ 4.03	12.02 $\pm$ 3.04	p < 0.05
linoleic acid	11.29 $\pm$ 3.89	13.82 $\pm$ 4.01	NS
linolenic acid	0.72 $\pm$ 0.38	0.61 $\pm$ 0.26	NS
arachidic acid	0.10 $\pm$ 0.04	0.14 $\pm$ 0.05	NS
eicosenoic acid	0.37 $\pm$ 0.36	0.34 $\pm$ 0.32	NS
eicosapentaenoic acid	0.56 $\pm$ 0.52	0.68 $\pm$ 0.42	NS
behenic acid	0.19 $\pm$ 0.05	0.23 $\pm$ 0.11	NS
docosapentaenoic acid	0.47 $\pm$ 0.17	0.39 $\pm$ 0.30	NS
docosahexaenoic acid	2.50 $\pm$ 1.23	3.32 $\pm$ 1.87	NS
lignoceric acid	0.10 $\pm$ 0.03	0.16 $\pm$ 0.13	NS
nervonic acid	0.12 $\pm$ 0.10	0.20 $\pm$ 0.17	NS
total	66.78 $\pm$ 18.53	64.14 $\pm$ 15.02	NS

Values expressed as means  $\pm$  SD (standard deviation). Fatty acids < 0.10 ( $\times 10^2$  mEq/l) are not shown.

serum phospholipids. There were no marked differences in the concentrations of other fatty acids between bone marrow aspirates and serum phospholipids. These data indicated that the concentrations of oleic acid and palmitic acid primarily differed between bone marrow aspirates and serum phospholipids.

#### *Osteoblast and adipocyte differentiation potential of ST2 cells*

Then, we selected oleic acid and palmitic acid, which showed significantly higher concentrations in bone marrow aspirates than in serum phospholipids (Table 2).

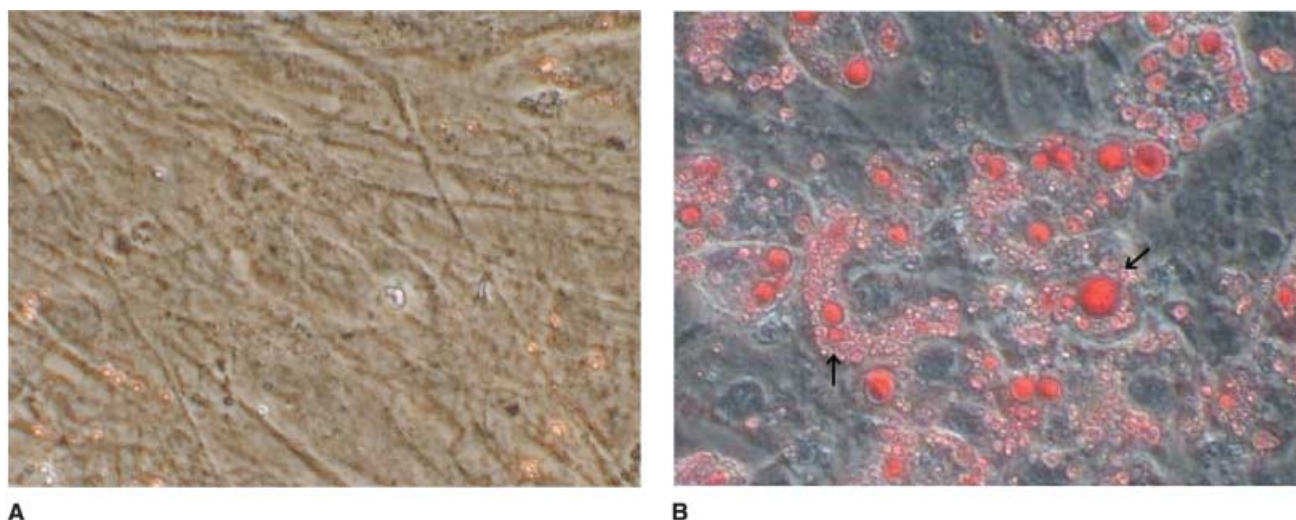
ALP assay and oil-red O staining of mesenchymal ST2 cells were performed with or without TGF- $\beta$  and retinoic acid, both of which were known to allow osteoblast differentiation,<sup>17</sup> in order to verify the osteoblast and adipocyte differentiation potentials of ST2 cells which we used. Fig. 1 shows the morphology of ST2 cells in the presence or absence of  $10^{-6}$  M rosiglitazone, and treatment of ST2 cells with rosiglitazone induced adipogenic differentiation with accumulation of lipid. The rates of ALP positive cells were significantly increased in a dose-dependent fashion by TGF- $\beta$  and retinoic acid (Fig. 2). In contrast, the rates of oil-red O positive cells were significantly decreased in a dose-dependent fashion by retinoic acid, but were unchanged by treatment with TGF- $\beta$  (Fig. 3).

#### *Effects of oleic acid and palmitic acid on osteoblast differentiation of ST2 cells*

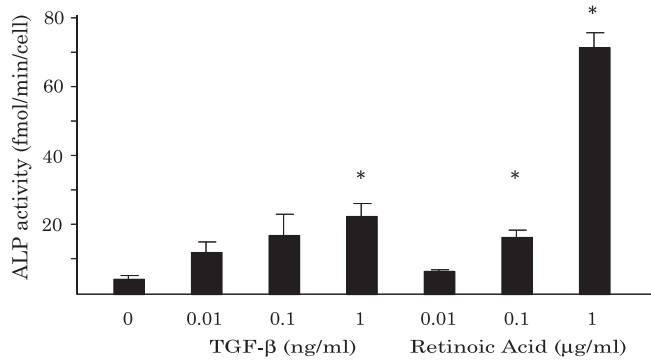
To test the effects of oleic acid and palmitic acid on osteoblast differentiation, we performed ALP assays using ST2 cells induced by BMP-2. The rate of ALP positive cells was significantly increased by 100  $\mu$ M of oleic acid plus 100 ng/ml of BMP-2. The rate of ALP positive cells was similar with or without 100  $\mu$ M of palmitic acid (Fig. 4), and the rate of oil-red O positive cells was also similar in all of these conditions (Fig. 5). These findings indicated that only oleic acid acted synergistically with BMP-2 to promote osteoblastic differentiation of ST2 cells.

### Discussion

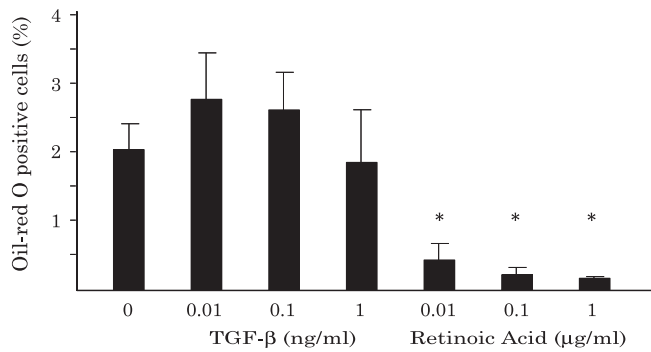
Mesenchymal stem cells in bone marrow, whose progeny become committed to specific lineages in re-



**Fig. 1** Oil-red O staining of lipids in ST2 cells in the absence (A), and in the presence (B) of  $10^{-6}$  M rosiglitazone. Arrows indicate lipids.



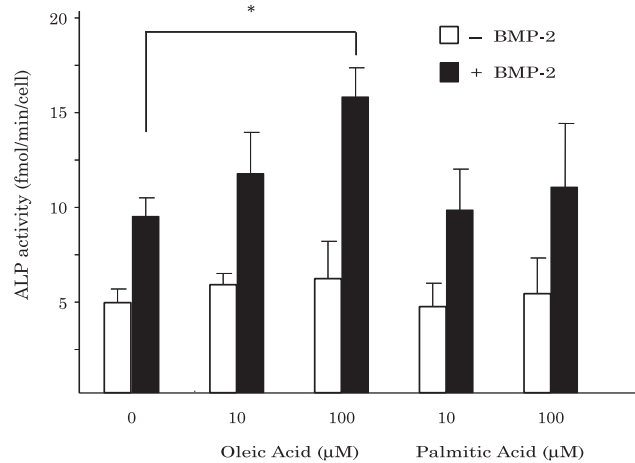
**Fig. 2** Osteoblast differentiation potential of ST2 cells with dose-dependent induction by TGF- $\beta$  and retinoic acid. \* $p < 0.05$ : Significantly different from basal value.



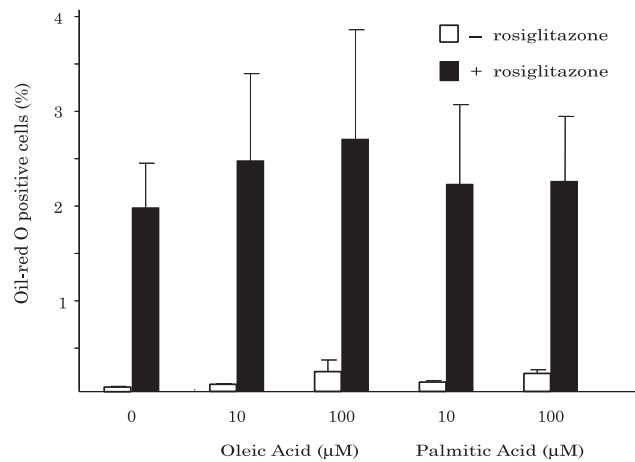
**Fig. 3** Effects of TGF- $\beta$  and retinoic acid on adipocyte differentiation of ST2 cells with induction by  $10^{-7}$  M rosiglitazone. \* $p < 0.05$ : Significantly different from basal value.

sponse to growth factors, hormones, and other signals, are pluripotential and differentiate into fibroblasts, adipocytes, chondrocytes, or osteoblast.<sup>18–23</sup> ST2 cell line, a clone of stromal cells, was isolated from the bone marrow of BC8 mice. This cell line has characteristics typical of preadipocytes and differentiates into osteoblasts and adipocyte-like mesenchymal stromal cells.<sup>23</sup> ST2 cells differentiate into osteoblasts when BMP-2, TGF- $\beta$ , and ascorbic acid are added to the culture medium. BMP-2 belongs to the TGF- $\beta$  superfamily, which induces the development and differentiation of bone cells. ST2 cells also differentiate into adipocytes when long-chain fatty acids are added to the culture medium.<sup>25,26</sup> Peroxisome proliferator-activated receptor  $\gamma$  simultaneously promotes the formation of adipocytes and suppresses the formation of osteoblasts,<sup>18</sup> and retinoic acid also simultaneously promotes the formation of osteoblasts and suppresses the formation of adipocytes.<sup>6</sup>

In this study, using ST2 cells, the effects of TGF- $\beta$  or retinoic acid on osteoblast and adipocyte differentiation were consistent with previous findings.<sup>6</sup> We analyzed



**Fig. 4** Effects of oleic acid and palmitic acid on osteoblast differentiation of ST2 cells with induction by 100 ng/ml BMP-2. \* $p < 0.05$  (BMP-2 vs BMP-2 plus oleic acid): Significant differences from the culture incubated with 100  $\mu$ M of oleic acid are indicated.



**Fig. 5** Effects of oleic acid and palmitic acid on adipocyte differentiation of ST2 cells with induction by  $10^{-7}$  M rosiglitazone.

the fatty acid composition of the culture medium used in this study, which contained the same lot of 10% FBS (data not shown). The medium was found to contain minimal amounts of oleic acid and palmitic acid. It was therefore considered to have had no appreciable effect on the results of our differentiation experiments. Our assay using ST2 cells was therefore considered a valid approach for examining the effects of fatty acids on osteoblast or adipocyte differentiation.

We had initially thought that oleic acid and palmitic acid have simultaneous differentiation effects to promote adipocyte differentiation and suppress osteoblast differentiation. Several reasons supported this assumption: 1) Mature adipocytes release PUFA, which impairs osteoblast proliferation,<sup>3</sup> and long-chain fatty acids, which promote adipocyte differentiation. 2) The bone



marrow microenvironment is a complex network of cells and extracellular matrix that maintains the hematopoietic system throughout the life of the individual. Regulating factors may maintain the homeostasis of mesenchymal cell differentiation in the bone marrow microenvironment. 3) Our results indicated that the ratios of oleic acid and palmitic acid were higher in bone marrow than in serum phospholipids.

Contrary to our expectation, our result showed that oleic acid acts synergistically with BMP-2 in promoting osteoblast differentiation of ST2 cells. In addition, the results of oil-red O staining of ST2 cells indicated no significant promotion or suppression of adipocyte differentiation by oleic acid. These results indicated that oleic acid may promote osteoblast differentiation of only pre-osteoblasts synergistically with BMP-2, because BMP-2 has osteoblast differentiation effect on both mesenchymal cells and pre-osteoblasts with the induction of Runx2 expression.<sup>27</sup> Oleic acid, a mono-unsaturated fatty acid, is found abundantly in palm oil and olive oil. Mono- or polyunsaturated fatty acids have been shown to have protective effects towards the development of cardiovascular disease; however, the relation between monounsaturated fatty acids and osteoporosis is still unknown.<sup>7</sup> From a clinical standpoint, in order to propose ideal eating habits and dietary recommendations to prevent osteoporosis, it is necessary to elucidate the role of oleic acid in bone metabolism.

To our knowledge, this is the first study to show differences in fatty acid composition between bone marrow aspirates and serum phospholipids. We studied subjects who had never received bone marrow transplants, which impair differentiation of bone marrow stromal cells into osteoblasts,<sup>28</sup> or had myelosuppressive chemotherapy within three months.

Our study had several limitations. First, it was a small study, with differences in age and menopausal status of the subjects. Second, we did not measure bone mineral density in our patients. Third, there could be considerable effects on the bone marrow function of our subjects who had hematologic diseases and received myelosuppressive chemotherapy previously, and the characteristics of our subjects do not reflect those of women in the general population. Indeed, the ideal subjects would have been healthy individuals, but bone marrow puncture is a painful procedure considered ethically unacceptable in a study of healthy volunteers. Despite these limitations, our results suggest a difference in fatty acid composition between bone marrow aspirates and serum phospholipids. Further studies, if possible using samples of bone marrow aspirates obtained from a bone marrow bank, are required to verify our results. In conclusion, our findings suggest that the fatty acid composition of bone marrow aspirates differs from that of serum phospholipids. This difference pre-

sumably affects osteoblast differentiation in the bone marrow microenvironment.

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## References

1. Blain H, Vuillemin A, Guillemin F: Serum leptin level is a predictor of bone mineral density in postmenopausal women. *J Clin Endocrinol Metab* 2002; 87: 1030–1035
2. Harada S, Rodan GA: Control of osteoblast function and regulation of bone mass. *Nature* 2003; 423: 349–355
3. Maurin AC, Chavassieux PM, Vericel E, Meunier PJ: Role of polyunsaturated fatty acids in the inhibitory effect of human adipocytes on osteoblastic proliferation. *Bone* 2002; 31: 260–266
4. Yamaguchi A, Ishizuya T, Kintou N: Effects of BMP-2, BMP-4, and BMP-6 on osteoblastic differentiation of bone marrow-derived stromal cell lines, ST2 and MC3T3-G2/PA6. *Biochem Biophys Res Commun* 1996; 220: 366–371
5. Ahdjoudj S, Lasmoles F, Holy X, Zerath E, Marie PJ: Transforming growth factor beta2 inhibits adipocyte differentiation induced by skeletal unloading in rat bone marrow stroma. *J Bone Miner Res* 2002; 17: 668–677
6. Skillington J, Choy L, Derynck R: Bone morphogenetic protein and retinoic acid signaling cooperate to induce osteoblast differentiation of preadipocytes. *J Cell Biol* 2002; 159: 135–146
7. Watkins BA, Lippman HE, Le Bouteiller L, Li Y, Seifert MF: Bioactive fatty acids: role in bone biology and bone cell function. *Prog Lipid Res* 2001; 40: 125–148
8. Evans DB, Thavarajah M, Kanis JA: Involvement of prostaglandin E2 in the inhibition of osteocalcin synthesis by human osteoblast-like cells in response to cytokines and systemic hormones. *Biochem Biophys Res Commun* 1990; 167: 194–202
9. Kajii T, Suzuki K, Yoshikawa M, Imai T, Matsumoto A, Nakamura S: Long-term effects of prostaglandin E2 on the mineralization of a clonal osteoblastic cell line (MC3T3-E1). *Arch Oral Biol* 1999; 44: 233–241
10. Igarashi K, Hirafuji M, Adachi H, Shinoda H, Mitani H: Role of endogenous PGE2 in osteoblastic functions of a clonal osteoblast-like cell, MC3T3-E1. *Prostaglandins Leukot Essent Fatty Acids* 1994; 50: 169–17
11. Watkins BA, Li Y, Lippman HE, Seifert MF: Biochemical and molecular actions of fatty acids in bone modeling. *World Rev Nutr Diet* 2001; 88: 126–140
12. Yamada Y, Fushimi H, Inoue T: Effect of eicosapentaenoic acid and docosahexaenoic acid on diabetic osteopenia. *Diabetes Res Clin Pract* 1995; 30: 37–42
13. Albertazzi P, Coupland K: Polyunsaturated fatty acids. Is there a role in postmenopausal osteoporosis prevention? *Maturitas* 2002; 42: 13–22
14. Ristic-Medic D, Ristic G, Tepsic V, Ristic GN: Effects of different quantities of fat on serum and liver lipids, phospholipid class distribution and fatty acid composition in alcohol-treated rats. *J Nutr Sci Vitaminol* 2003; 49: 367–374
15. Holub BJ, Skeaff CM: Nutritional regulation of cellular phosphatidylinositol. *Methods Enzymol* 1987; 141: 234–244
16. Stark KD, Park EJ, Holub BJ: Fatty acid composition of serum phospholipid of premenopausal women and postmenopausal women receiving and not receiving hormone replacement therapy. *Menopause* 2003; 10: 448–455
17. Otsuka E, Yamaguchi A, Hirose S, Hagiwara H: Characteriza-

- tion of osteoblastic differentiation of stromal cell line ST2 that is induced by ascorbic acid. *Am J Physiol* 1999; 277: C132–138
18. Cheleuitte D, Mizuno S, Glowacki J: In vitro secretion of cytokines by human bone marrow: effects of age and estrogen status. *J Clin Endocrinol Metab* 1998; 83: 2043–2051
  19. Lecka-Czernik B, Gubrij I, Moerman EJ: Inhibition of *Osf2/Cbfa1* expression and terminal osteoblast differentiation by *PPARgamma2*. *J Cell Biochem* 1999; 74: 357–371
  20. Cooper SC, Roncari DA: 17-beta-estradiol increases mitogenic activity of medium from cultured preadipocytes of massively obese persons. *J Clin Invest* 1989; 83: 1925–1929
  21. Franceschi RT, Young J: Regulation of alkaline phosphatase by 1,25-dihydroxyvitamin D3 and ascorbic acid in bone-derived cells. *J Bone Miner Res* 1990; 5: 1157–1167
  22. Bennett JH, Joyner CJ, Triffitt JT, Owen ME: Adipocytic cells cultured from marrow have osteogenic potential. *J Cell Sci* 1991; 99: 131–139
  23. Nuttall ME, Patton AJ, Olivera DL, Nadeau DP, Gowen M: Human trabecular bone cells are able to express both osteoblastic and adipocytic phenotype: implications for osteopenic disorders. *J Bone Miner Res* 1998; 13: 371–382
  24. Ogawa M, Nishikawa S, Ikuta K, Yamamura F, Naito M, Takahashi K: B cell ontogeny in murine embryo studied by a culture system with the monolayer of a stromal cell clone, ST2: B cell progenitor develops first in the embryonal body rather than in the yolk sac. *Embo J* 1988; 7: 1337–1343
  25. MacDougald OA, Lane MD: Transcriptional regulation of gene expression during adipocyte differentiation. *Annu Rev Biochem* 1995; 64: 345–373
  26. Amri EZ, Ailhaud G, Grimaldi PA: Fatty acids as signal transducing molecules: involvement in the differentiation of preadipose to adipose cells. *J Lipid Res* 1994; 35: 930–937
  27. Lee KS, Kim HJ, Li QL, Chi XZ, Ueta C, Komori T, Wozney JM, Kim EG, Choi JY, Ryoo HM, Bae SC: *Runx2* is a common target of transforming growth factor beta1 and bone morphogenetic protein2, and cooperation between *Runx2* and *Smad5* induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. *Mol Cell Biol* 2000; 20: 8783–8792
  28. Lee WY, Cho SW, Oh ES: The effect of bone marrow transplantation on the osteoblastic differentiation of human bone marrow stromal cells. *J Clin Endocrinol Metab* 2002; 87: 329–335