Bone morphogenetic protein-2 stimulates adipogenic differentiation of mesenchymal precursor cells in synergy with BRL 49653 (rosiglitazone)

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Received 21 March 2000; received in revised form 18 May 2000

Edited by Veli-Pekka Lehto

Abstract Bone morphogenetic proteins (BMPs) were discovered as potent bone-inducing molecules. Their effect on adipogenic differentiation is not well understood, both stimulation and inhibition of the process have been described. We show here that BMP-2 strongly stimulates adipogenic differentiation of murine 3T3-L1 preadipocytes if applied together with an agonist of peroxisome proliferator-activated receptor γ (PPARγ). On its own, BMP-2 (500 ng/ml) did not stimulate adipogenesis as quantified by flow cytometry with the lipophilic dye Nile Red. However, the protein strongly potentiated adipogenesis stimulated by the thiazolidinedione BRL 49653 as well as glycerol-3-phosphate dehydrogenase activity and induction of mRNAs for the adipogenic markers PPARγ and adipsin. We confirmed the synergistic action of BMP-2 and BRL 49653 with primary cultures of rat bone marrow stromal cells. Our data demonstrate that BMP-2 can act as a potent adipogenic agent if presented together with activators of PPARγ. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Cell differentiation; Peroxisome proliferator-activated receptor; Osteoblast; Adipocyte

1. Introduction

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor β family of growth and differentiation factors. First isolated from bone as potent inducers of ectopic ossification [1–3], BMPs are today known to exert a variety of functions during embryonal development and in adult organisms [4,5].

The BMP family of proteins comprises more than 20 molecules. Among these, BMP-2, BMP-4, BMP-6 and BMP-7 (OP-1) have been extensively characterized as agents inducing bone formation in vitro and in vivo [2–5].

Like osteoblasts and chondrocytes, adipocytes arise from mesenchymal precursor cells through a differentiation process that can be recapitulated in vitro. The nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPARγ) has been shown to be a key regulator of this process [6,7] and PPARγ activators like certain prostaglandin derivatives, lipid messengers and the synthetic insulin-sensitizing thiazolidinediones are known as strong inducers of adipogenesis [8–10].

As adipocytes and osteoblasts originate from common precursor cells [11,12] it was tempting to speculate that PPARγ activators and BMPs would oppose each other with respect to cell differentiation, inducing adipogenic and osteogenic signals, respectively. Recent publications indeed supported this notion. Gimble et al. reported that BMP-2 inhibits adipogenic differentiation of a bone marrow stromal cell line [13] and Gori et al. [14], studying an immortalized human mesenchymal precursor cell line, showed that BMP-2 directs cell differentiation away from the adipogenic towards the osteogenic pathway. However, in both series of experiments, no potent PPARγ activators were used to induce adipogenic differentiation. In contrast, work from other laboratories has indicated that BMPs can induce both osteogenesis and adipogenesis in the murine pluripotent cell line C3H10T1/2 under specific culture conditions [15,16]. More recently Chen et al. demonstrated that BMP-2 transmits osteogenic and adipogenic signals through different receptor subunits [17].

We have re-evaluated the adipogenic action of BMP-2 using as experimental model the well known preadipocyte cell line 3T3-L1 as well as primary cultures of rat bone marrow stromal cells. We show that BMP-2 becomes a strong inducer of adipogenic differentiation if associated with a thiazolidinedione activator of PPARγ. Our results provide a rationale to explain diverging results obtained with BMP-2 as an adipogenic agent. The strong synergy observed between BMP-2 and PPARγ agonists may also be relevant for our general understanding of cell differentiation processes stimulated by both types of factors.

2. Materials and methods

2.1. Materials

If not otherwise stated, fine chemicals were obtained from Sigma, Buchs, Switzerland. BMP-2 and BRL 49653 were produced at Novartis Pharma AG, Basel, Switzerland.

2.2. Cell culture

The 3T3-L1 cells were grown in MEMα/Ham's F12 medium (1:1) (Life Technologies, Basel, Switzerland) supplemented with 10% fetal calf serum (Life Technologies).

Primary cultures of rat bone marrow mesenchymal cells were prepared according to a published method [18]. Briefly, bone marrow was flushed out of femurs and tibias of young adult female Wistar rats using a syringe and culture medium (MEMα/Ham's F12 as described above). Primary cultures were established using approximately 60×10^6 bone marrow cells per T75 culture flask and mesenchymal cells were allowed to adhere to the substratum for 2 days. Hematopoietic cells were then eliminated by washing and medium change. Cultures were grown to confluence, passaged 1:4 and frozen at the

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Abbreviations: BMP, bone morphogenetic protein; GPDH, glycerol-3-phosphate dehydrogenase; IBMX, 3-isobutyl-1-methylxanthine; NADH, nicotinamide adenine dinucleotide, reduced form; PPARγ, peroxisome proliferator-activated receptor γ

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PII: S0014-5793(00)01655-0

end of passage 1. Routine experiments were carried out with cells taken from frozen stocks and used between passages 2 and 6.

For adipogenic differentiation experiments, cells were seeded at a density of 10^4 cells per cm^2 in either six-well plates (cytofluorometry), 24-well plates (glycerol-3-phosphate dehydrogenase (GPDH) assay) or 10 cm culture plates (RNA extraction) and grown to confluence for 3 to 4 days. Cultures were then treated with BRL 49653, BMP-2, 3-isobutyl-1-methylxanthine (IBMX) or combinations thereof in full medium supplemented with 10 mM β-glycerophosphate and 50 μM ascorbic acid phosphate. The latter are components of osteogenic differentiation media which we included in this series of experiments in order to have similar experimental conditions in osteogenic and adipogenic differentiation assays (to be reported elsewhere). Ascorbic acid promotes extracellular collagen matrix formation and supports both osteogenic and adipogenic differentiation, β-glycerophosphate constitutes a phosphate reserve supporting mineral deposition in osteogenic differentiation assays and has no effect on adipogenic differentiation ([19] and unpublished results). Cultures were maintained under differentiating conditions for up to 2 weeks as indicated in Section 3, with twice weekly medium change.

The presence of mature adipocytes was assessed either by Oil Red O staining of cultures or by cytofluorometry with Nile Red (see Section 2.4). For Oil Red O staining, cell monolayers were washed with calcium- and magnesium-free phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde. The fixed cells were then covered with 3 mg/ml Oil Red O dissolved in 60% isopropanol (v/v) for 10 min, before excess dye was washed away with H_2O.

2.3. GPDH activity assay

For biochemical determination of GPDH activity we used a protocol adapted from Pairault and Green [20]. Briefly, cells were washed with PBS, covered with a solution containing 20 mM Tris, 1 mM EDTA, 1 mM β-mercaptoethanol (BioRad, Glattbrugg, Switzerland) and harvested in a tube maintained on ice. After passing six times through a G20 needle to break the cells, the suspension was centrifuged for 3 min at $5000 \times g$ at 4°C. Aliquots of the supernatant were incubated at 37°C in 1 ml of assay solution containing 0.1 M triethanolamine, 2.5 mM EDTA, 0.1 mM β-mercaptoethanol, 125 μM nicotinamide adenine dinucleotide, reduced form (NADH) (Roche, Basel, Switzerland) and 200 μM dihydroxyacetonephosphate. Enzyme activity is reflected by the disappearance of NADH measured by absorption at 340 nm over 10–20 min. The protein content of each sample was analyzed in parallel using the $BioRad^D_C$ Protein Assay. Results are expressed as mU/μg protein (1 mU = 1 nmol NADH/min). All determinations were done in duplicate (with the exception of the data in Table 1, experiment 1, which were single determinations). Error bars indicate mean ± range. For statistical analysis of data we used Student's *t*-test as provided by Microsoft Excel (two-tailed, two samples with equal variance).

2.4. Flow cytometry

The quantification of adipocytes in culture using the lipophilic fluorescent dye Nile Red was carried out essentially as described by Gimble et al. [13]. Cells were trypsinized carefully and centrifuged for 5 min at $200 \times g$ at 4°C. The cell pellet was resuspended and fixed with 4% paraformaldehyde at 4°C. For flow cytometry, the cells were again sedimented as described above, resuspended in cold PBS with a final Nile Red concentration of 1 μg/ml and kept on ice for 30 min. The samples were then analyzed with a FACScan™ flow cytometer (Becton Dickinson, Basel, Switzerland). Nile Red fluorescence was measured on the FL2 emission channel through a 585 ± 21 nm band pass filter, following excitation with an argon ion laser source at 488 nm. Using a forward scatter (FSC)/side scatter representation of events, a first R1 region was defined to exclude cellular debris from the analysis. A selection window called R2 was then established on the FL2/FSC blot of the R1 population as indicated in Fig. 1 in order to count cells with high FL2 values (adipocytes). Data analysis was performed using CellQuest™ 3.1 software (Becton Dickinson). For each sample 20 000 events were collected. The results are expressed as the percentage of cells appearing in the R2 region.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted from cell cultures using the RNeasy Midi Kit (Qiagen, Basel, Switzerland) following the instructions of the manufacturer. After DNase treatment of the samples (Promega, Basel, Switzerland), reverse transcription was carried out using MMLV reverse transcriptase (Promega). Preparations of cDNA were quality-controlled with respect to the absence of genomic DNA using appropriate PCR primers. PCR reactions (20 μl) were set up using Platinum Taq DNA Polymerase (Life Technologies) and run on a GeneAmp PCR System 9600 (Perkin Elmer, Rotkreuz, Switzerland). PCR conditions were: 30 s denaturation at 94°C, 45 s annealing at 60°C and 1 min extension at 72°C. For adipsin and PPARγ 26 PCR cycles were carried out and for clathrin 30 cycles were run. The following primer pairs were used (forward/reverse):

PPARγ: 5′ aaactctgggagattctcct 3′/5′ tcttgtgaatggaatgtctt 3′;
Adipsin: 5′ ctgctggacgagcagtgg 3′/5′ gatgacactcgggtat 3′;
Clathrin: 5′ gacagtgccatcatgaatcc 3′/5′ tttgtgcttctggaggaaagaa 3′

3. Results

The murine mesenchymal precursor cell line 3T3-L1 can be efficiently stimulated to undergo adipogenic differentiation using a combination of thiazolidinediones and the phosphodiesterase inhibitor IBMX [21]. We initially observed that high concentrations of BMP-2 failed to inhibit adipogenesis under these conditions, also when other cell systems were used. A more careful analysis unexpectedly demonstrated a strong synergy between BMP-2 and BRL 49653 in such differentiation assays. Fig. 1 shows a representative experiment of three independent experiments carried out. Treatment of confluent 3T3-L1 cell cultures for 14 days with either BMP-2 (500 ng/ml) or BRL 49653 (1 μM) alone led only to a weak increase in the number of adipocytes as assessed by Nile Red flow cytometry or Oil Red O staining (Fig. 1). However, combining BMP-2 and thiazolidinedione resulted in strong adipogenic differentiation, comparable to the effects of the standard adipogenic treatment BRL 49653+IBMX (0.1 mM).

An almost identical picture emerged when GPDH activity, a late adipocyte differentiation marker, was measured biochemically. GPDH activity was undetectable or weak in control cells or cells treated with BMP-2 or BRL 49653 alone, respectively (Fig. 2, top). However, treatment with both

Table 1
Induction of adipogenesis in rat bone marrow mesenchymal cells by BMP-2, BRL 49653/IBMX and combinations

Treatment	Experiment 1		Experiment 2
	cytofluorometry (% cells in R2)	GPDH activity (mU/mg protein)	GPDH activity (mU/mg protein)
Control	0.05	< 1	< 1
BMP-2 (500 ng/ml)	0.95	< 1	< 1
BRP 49653 (1 μM)+IBMX (200 μM)	7.4	5.62	5.81 ± 0.16
BMP-2+BRL+IBMX	14.0	26.02	1372(**) ± 0.80

Results of flow cytometric analysis and GPDH activity measurements from two independent experiments. Cells were treated for 11 days. Asterisks indicate statistical significance ($P < 0.01$) compared to the treatment with BRL 49653/IBMX alone.

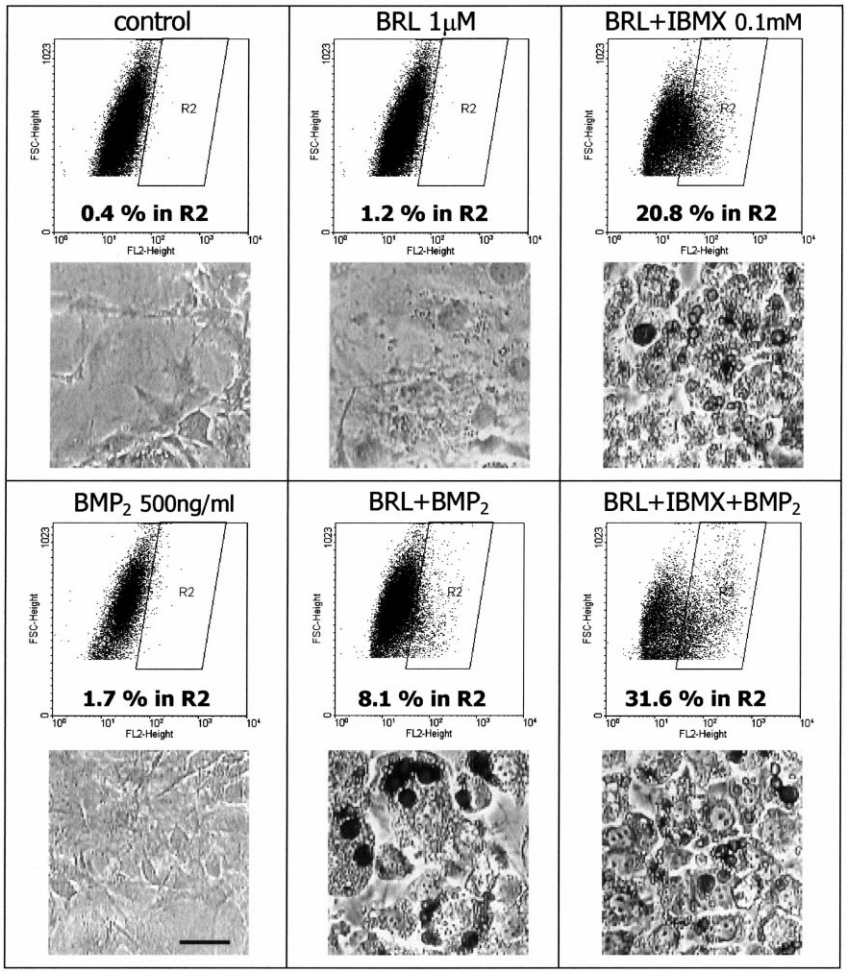

Fig. 1. Induction of adipogenesis in 3T3-L1 cell cultures by BMP-2, BRL 49653, IBMX and combinations of these (14 days of treatment). Results of flow cytometric analysis and Oil Red O staining of cultures. Bar: 50 μm.

agents together resulted in a strong synergistic response, which was clearly significant ($P < 0.015$) in four of four independent experiments. A concentration–response curve for BMP-2 applied in the presence of BRL 49653 (1 μM) is shown in Fig. 2, bottom. The protein stimulated GPDH activity with an EC_{50} of approximately 200 ng/ml, which is in good agreement with published data [22] and own experimental results for osteogenic differentiation of preosteoblasts (not shown).

We further assessed expression of the adipocyte markers PPARγ and adipsin by RT-PCR under the experimental conditions described above. Two independent sets of RNA were prepared that gave identical results. Both PPARγ and adipsin were detectable following a 10 day treatment with the thiazolidinedione alone, whereas the corresponding bands could not be detected in control cells or cells treated with BMP-2 (Fig. 3). Again, there was a substantial increase in the expression of both PPARγ and adipsin visible following a combined treatment with both agents.

In order to demonstrate the relevance of our findings for cell systems other than 3T3-L1 we used primary mesenchymal precursor cells isolated from rat bone marrow. As is shown in Table 1, combined treatment with IBMX, BMP-2 and BRL 49653 resulted in a significant synergistic response in adipocyte differentiation, measured by flow cytometry and the GPDH activity of cultures.

4. Discussion

Adipogenesis is regulated by cell density and by growth and differentiation factors [23]. The nuclear hormone receptor PPARγ is required for this process [6,7]. However, in most experimental systems PPARγ activators alone are not sufficient to stimulate differentiation significantly, but need to be associated with glucocorticoids, insulin and/or cAMP-elevating agents like IBMX [7,9,13,21,24]. The precise interplay of these factors inducing the adipogenic program is not fully understood.

We show here that BMP-2 strongly stimulates adipogenesis in vitro when associated with the thiazolidinedione BRL 49653. These results came unexpected as, based on earlier studies, we had rather anticipated a pro-osteogenic and anti-adipogenic effect of this molecule [12–16]. We believe that our results provide a rationale to explain diverging experimental data obtained in the past with BMPs [12–17], as described in Section 1. In fact, the adipogenic effects observed with BMPs likely depend on the expression and activity state of PPARγ in a given cell type, which in turn can be modulated by culture conditions and autocrine production of lipophilic messengers that may act as PPARγ agonists [8,10].

Our results may also have important implications for our understanding of osteoporosis, which is associated with the

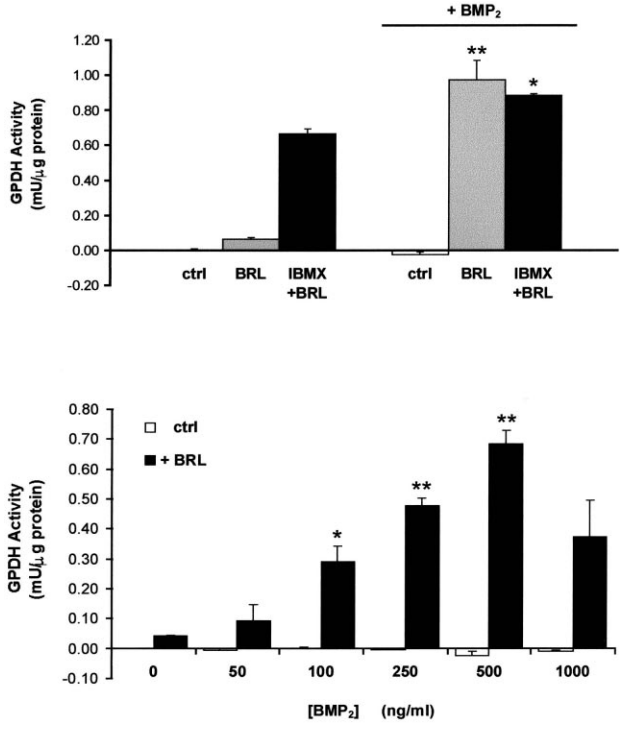

Fig. 2. Induction of GPDH activity in 3T3-L1 cell cultures. Top: cells were treated for 14 days in the absence (ctrl) or presence of BMP-2 (500 ng/ml), BRL 49653 (1 μM) and IBMX (100 μM). Bottom: concentration–response curve for BMP-2 measured in the absence (ctrl) or presence of 0.1 μM of BRL 49653 after 14 days of treatment. Asterisks indicate statistical significance compared to the corresponding experimental condition not treated with BMP-2 (**$P < 0.015$; *$P < 0.05$).

unwanted adipogenic differentiation of precursor cells in bone marrow [12,25,26], where BMPs are known to be produced. The signalling status of PPARγ may influence BMP action significantly in this biological context. It is conceivable that antagonists or partial agonists of PPARγ [27,28] may have beneficial effects in osteoporosis, provided a potential induction of insulin resistance would not occur [24].

Acknowledgements: We thank our colleagues F. Bassilana, N. Cerletti and C. Darimont for supplying reagents and for valuable advice.

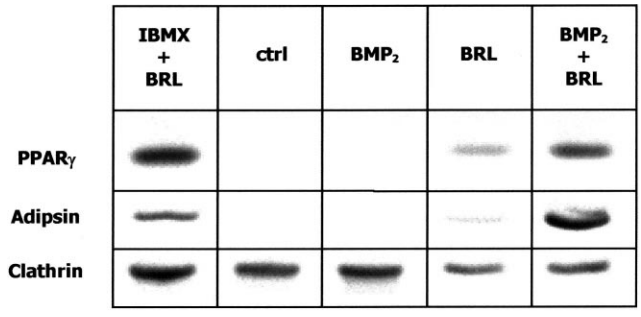

Fig. 3. Expression of markers of adipogenic differentiation (PPARγ, adipsin) as assessed by RT-PCR. RNA was extracted from cells after 10 days of treatment with BMP-2 (500 ng/ml), BRL 49653 (1 μM), IBMX (100 μM) and indicated combinations. Clathrin was used as a control of cDNA quality.

References

[1] Hanamura, H., Higuchi, Y., Nakagawa, M., Iwata, H., Nogami, H. and Urist, M.R. (1980) Clin. Orthop. 148, 281–290.
[2] Wozney, J.M., Rosen, V., Celeste, A.J., Mitsock, L.M., Whitters, M.J., Kriz, R.W., Hewick, R.M. and Wang, E.A. (1988) Science 242, 1528–1534.
[3] Ozkaynak, E., Rueger, D.C., Drier, E.A., Corbett, C., Ridge, R.J., Sampath, T.K. and Oppermann, H. (1990) EMBO J. 9, 2085–2093.
[4] Wozney, J.M. and Rosen, V. (1998) Clin. Orthop. 346, 26–37.
[5] Hogan, B.L. (1999) Cell 96, 225–233.
[6] Barak, Y., Nelson, M.C., Ong, E.S., Jones, Y.Z., Ruiz-Lozano, P., Chien, K.R., Koder, A. and Evans, R.M. (1999) Mol. Cell 4, 585–595.
[7] Rosen, E.D., Sarraf, P., Troy, A.E., Bradwin, G., Moore, K., Milstone, D.S., Spiegelman, B.M. and Mortensen, R.M. (1999) Mol. Cell 4, 611–617.
[8] Forman, B.M., Tontonoz, P., Chen, J., Brun, R.P., Spiegelman, B.M. and Evans, R.M. (1995) Cell 83, 803–812.
[9] Tontonoz, P., Hu, E. and Spiegelman, B.M. (1994) Cell, 79, 1147–1156 (published erratum appears in (1995) Cell, 80(6), following 957).
[10] Nagy, L., Tontonoz, P., Alvarez, J.G., Chen, H. and Evans, R.M. (1998) Cell 93, 229–240.
[11] Gimble, J.M., Robinson, C.E., Wu, X. and Kelly, K.A. (1996) Bone 19, 421–428.
[12] Prockop, D.J. (1997) Science 276, 71–74.
[13] Gimble, J.M., Morgan, C., Kelly, K., Wu, X., Dandapani, V., Wang, C.S. and Rosen, V. (1995) J. Cell Biochem. 58, 393–402.
[14] Gori, F., Thomas, T., Hicok, K.C., Spelsberg, T.C. and Riggs, B.L. (1999) J. Bone Miner. Res. 14, 1522–1535.
[15] Wang, E.A., Israel, D.I., Kelly, S. and Luxenberg, D.P. (1993) Growth Factors 9, 57–71.
[16] Asahina, I., Sampath, T.K. and Hauschka, P.V. (1996) Exp. Cell Res. 222, 38–47.
[17] Chen, D., Ji, X., Harris, M.A., Feng, J.Q., Karsenty, G., Celeste, A.J., Rosen, V., Mundy, G.R. and Harris, S.E. (1998) J. Cell Biol. 142, 295–305.
[18] Beresford, J.N., Bennett, J.H., Devlin, C., Leboy, P.S. and Owen, M.E. (1992) J. Cell Sci. 102, 341–351.
[19] Ono, M., Aratani, Y., Kitagawa, I. and Kitagawa, Y. (1990) Exp. Cell Res. 187, 309–314.
[20] Pairault, J. and Green, H. (1979) Proc. Natl. Acad. Sci. USA 76, 5138–5142.
[21] Young, P.W., Buckle, D.R., Cantello, B.C., Chapman, H., Coyle, P.J., Haigh, D., Hindley, R.M., Holder, J.C., Kallender, H., Latter, A.J., Lawrie, K.W.M., Mossakowska, W., Murphy, G.J., Roxbee-Cox, L. and Smith, S.A. (1998) J. Pharmacol. Exp. Ther. 284, 751–759.
[22] Thies, R.S., Bauduy, M., Ashton, B.A., Kurtzberg, L., Wozney, J.M. and Rosen, V. (1992) Endocrinology 130, 1318–1324.
[23] Mandrup, S. and Lane, M.D. (1997) J. Biol. Chem. 272, 5367–5370.
[24] Kubota, N., Terauchi, Y., Miki, H., Tamemoto, H., Yamauchi, T., Komeda, K., Satoh, S., Nakano, R., Ishii, C., Sugiyama, T., Eto, K., Tsubamoto, Y., Okuno, A., Murakami, K., Sekihara, H., Hasegawa, G., Naito, M., Toyoshima, Y., Tanaka, S., Shiota, K., Kitamura, T., Fujita, T., Ezaki, O., Aizawa, S., Nagai, R., Tobe, K., Kimura, S. and Kadowaki, T. (1999) Mol. Cell 4, 597–609.
[25] Meunier, P., Aaron, J., Edouard, C. and Vignon, G. (1971) Clin. Orthop. 80, 147–154.
[26] Nuttall, M.E., Patton, A.J., Olivera, D.L., Nadeau, D.P. and Gowen, M. (1998) J. Bone Miner. Res. 13, 371–382.
[27] Elbrecht, A., Chen, Y., Adams, A., Berger, J., Griffin, P., Klatt, T., Zhang, B., Menke, J., Zhou, G., Smith, R.G. and Moller, D.E. (1999) J. Biol. Chem. 274, 7913–7922.
[28] Oberfield, J.L., Collins, J.L., Holmes, C.P., Goreham, D.M., Cooper, J.P., Cobb, J.E., Lenhard, J.M., Hull-Ryde, E.A., Mohr, C.P., Blanchard, S.G., Parks, D.J., Moore, L.B., Lehmann, J.M., Plunket, K., Miller, A.B., Milburn, M.V., Kliewer, S.A. and Willson, T.M. (1999) Proc. Natl. Acad. Sci. USA 96, 6102–6106.