Leptin receptor isoform expression in rat osteoblasts and their functional analysis

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Abstract The genetic defect in producing the adipose hormone leptin results among others in a drastic increase of bone mass. The current understanding is that under normal circumstances, osteoblast activity is indirectly suppressed by a hypothalamic relay induced by leptin-signalling in the brain. To investigate whether leptin might also regulate osteoblast activity in a direct manner, expression of leptin receptors in rat osteoblasts was determined and their functionality was analyzed upon recombinant leptin treatment. Reverse transcription-PCR confirmed the expression of four among the six currently described receptor isoforms, which were also able to transduce cell signalling as shown by STAT3 phosphorylation after activation.

Key words: Bone; Flow cytometry; Leptin; Leptin receptor; Osteoblasts; STAT3

1. Introduction

Identification of the regulatory mechanism of osteoblast activity is a key component in understanding the process of bone formation and remodeling [1]. Recently, a novel pathway for modulating osteoblast activity has been described [2], which involves the action of leptin, a small cytokine-like product from the *ob* gene [3]. Leptin is a 16 kDa non-glycosylated protein, that is produced in adipocytes and which was originally described to be involved in appetite suppression, energy expenditure, and body weight regulation [4]. Interestingly, the recent analysis of naturally occurring leptin- or leptin receptor-deficient (OB-R) mice [2] revealed also a significant increase in bone mass, which reason could be tracked back to a drastically enhanced osteoblast activity. These findings consequently lead to the assumption that leptin might be a novel key regulatory molecule in bone formation.

Further investigations on these mutant mice clearly showed that under normal conditions leptin indeed suppressed osteoblast activity and that this hormone is necessary for the proper control of bone formation and overall bone mass. Nevertheless, its mode of action on osteoblasts is still under dispute. So far, evidences for both a central regulatory pathway using the hypothalamic relay [5] as well as for a direct action mechanism via endocrine/paracrine pathways [6] have been reported. The failure in detecting leptin receptor expression on osteoblasts as well as a non-responsiveness to exogenous leptin in these cells were regarded as strong evidences against a direct regulatory role of leptin in osteoblasts [2], so that this is currently accepted as the general working model [5]. On the other hand, there have been also reports on the isolation of leptin receptor proteins and their activation by leptin in some mouse osteoblasts, which rather support the possibility of a direct effect of leptin [6]. To clarify this issue, in the present study, mRNA were extracted from an established osteoblast cell line or from primary rat osteoblasts, which have been isolated in high purity from neonatal rat calvariae, and leptin receptor expression was determined and confirmed by reverse transcription (RT)-PCR in these cells. This observation was further confirmed by the cell surface staining with leptin receptor-specific antibodies using immunofluorescence analysis. Furthermore, incubation of rat osteoblasts with recombinant rat leptin resulted in a specific STAT3 (signal transducers and activators of transcription) phosphorylation in those cells, implicating a direct regulatory role of leptin on osteoblasts.

2. Materials and methods

2.1. Isolation of primary osteoblasts from neonatal rat calvariae

Primary osteoblasts were isolated from the calvariae of 19-day-old fetal Sprague–Dawley neonate rats by sequential enzymatic digestion as originally described by Luben et al. [7]. In brief, calvariae were aseptically dissected, minced and then incubated at 37°C for 20 min in a 5.0% CO_2 incubator with gentle shaking in an enzymatic solution containing 0.1% collagenase (Gibco BRL, Grand Island, NY, USA), 0.05% trypsin (Gibco BRL) and 4 mM EDTA (Sigma, St. Louis, MO, USA) in Hanks' balanced salt solution (Gibco BRL). This procedure was repeated five times. After cells from the first three digests were discarded, cells treated for the fourth and fifth time under same conditions were collected together and cultured in DMEM media (Gibco BRL) during the first 2 days and then changed to BGJb media (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL), in which they were maintained for 5 weeks before analysis.

2.2. Isolation of RNA and RT-PCR

Total RNA was isolated using the guanidium–thiocyanate method as originally described by Chomzynski and Sacchi [8]. In brief, osteoblasts were harvested, and the cell pellet was resuspended in an acidic guanidium–isothiocyanate solution. Protein and DNA fractions were isolated from the homogenate by acidic phenol (pH 4.0) extraction, and the upper aqueous phase was transferred into a new reaction tube to precipitate RNA by addition of 2.5 vol. absolute ethanol. Possible

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contamination of genomic DNA was removed by DNase I treatment. Purified total RNA was analyzed in a MOPS-buffered 1.5% agarose gel containing formaldehyde as denaturing agent. The integrity of the isolated RNA was then confirmed by visualization with EtBr-staining and UV illumination. For cDNA synthesis, each 1 μg of the whole RNA was taken and reverse transcribed in a 40 μl reaction using SuperScript™ II reverse transcriptase (Life Technology Inc., Grand Island, NY, USA) and oligo-dT_{15} primers (Promega, Madison, WI, USA) following the protocol as provided by the enzyme supplier. For the detection of leptin receptor isoform mRNA signals, the following combination of PCR primers was used (Fig. 1). OB-Ra (5′-aatgaagtggcttagaatcc-3′, 5′-tcaaagagtgtccgctctc-3′), OB-Rb (5′-aatgaagtggcttagaatcc-3′, 5′-agaccatagctgctggtac-3′), OB-Rc (5′-attgtaccggtaattatttcct-3′, 5′-ctgcaaccttagatatcttgg-3′), OB-Rd (5′-aatgaagtggcttagaatcc-3′, 5′-ctatctgaaaataaaaacttcat-3′), OB-Re (5′-aatgaagtggcttagaatcc-3′, 5′-gtaaaagcacagtacacatacc-3′), and OB-Rf (5′-aatgaagtggcttagaatcc-3′, 5′-ctaatttctgccaggcatt-3′). The expression of alkaline phosphatase and osteocalcin was confirmed using the following PCR primers: alkaline phosphatase (5′-tggaatatgaactggatgaga-3′, 5′-gttgttgtgagcataatccac-3′), osteocalcin (5′-tctgagtctgacaaagcctt-3′, 5′-ctaaacggtggtgccatagat-3′). For the detection of GAPDH signals, the following primer pair was used: (5′-caactccctcaagattgtcagc-3′, 5′-gggagttgctgttgaagtcaca-3′). For the detection of rat CD3 and CD11b the following primer pairs were used, respectively: rat-CD3 (5′-ctgcatccttcaagtgcag-3′, 5′-catatgcagggcgtcatag-3′), rat-CD11b (5′-cacagtacaccagaactgca-3′, 5′-atcaccagctggcttagac-3′). PCR was performed using 1 μl of the cDNA for 37 cycles under the following conditions: denaturation at 95°C for 30 s, annealing at 54°C for 45 s, elongation at 72°C for 60 s, and after the last cycle an additional elongation step for 5 min at 72°C. The RT-PCR products were then loaded in an 1.8% TAE-buffered agarose gel and analyzed after gel electrophoresis by EtBr-staining and UV light illumination.

2.3. Cloning and sequencing of PCR products

Every PCR product as amplified with the OB-R isotype-specific primers were cloned into plasmid vectors for determining the identity of each individual nucleotide sequence. For this, the PCR amplicons were loaded and separated in an 1.8% TAE-buffered agarose gel, and then eluted from the gel using the QIAquick gel extraction kit (Qiagen, Hilden, Germany) and subcloned into the pGEM-T vector (Promega). Nucleotide sequencing was performed at the Genome Research Center of the Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea, using an ABI PRISM DNA sequencing kit and ABI PRISM 377 DNA sequencer (Perkin Elmer).

2.4. Fluorescence-activated flow cytometry

Each 4×10^5 cells were used for one staining reaction. After harvest from a stationary culture, ROS 17/2.8 cells were stained with either a commercially available OB-R-specific antiserum (Research Diagnostics Inc., Flanders, NJ, USA) or the same concentration of a control goat serum in staining buffer (0.1% BSA, 0.02% Na-azide in PBS) for 30 min on ice. After washing off excess antibodies with staining buffer, specific binding of the primary antibodies was detected with FITC-conjugated anti-goat IgG secondary antibodies (DAKO A/S, Glostrup, Denmark). Cells were then analyzed in a FACScalibur flow cytometer (Becton Dickinson Flow Cytometry Systems, San Jose, CA, USA).

2.5. STAT3 immunoblot analysis

The detection of leptin-mediated signaling in osteoblasts was performed after treatment of ROS17/2.8 cells and primary osteoblasts with recombinant rat leptin [9]. STAT3 signaling was analyzed using a commercial kit (PhosphoPlus Stat3 (Tyr705) Antibody Kit #9130; New England Biolabs, Beverly, MA, USA) following the manufacturer's instruction. In brief, 1×10^5 ROS17/2.8 cells/well were seeded into a 6-well plate and cultured in DMEM (+5%FBS) for 3 days before analysis. Culture media was then changed into DMEM media without serum for inducing serum starvation 3 h before leptin treatment. Purified recombinant leptin (400 ng/4 ml/well) was then added to the cells, and after each 0, 3, 10, 15, 30, 60 min incubation, cells were harvested by lifting the media and by adding 150 μl SDS sample buffer to scrape the cells off the plate. The cell suspension was then heated at 100°C for 7 min, and a 20 μl aliquot was loaded and separated in a reducing 12.5% SDS–PAGE. Proteins were then transferred from the gel by electroblot onto a nitrocellulose membrane (Amersham-Pharmacia, Uppsala, Sweden), and blocked with 3% skim milk (Sigma) in PBS at 4°C overnight under continuous shaking. Next day, phosphorylated STAT3 was detected using phospho-STAT3-specific antibodies (Tyr705) diluted 1:1000 in TBS/0.1% Tween 20 buffer by incubation for 2 h at room temperature. Non-specific binding was washed out with TBS/0.1% Tween 20, and specifically bound antibodies were detected with horseradish peroxidase-conjugated anti-rabbit immunoglobulin antibodies. The same procedure was applied for detection of STAT3 using STAT3-specific antibodies. Finally, protein bands were visualized by a commercial chemiluminescence kit (New England Biolabs). Detection of leptin-mediated STAT phosphorylation in primary osteoblasts was performed essentially under the same experimental conditions.

3. Results and discussion

To address the central question about leptin receptor expression in osteoblasts, two different target cells were used for analysis. The ROS17/2.8 is an established end-differentiated osteoblast cell line, which has been extensively characterized [10] and which has been used in various studies to investigate the mechanism of bone formation. While this cell line is clearly the first choice for analyzing leptin effect on osteoblasts, on the other hand, it is also arguable that cell lines sometimes do not exactly represent the situation in vivo, which led to the further analysis of also primary osteoblasts as isolated from neonatal rat calvariae. In contrast to the ROS17/2.8 cells, primary osteoblasts had to be freshly isolated for each individual experiment, and isolation processes and cell culture of these cells are described in details in Section 2. The identity of the isolated osteoblasts was confirmed among others by the specific detection of osteocalcin [11] and alkaline phosphatase [12] mRNA expression (Fig. 2), which are classical osteoblast-specific marker genes. Microscopic analysis on the cell morphology also confirmed the identity of the isolated cells (data not shown). After proving the successful isolation of osteoblasts, RT-PCR was performed to analyze the expression of leptin receptor isoforms in both ROS17/2.8 and primary osteoblasts, for which six sets

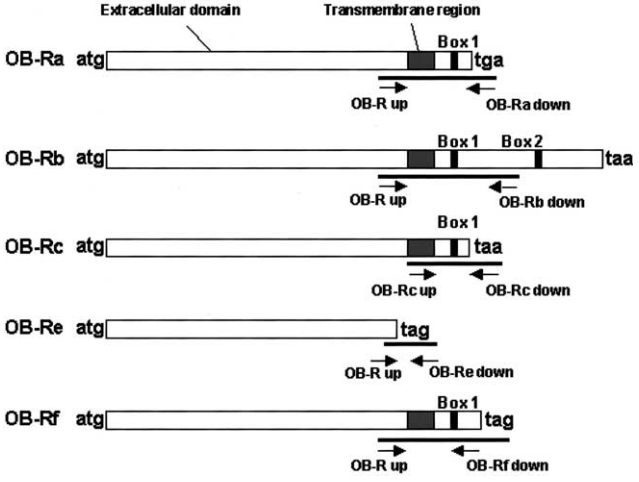

Fig. 1. Schematic presentation of rat leptin receptor isoforms. So far, six isoforms of the leptin receptor have been reported, which comprise the OB-Ra, -Rb, -Rc, -Rd, -Re, and -Rf isoforms. Discrimination of the isoform mRNA is possible due to the unique C-terminal variance between each receptor isotype. The position of the oligonucleotide primers as used for the specific detection of each receptor isoforms are indicated in arrows below each receptor isoforms. Their sequences are listed in Section 2.

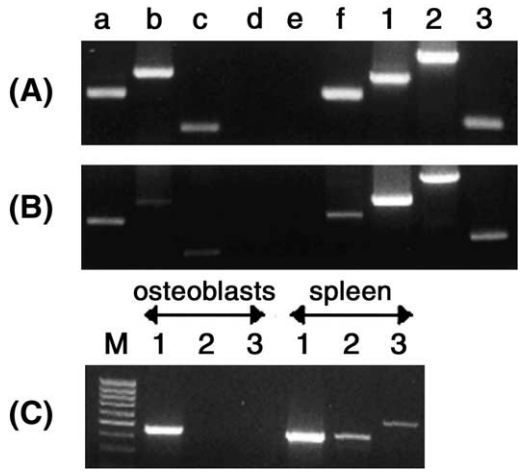

Fig. 2. Determination of leptin receptor isoform expression in primary rat osteoblasts. Using OB-R isoform-specific primers, the expressions of leptin receptor isoforms were determined in (A) primary rat osteoblasts, or (B) in the rat osteoblast cell line ROS 17/2.8 using RT-PCR. a, OB-Ra; b, OB-Rb; c, OB-Rc; d, OB-Rd; e, OB-Re; f, OB-Rf; 1, GAPDH; 2, alkaline phosphatase; 3, osteocalcin. C: To confirm the purity of the isolated primary osteoblasts, RT-PCR was performed with either primary osteoblasts RNA or rat whole spleen RNA. M, molecular weight marker lane; 1, GAPDH; 2, CD3; 3, CD11b.

of primer pairs, corresponding to the six isoforms of rat leptin receptors, were used. The positions of the primers are shown in Fig. 1, where all the differentially spliced leptin receptor (OB-R) isoforms as reported to date are schematically displayed.

Fig. 2 shows the result of this experiment. The identities of the amplified PCR products were confirmed by molecular cloning and subsequent nucleotide sequencing analysis of every individual clone upon their correctness (data not shown) so that the possibility of PCR artifacts could be excluded. Also a possible contaminating signal from genomic DNA can be excluded since control PCR with cDNA reactions without addition of reverse transcriptase showed no specific signals (data not shown). When using cDNA from primary osteoblasts (Fig. 2A) for RT-PCR, four among the six reported OB-R isoforms were detected, which are the OB-Ra, -Rb, -Rc, and the -Rf receptors. No signal for the OB-Rd or -Re isoforms was detectable in the RT-PCR. Judging from these results, it can be assumed that four different isoforms of leptin receptors are expressed in the osteoblast cells, and, based on the mRNA signal strength, all four isoforms must be of a similar expression level whereas the OB-Rf form seems to be the dominant one. The exact functional role of the OB-Rf isoforms is still not clear so far. However its detection in osteoblasts, as shown in this study, is in concordance with its broad tissue distribution [13] which includes testis, adrenal gland, and brain, etc.

Interestingly, high-level expression of both the OB-Ra and -Rb receptors were detected by RT-PCR, which was also observed in ROS17/2.8 rat osteoblast cells (Fig. 2B). Indeed both cell types showed the same patterns of OB-R isoform expression, indicating that this expression profile is characteristic for osteoblast cells. The rather low level of mRNA expression in ROS17/2.8 cells in comparison to primary osteoblasts might have been resulted due to repetitive passages of the cell line or would represent the end-differentiated state of ROS17/2.8 cells. Furthermore, a possible contamination of macrophages or T-lymphocytes as a source of OB-R mRNA in the primary osteoblast preparation could not account for the present observation, since RT-PCR for macrophage-specific genes (CD11b) or T-cells specific markers (CD3) failed to amplify specific PCR products (Fig. 2C) from the same RNA preparation as used in Fig. 2A.

This present observation on OB-Ra and OB-Rb expression in osteoblasts has a large impact on understanding the biological mechanism of leptin-mediated modulation of bone forming activity. Among the six OB-R isoforms, only the OB-Ra and OB-Rb are able to mediate leptin-induced signal transduction into the cell [14]. Furthermore, only the OB-Rb isoform is reported to contain the potential Janus kinase (JAK) binding domains, box 1 and box 2 (see Fig. 1), and a potential consensus sequence (YXXQ) for the STAT binding in its cytoplasmic domain. These characteristics have led to the assumption that OB-Rb is the major signal-transducing unit, and all prior analyses on leptin receptor expression in osteoblasts have been focused on the expression of this receptor isotype [2,6]. Nevertheless, reports on OB-Rb expression in osteoblast have been contradictory, leading to a dispute upon either an indirect or direct role of leptin on osteoblasts. The current observation of signal-transduction capable leptin receptor (OB-Ra as well as -Rb) expression in osteoblasts thus implies a direct regulatory role of leptin in bone morphogenesis and this is also in accordance to a recent study by Steppan et al. [6], who were able to detect OB-Rb expression in mouse osteoblasts. The reason why others [2] were not able to detect OB-Rb expression in primary osteoblasts remains elusive. However regarding the fact that tissue-specific expression of OB-Rb has been disputed also in other organs, it might be assumed that detection of OB-Rb expression is largely dependent on the experimental procedure.

Indeed, there have been also some controversies regarding the presence of OB-Rb, for example in the kidney, where some investigators could [15] or could not [16] detect OB-Rb expression. Also, both the absence and presence of OB-Rb expression in muscle tissues have been reported by different groups [15–17] as well as are there controversies in other tissues including the adrenal gland, spleen, etc. It has been proposed that the reason for such inconsistencies might be due to a sensitivity problem of the applied detection methods or either due to differential physical or nutritional state of the laboratory animals. In the latter case, different conditions might directly affect the serum leptin level and this in turn would modulate leptin receptor level resulting in the differences of the read-out.

The next question in the present study regarded whether leptin receptor mRNA products as detected by RT-PCR are indeed expressed and transported to the cell surface. To address this issue, indirect immunofluorescence analysis was performed on osteoblasts cells using leptin receptor-specific antibodies, which are directed to the 32–51 amino acid region at the amino terminus of the mouse leptin receptor. This commercially available antibody (Research Diagnostic Inc.) has been proved on prior occasions to specifically interact also rat leptin receptors ([18] and communication from manufacturer). Fig. 3 shows the result from flow cytometric analysis, where ROS17/2.8 cells were first stained with these leptin receptor-specific antibodies and then the specific stainings were visualized by fluorescence (FITC)-conjugated anti-goat immu-

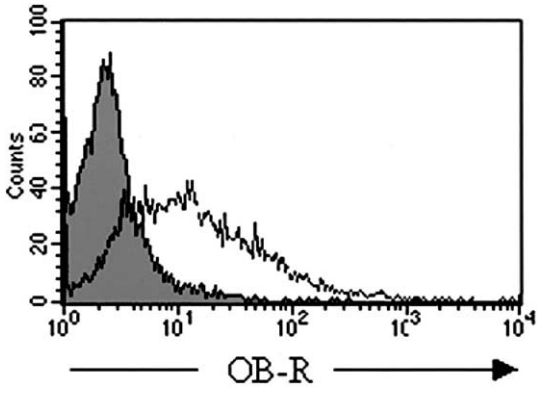

Fig. 3. Flow cytometric analysis of OB-R expression in rat osteoblasts. Using commercially available leptin receptor-specific antibodies, the cell surface expression of leptin receptor in ROS 17/2.8 osteoblasts was analyzed. The FACS analysis data is a representative result from five independently performed experiments. Indirect immunofluorescence with FITC-conjugated rabbit anti-goat antibodies as secondary antibodies enabled the detection of specifically bound primary antibodies. Open histogram indicates the distribution of the relative fluorescence intensity of anti-OB-R staining, while filled histogram shows the staining with a control antibody.

noglobulin secondary antibodies. Here it is to see that the log fluorescence of osteoblasts stained with the anti-leptin receptor antibodies (open histogram) drastically shifts to the right in the *x*-axis indicating a high and specific staining of the cells by this antibody. On the other hand, the same osteoblasts stained with a control goat antibody (specific for an irrelevant human immunodeficiency virus envelope protein) showed no increased fluorescence signals (filled histogram). Upon confirmation of the cell surface expression of leptin receptors, the leading question was whether they would be also functional for leptin-mediated signal transduction. It is currently accepted that ligand-binding to the leptin receptor induces the expression of several genes including the *socs*-3 (suppressor-of-cytokine-signaling), and c-*fos* as well as activates some other intracellular signaling components such as STAT, phosphatidylinositol 3-kinase [19] and MAPK (ERK) activity [20]. The determination of induction of either such target genes or activation of downstream signaling pathways would be a good proof for the activity of ligand-bound leptin receptors. In this regard, the current study focused on a possible activation of down-stream components in the leptin receptor signaling, which would prove the functionality of these receptors.

Specific phosphorylation of STAT proved to be the most direct and affirmative way in detecting leptin receptor signaling [14]. However, while several different STAT isoforms have been reported to be activated by leptin, so far only STAT3 has been proved to be active after leptin-mediated signaling in the hypothalamus, the major action site of leptin. This then lead to the suggestion that STAT3 might be the most crucial component in leptin receptor signaling [20], and that detection of STAT3 phosphorylation would serve as the conclusive proof for the direct leptin activity in osteoblasts as aimed in the current study. For activating ROS17/2.8 rat osteoblasts, recombinant rat leptin was used which had been expressed in *E. coli* [9], and which biological activity had been previously confirmed by an in vitro assay. Since leptin receptor-transfected cells showed a saturation of leptin-mediated signaling after a concentration of 20 ng/ml using this recombinant protein [18], in the present study, osteoblasts were stimulated with leptin to a final concentration of 100 ng/ml to ensure a successful binding of leptin to their cognate receptors. As shown in Fig. 4A, leptin treatment indeed initiated a strictly time-dependent phosphorylation of STAT3 indicating that the JAK-mediated signaling to STAT3 activation was intact and functional. While no densitometric analysis of the signal intensity has been performed, it is clear to see that STAT3 phosphorylation is induced by leptin treatment and that this signal peaks after 30 min. Cross-reactivity of bovine leptin derived from culture media serum can be largely excluded since the cells have been prior starved under serum-devoid conditions, and since cells without exogenous leptin treatment showed no increase in phospho-STAT3 signals. The same observation was confirmed when using primary osteoblasts (Fig. 4B), where the incubation with leptin also induced STAT3 phosphorylation with a similar kinetic as ROS17/2.8 cells. Thus, it is concluded that both osteoblast cell lines as well as primary osteoblasts are able to respond to exogenous leptin, and that there are indeed signal transduction-capable receptors for leptin are expressed on those cells.

After determining the leptin-mediated signal transduction in osteoblasts, it is now of course of great interest, which genes would be down-stream to the STAT3 signal in these cells. It is our belief that such information will provide further clues on

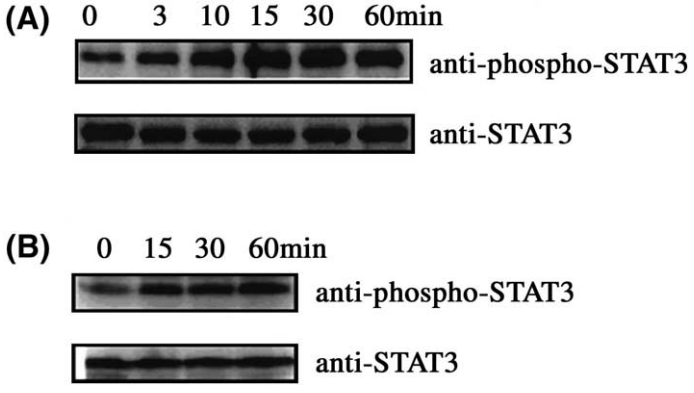

Fig. 4. Determination of leptin-mediated cell signaling in ROS 17/2.8 cells and primary osteoblasts. Analysis of STAT3 phosphorylation in leptin stimulated rat osteoblasts. A: Recombinant leptin-treated ROS 17/2.8 cells were harvested in a time-dependent manner, and the cell lysate was probed in an immunoblot with either STAT3 or phosphorylated STAT3-specific antibodies. B: Same experiment was performed with primary osteoblasts which have been cultured for 2 weeks after isolation from neonatal rat calvariae.

the actual role of leptin in bone remodeling as well as is it expected to help the further identification of genes directly involved in this process, which will be the subject of our next studies.

In conclusion, the current study shows the physical presence and cell surface expression of leptin receptors in osteoblasts and thereby suggests a direct regulatory role of leptin in the bone formation processes. Furthermore, the phosphorylation of STAT3 by leptin treatment indicated that the expressed leptin receptors are functionally active and that they successfully mediate signal transduction into the cytoplasm. While the possibility of an alternative mechanism of a central endocrine or neuroendocrine pathway using a hypothalamic relay can not be excluded [2,6], it is evident that the current study rather supports the postulation on a direct effect of leptin on bone growth as also suggested by others [6,21].

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