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Ann-Christin Kappfjell

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Veileder
Yngve Figenschau,
Klinisk Kjemi, UNN

Human Auricular Chondrocytes Express Functional Leptin Receptors

Ann-Christin Kappfjell¹, Signy Bendiksen², Baldur Sveinbjörnsson³ Ole Vik⁴, Yngve Figenschau^{1,5}

¹Department of Clinical Chemistry, University Hospital of Northern Norway, N-9038 Norway. ²Department of Molecular Genetics, University of Tromsø, N-9037 Norway.

³Department of Experimental Pathology, University of Tromsø, N-9037 Norway.

⁴Department of Ear, Nose, and Throat Diseases, University Hospital of Northern Norway, N-9038 Norway.

⁵Address correspondence to:

Yngve Figenschau, MD, PhD
Dept. of Clinical Chemistry
9038 UNN, Tromsø
Norway

eMail: yngve.figenschau@unn.no
Fax: +(47) 77626711
Tel: +(47) 77676624

Abstract.

Leptin is a hormone produced in adipose tissue by adipocytes. Its serum level correlates with the fat storage in the body and hence reflects the size of fat deposits. The effects of leptin are mediated by targeting cells directly, and by interactions with several physiological regulatory systems, such as the cytokine network. The leptin receptor is a member of the hematopoietin receptor family, and its signalling pathway resembles that induced by cytokines of this class. Several splice variants of the leptin receptor have been found, but only one isoform is considered functional, i.e. the one possessing a long cytoplasmatic tail named Ob-R_L. We demonstrated that human auricular chondrocytes in native cartilage possess leptin receptors (Ob-R), and that these receptors also were present on serially cultured cells. We demonstrated that the cells expressed mRNA encoding the functional receptor Ob-RL. We have previously shown that leptin promotes cell proliferation and matrix synthesis in articular hyaline cartilage, i.e collagen and proteoglycan synthesis. However, the biological function of the receptor in auricular cartilage was not investigated here, but provided that auricular chondrocytes express functional receptors, leptin could affect these cells similarly as articular cartilage

INTRODUCTION

Human leptin is a 16 kDa non-glycosylated polypeptide constituted of 146 amino acid residues. Leptin regulates food intake and energy expenditure, mainly by a mechanism involving hypothalamic neuronal receptors (1). The levels of leptin in serum correlate directly with adipose tissue mass, and reflects the amount of fat in the body (2). Leptin affects the release of a neurotransmitter, neuropeptide Y, from hypothalamic neurons by negative feedback. When adipocytes shrink, less leptin is secreted into the circulation, which in turn boosts the release of neuropeptide Y, resulting in an increased appetite (3). Simultaneously, less α -melanocyte-stimulating hormone (α -MSH), a hormone which blocks the feeling of hunger, is released. Hence, both hunger and appetite are increased as a result of reduced serum level of leptin (4).

The human leptin receptor is a 150 kDa type I transmembrane glycoprotein constituted of 1144 amino acid residues. Several alternatively spliced forms of the leptin receptor (Ob-R) have been identified: Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, and Ob-Re. Only the long isoform named OB-R_L or Ob-Rb, which possess a long cytoplasmatic tail of approximately 300 amino acid residue, has been considered functional. Upon ligand binding, the functional leptin receptor activates intracellular signal transduction which involves Janus kinases (JAK's), whereas the other receptors do not (5). JAK's are a group of tyrosine kinases, which phosphorylate and activate signal transducers and activators of transcription (STAT's), namely STAT1, STAT3 and STAT5 (6, 7, 8). However, it has been suggested that leptin binding to a shortened form of the mouse receptor, OB-Ra (equivalent to OB-RS1 in humans), may initiate gene transcription (9).

The leptin receptor is classified as a class I cytokine receptor which comprises a family of receptors that mediates effects of more than 20 different cytokines. Its sequence is most

similar to the α -chain of leukemia inhibiting factor (LIF) receptor, granulocyte colony stimulating factor (G-CSF) receptor, and to the gp130 signal-transducing component of interleukin (IL)-6 receptor (5). Hence, due to the structural and functional similarities to cytokines, leptin is considered a cytokine-like hormone.

Leptin, encoded by the obese gene (*ob*), is produced by adipocytes, mainly in the white adipose tissue type (10). The expression of functional receptors in peripheral cells, tissues and organs, indicates that leptin affects a variety of cell types (11). Mice deficient in leptin production exhibit hyperglycemia, hyperinsulinemia, hyperphagia, obesity, infertility, decreased brain size and decreased stature. After leptin administration, these mice achieved a significant increase in bone mineral content and bone density, femoral length and total body bone area, as compared to that of vehicle treated controls (12). Bone mass is positively correlated to body fat (13,14), and there is a positive correlation between bone mass and serum levels of leptin in humans (15). Functional leptin receptors have been identified on primary adult osteoblasts (12), and it has been shown that leptin inhibits generation of osteoclast.

Previously, we demonstrated that human chondrocytes in native hyaline cartilage taken from human knee-joints express functional leptin receptor, as did the cultured cells (16). The elastic cartilage of the human auricle is not a part of the skeletal structure, neither has it a weight bearing function. It appears that hyaline cartilage subjected to weight bearing strain can be affected by hormonal signalling from adipocytes directly, yet it is unknown whether auricular chondrocytes, exhibiting a phenotype different from articular hyaline cartilage, is subjected to leptin regulation. The aim of this study was to identify leptin receptors on auricular cartilage, and to clarify whether the leptin receptors were the functional type.

MATERIALS AND METHODS

Isolation and cultivation of chondrocytes

Human auricular cartilage was obtained from two patients subjected to auricular plastic surgery. They were aged < 20 years, and participated by informed consent. The biopsies, weighing approximately 100 mg, were transferred to sterile 50 ml tubes (cat. no 373687, Nunc, Roskilde, Denmark) containing 10 ml cultivation medium. Thereafter, the cartilage was inspected in an inverted microscope, and part of the tissue was subjected to immunohistochemistry, while the remaining tissue was cut into 1mm³ pieces in a sterile petri-dish using a scalpel. The cartilage was digested for 18 hours (h) in a vessel (cat. no. 3108, Falcon, Becton Dickinson Labware, NJ, USA) containing 5 ml cultivation medium supplemented with 0,8 mg/ml collagenase (C-9407, Sigma, St.Louis, MO, USA) and 1,0 mg/ml DNase (Sigma, cat. no. D-5025). Subsequently, the content in the vessel was transferred to a sterile tube and centrifuged at 250 x gravity (G) for 5 minutes (min), which was repeated twice with medium replacement. The pellet was then resuspended in 5 ml cultivation medium supplemented with 20 % human serum and seeded in a cultivation vessel (cat. no. 3108, Falcon).

After a cultivation period of 4 days, at 37°C in humidified air containing 5% CO₂, the medium was exchanged, and further cultivation was carried out using 10% serum. To remove remnants of digested tissue, the adherent cells were washed prior to first medium exchange with 5 ml warm phosphate buffered saline (PBS, cat. no. 14190185, Gibco). The culture was inspected regularly, and the cells were transferred into larger vessels (cat. no. 3110, Falcon) before they reached confluence, as judged by microscopy. The culture was washed with warm PBS, and subsequently a trypsin solution was added (cat. no. 25300-054, Gibco). The cells detached after typically 5 min, then fresh media was added, and the suspension was

transferred to a tube that was centrifuged at 250G for 5 min. The pellet was resuspended in fresh medium, and centrifugation was repeated twice. Further culture expansion was performed by transferring cells to larger culture vessels (cat. no. 353112, Falcon). Cells were used in experiments when an appropriate number of cells was obtained.

Human serum

The human serum used in our experiments was collected from healthy volunteer donors. Blood was collected by vein puncture into a plain vacuum tube (BD Vacutainer™, Puls, Oslo, Norway). After at least four hours in room temperature, the vacuum tubes were centrifuged at 1100G for 10 min, and the resulting serum was filtered (0,22 µm, cat. no. SCHA02OS, Millipore, Bedford, MA, USA), and thereafter added to the medium.

Culture medium

The medium used was DME/F12 (cat. no. 31330-38, Gibco, Paisley, Scotland) supplemented with 50 µg/ml ascorbic acid (cat. no. A-4034, Sigma), and 50 µg/ml gentamycin (cat. no. G-1264, Sigma), and 2,5 µg/ml amphotericin (cat. no. A-9528, Sigma).

RT-PCR.

mRNA from chondrocytes was extracted with Qiagen RNeasy total RNA kit (cat. no. 74104, Qiagen, VWR International, Oslo, Norway). cDNA was synthesised by using OneStep RT-PCR kit (cat. no. 210210, Qiagen). Initially a 50 µl reaction mixture containing 0.6 µM of PCR primers was heated in a thermal cycler to 50 °C for 30 min to complete reverse transcription, thereafter the HotStar Taq DNA polymerase was activated by heating the mixture to 95 °C for 15 min. A 3-step cycling was performed at 94 °C for 1 min (denaturation), 55 °C for 1 min (annealing), and 72 °C for 1 min (extension). 35 cycles were

used with a 10 min final extension at 72 °C. PCR products were analyzed by agarose gel (2%) electrophoresis and photographed under UV-light.

Nucleotide sequences of PCR primers were designed to detect intracellular and extracellular domains of the functional leptin receptor (20), and these were respectively: Exon 3 (primer1), 5'-CCTTTTCCCAGGTGTA CTTCTCTG-3' (sense), and (primer 2) 5'-CACCATCCAGGTTGTCTTTAGGAG-3' (antisense); a 285-bp fragment was expected. Exon 20 (primer 3), 5'-GTGGTCCTCTTCTTTGGAGCC-3' (sense), and (primer 4), 5'-AGCCCTTGTTCTTCACCAGTTTC-3' (antisense); a 289bp fragment was expected. To test the quality of the mRNA, the presence of APRT-gene transcripts was assayed. For APRT-PCR (annealing temperature 61°C) the following primers were used: 5'-CCCGAGGCTTCCTCTTTGGC-3' (sense), and 5'-CTCCCTGCCCTTAAGCGAGG-3' (antisense), corresponding to the sequences 1940 to 1959 in exon 3, and to the sequences 2725 to 2744 in exon 5 of the adenine phosphoribosyl transferase (APTR) gene, (gene bank accession number Y00486). Contaminating DNA will generate a fragment of 800 bp, while mRNA will generate a 300 bp fragment. All reactions were run using a GeneAmp 2400 thermocycler (Perkin Elmer, Cambridge, UK).

Immunocytochemistry/Immunohistochemistry.

To identify leptin receptor on serially cultured human auricular chondrocytes, we used monoclonal mouse anti-human leptin receptor antibody (cat. no. MAB867, R&D Systems, Minneapolis, MN, USA). Cells were grown on fibronectin coated chamber slides (cat. no. 177402, Nunc) for 24h. Cultures were then washed twice with PBS (phosphate buffered saline) and fixed for 4h in cold PBS containing 0,2 M sucrose and 4% paraformaldehyde. After washing twice with PBS, the slides were incubated for 15 min in a 1% albumin solution (bovine albumin in PBS) allowing to block unspecific protein binding. Subsequently, primary

antibody diluted 1:250 in 1% albumin solution was incubated for 20 min in room temperature. After rinsing three times in PBS, the slides were incubated for 30 min in room temperature with secondary antibody (biotin-rabbit anti-mouse IgG, Zymed, San Francisco, CA, USA) that was diluted 1:200 in a 1% albumin solution. After rinsing twice in PBS, FITC-conjugated streptavidin (Zymed) diluted 1:500 in 1 % albumin solution was added and 20 min incubation was allowed. Negative controls were arranged by omitting the primary antibody in this procedure. The labelled cell cultures were examined in a Zeiss Axiophot photomicroscope equipped with phase contrast and incident-light fluorescence optics (Carl Zeiss, Oberkochen, Germany), and micrographs were taken with a Nikon Coolpix 995 digital camera.

To study whether leptin receptor was present in situ, we used sections of a cartilage taken from biopsies that were used to establish cell cultures. Paraformaldehyde (4%) containing 0.2 M sucrose in PBS was used as fixative, and after 48h the biopsy was embedded in paraffin and sectioned at 5 μ m thickness onto poly-L-lysine (0.01%, Sigma) coated slides. Sections were deparaffinized by graded alcohol and xylene washes, and immersed in distilled water, following an incubation in 1% albumin solution for 30 min. Addition of monoclonal antibody (diluted 1:100) overnight at 4 °C was allowed. After rinsing twice in PBS, sections were incubated for 45 min with secondary biotinylated antibody, and subsequently for 2h incubated with FITC-conjugated streptavidin. Micrographs were taken with a Nikon Coolpix 995 digital camera.

RESULTS

Immunohistochemistry/Immunocytochemistry.

Immunohistochemistry was performed to identify leptin receptors on chondrocytes in native cartilage. In presence of primary antibody, the sections showed brightly stained as

compared to the unlabelled controls. This indicates that human auricular chondrocytes express leptin receptor *in vivo* (Figure 1A and 1B).

Similarly, cultivated auricular chondrocytes stained well in presence of primary antibody in contrast to that of the controls (Figure 2A and 2B). This suggests that the leptin receptors are present on serially cultured human auricular chondrocytes.

RT-PCR.

RT-PCR analysis was used to investigate whether chondrocytes synthesise the functional isoform of leptin receptor. The PCR products, i.e cDNA, visualised by gel electrophoresis indicated that mRNAs corresponding to 285bp (Exon 3) and 289bp (Exon 20) were present (Figure 3, lanes 2 and 5). These primers were designed to detect exons coding for the extracellular and intracellular domains of the functional leptin receptor, respectively. Lanes reflecting runs without primers present, i.e. negative controls (Fig. 3, lanes 3 and 6), were negative. The products generated using APRT-primers indicated that no genomic contamination was present (Figure 3, lane 8). This suggests that human auricular chondrocytes synthesise functional leptin receptor.

DISCUSSION

The human obesity gene (*ob*) was identified in 1994 by positional cloning, followed by identification of the structures of both the encoded product (leptin) and its receptor (Ob-R) (1, 17). Leptin was initially considered a satiety factor primarily regulating food intake and energy balance. This assumption was based on the appearance and behaviour of animals deficient in leptin production (*ob/ob* mice), and leptin receptor deficient animals such as fatty Zucker rat *fa/fa* and *db/db* mice. However, reports suggest that leptin has a multitude of other

effects mediated by interaction with several physiological regulatory systems, including the cytokine network, and by targeting cells directly. Functional leptin receptors have been identified in several organs, tissues and cell types such as platelets, hematopoietic progenitors (CD34⁺-cells), lymphocytes, bone marrow stromal cells, adipocytes, osteoblasts and articular chondrocytes (11,12,16).

Studies of animals exhibiting impaired leptin production or receptor function have recognised leptin as a regulator of bone generation and remodelling. In mice, functional receptors have been found in skeletal growth centres using a mouse model for endochondral ossification (19). In humans, functional leptin receptors have been identified on primary adult osteoblasts (12), and on articular chondrocytes (16), indicating that leptin affect cartilage and bone generation directly, although it has been suggested that leptin signals to a central relay (20, 21), i.e. hypothalamus, which in turn affects the homeostasis of skeletal structure (Ducy, 2000).

The leptin receptor is a member of the class I cytokine receptor family which utilises the JAK-STAT signalling pathway, as do other members of this receptor family (22, 23). The receptor is a tyrosine kinase which upon ligand binding autophosphorylate and activate receptor associated Janus kinases, which in turn phosphorylate STAT1, STAT3 and STAT5 (6,7,8). STATs constitute a group of DNA binding proteins regulating DNA transcription, hence leptin activation leads to upregulation of DNA transcription. STAT 3 appears to be the signal transducer in hypothalamus in mice (24), whereas leptin failed to activate STAT3 in primary osteoblasts (21). We have previously demonstrated that leptin triggers STAT1 and STAT5 activation in articular chondrocytes, but we could neither demonstrate STAT1 nor STAT5 activity in auricular chondrocytes upon leptin stimulation. However, we are continuing this work in attempt to demonstrate STAT activation.

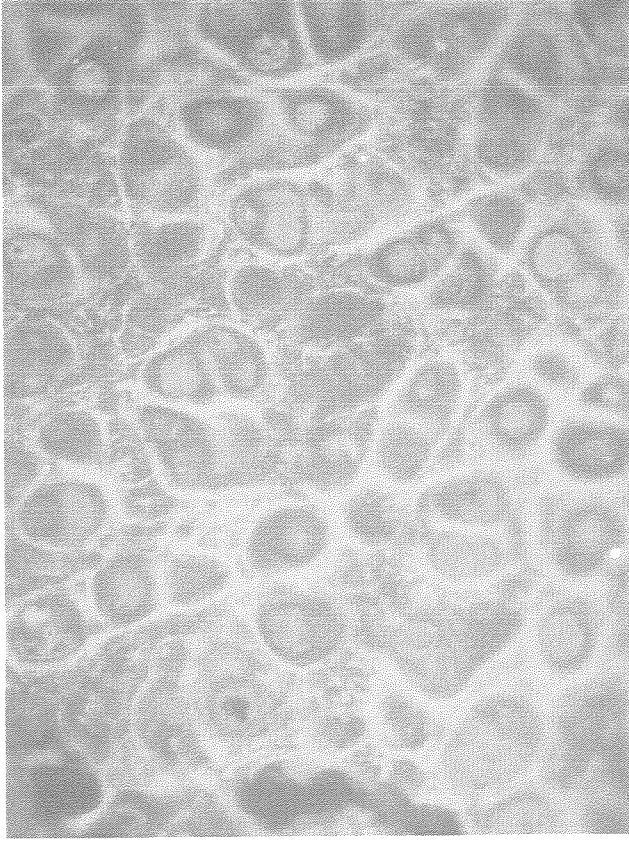
Despite the close relationship regarding their mesenchymal origin, articular and auricular chondrocytes exhibit different phenotypes, which is evident by the by the different organisation of the tissues. The former produces a rigid hyaline cartilage, whereas the latter synthesise an elastic tissue type. In addition to the morfological difference, the physiological function is various. Elastic auricular cartilage is in contrast to hyaline cartilage in knee-joints, not a part of the skeletal structure and has no weight bearing function. One theory is that mechanical strain entail leptin signalling from adipocytes to chondrocytes, e.g. enhanced matrix synthesis to compensate excessive tear force imposed by higher body mass index. Hence, in this sense the biological interpretation of the presence of functional leptin receptors on auricular chondrocytes is obscure. Due to restricted availability of auricular tissue, we were prevented from studying potential leptin mediated biological effects. On the other hand, several studies have demonstrated that leptin has a proliferativ effect on many cell types, such as hematopoeitic multilineage progenitors (25), gastric mucosa cells (26), pancreatic β -cells (27), T- lymphocytes (28), and tracheal epithelial cells (29). This parallels our previous findings using articular chondrocytes. We observed an augmented matrix synthesis as a result of leptin action. However, extracellular matrix is subjected to continuous synthesis and breakdown. Leptin most likely affects this turnover in auricular cartilage, and regulates homeostasis in auricular as well in articular cartilage. Provided a regulatory mechanism affecting proliferation only, a change in size and shape of the auricles would be expected paralleling an increased body mass index.

In summary, the present study on human auricular chondrocytes demonstrates presence of leptin receptors both *in vivo* and *in vitro*. Furthermore, RT-PCR analysis demonstrated that cultured cells contained mRNA coding the functional isoform of the leptin receptor. We could not show STAT phosphorylation as a result of leptin challenge in auricular chondrocytes. Consequently we could not prove that leptin stimulation elicits intracellular signalling

cascades resulting in DNA transcription. The role of functional leptin receptors in the human auricle remains obscure, and work will continue after this rapport to reveal the role of leptin in auricular cartilage generation and regeneration.

Histochemistry

A



B

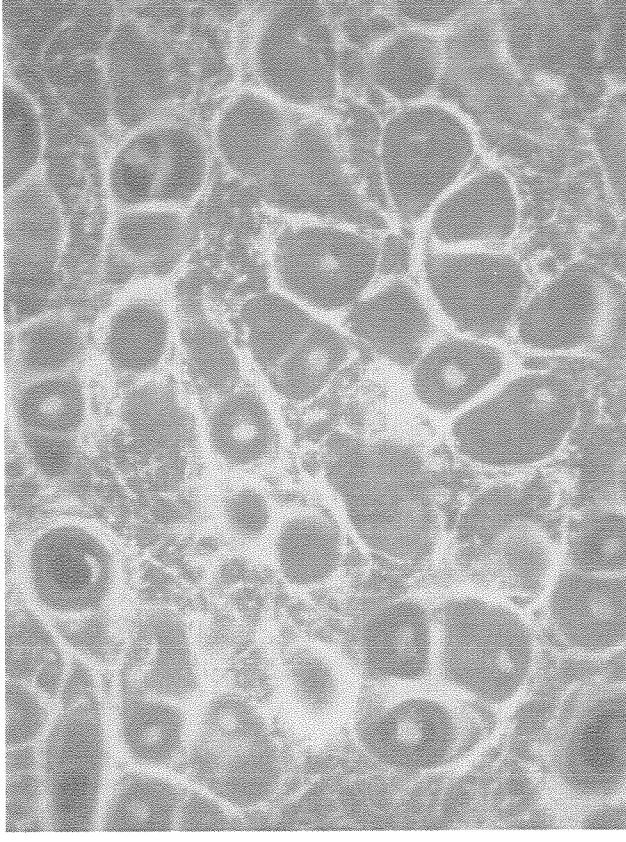


Fig. 1. Section of human auricular cartilage stained with monoclonal mouse anti-human leptin receptor antibody (A), and a negative control (B) that had no primary antibody. The chondrocytes are localised in lacunas within the cartilage, and several brightly stained cells can be seen in section A. Magnification is 400x.

Cytochemistry

A



B

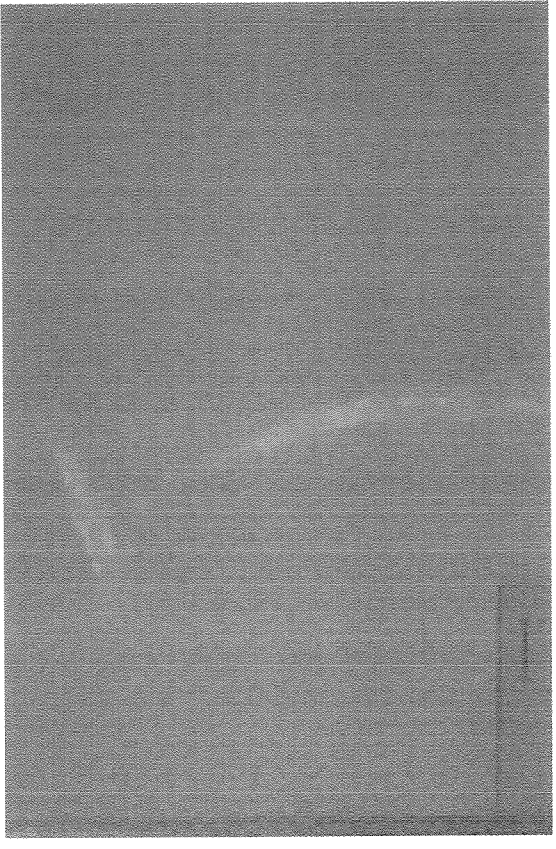


Fig. 2. Micrographs of cultured human auricular chondrocytes stained with monoclonal mouse anti-human leptin receptor antibody (primary antibody). Chondrocytes were brightly labelled in presence of antibody. The negative control that had no primary antibody was unlabelled (not shown). Magnification is 400x.

PCR

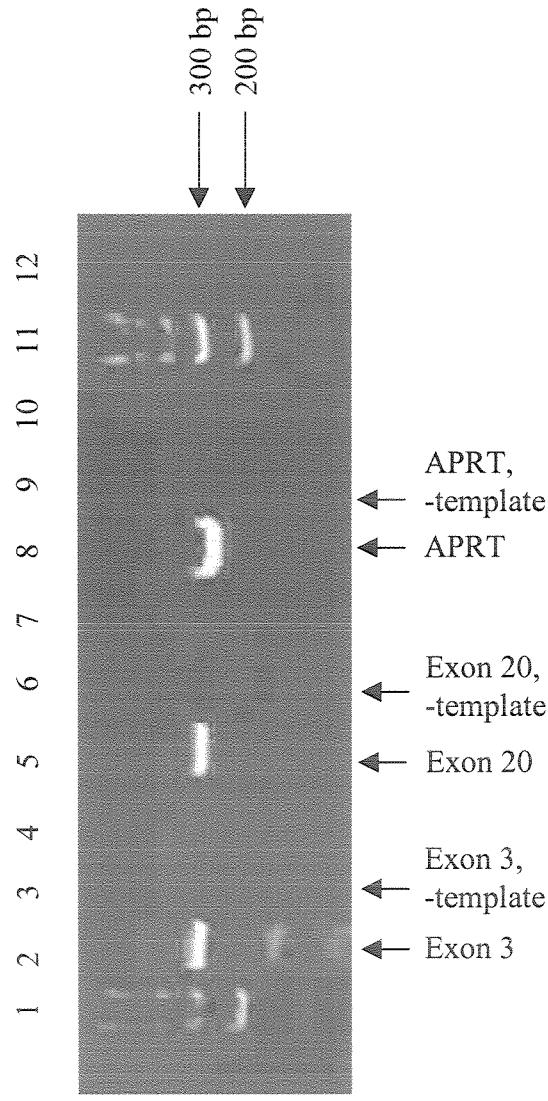


Fig. 3. RT-PCR analysis was performed to detect expression of the functional leptin receptor in cultured human auricular chondrocytes. PCR products occurred in lanes 2 and 5 reflecting runs with primers used to detect exon 3 (285) and 20 (289 bp) that code the extracellular and intracellular domain of the functional leptin receptor, respectively. Lanes and lane 6 reflect runs without the respective primers (negative control). APRT primers resulted in a 300bp product as expected when no genomic contamination is present.

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