# Silencing of OB-RGRP in mouse hypothalamic arcuate nucleus increases leptin receptor signaling and prevents diet-induced obesity

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**Obesity is a major public health problem and is often associated with type 2 diabetes mellitus, cardiovascular disease, and metabolic syndrome. Leptin is the crucial adipostatic hormone that controls food intake and body weight through the activation of specific leptin receptors (OB-R) in the hypothalamic arcuate nucleus (ARC). However, in most obese patients, high circulating levels of leptin fail to bring about weight loss. The prevention of this "leptin resistance" is a major goal for obesity research. We report here a successful prevention of diet-induced obesity (DIO) by silencing a negative regulator of OB-R function, the OB-R gene-related protein (OB-RGRP), whose transcript is genetically linked to the OB-R transcript. We provide *in vitro* evidence that OB-RGRP controls OB-R function by negatively regulating its cell surface expression. In the DIO mouse model, obesity was prevented by silencing OB-RGRP through stereotactic injection of a lentiviral vector encoding a shRNA directed against OB-RGRP in the ARC. This work demonstrates that OB-RGRP is a potential target for obesity treatment. Indeed, regulators of the receptor could be more appropriate targets than the receptor itself. This finding could serve as the basis for an approach to identifying potential new therapeutic targets for a variety of diseases, including obesity.**

leptin receptor overlapping transcript | leptin resistance | gene therapy | receptor trafficking | metabolic syndrome

Leptin and its receptor (OB-R) were initially identified and characterized because of their involvement in the regulation of energy balance, metabolism, and neuroendocrine responses to food intake (1). Subsequently, leptin has also been shown to be important in wound healing (2), angiogenesis (3), and bone mass and immune system regulation (4, 5). OB-R belongs to the class I cytokine receptor family, which typically uses the JAK/STAT signaling pathway (6). The human OB-R gene on chromosome 1 generates multiple transcripts. Several of these transcripts encode at least five OB-R isoforms, including the short OB-Ra isoform and the long signaling-competent OB-Rb isoform (1). An additional transcript is generated from the same locus encoding a protein called OB-RGRP [or leptin receptor overlapping transcript (LEPROT)], which does not share any sequence similarity with OB-R (7). *In situ* hybridization experiments show coexpression of the OB-R transcript and the associated OB-RGRP transcript in the mouse brain, including hypothalamic regions involved in body weight regulation (8). Evolutionarily conserved coexpression of two ORFs is often observed in prokaryotes and viruses; however, there are few known cases in eukaryotes (9). Mammals have a single OB-RGRP homologue called LEPROTL1 (leptin receptor overlapping transcript-like 1), that has 70% amino acid sequence similarity with OB-RGRP and whose gene maps on chromosome 8 in humans (10). In yeast, Vps55p, a functional homologue of OB-RGRP, plays a role in protein transport from the Golgi to the vacuole and in the late endocytic pathway (11). LEPROTL1 and OB-RGRP may, by analogy, be involved in protein trafficking. However, the ORFs of OB-R and OB-RGRP are genetically linked, so we investigated whether OB-RGRP is specifically involved in the control of the intracellular transport of OB-R.

Paradoxically, most obese individuals display high levels of circulating leptin but do not respond appropriately (12). Possible mechanisms underlying this pathological state, termed leptin resistance, are impaired leptin bioavailability and transport across the blood–brain barrier, up-regulation of negative feed-back regulators of OB-R signaling, and defects in OB-R trafficking and signaling (13–15). Importantly, at steady state, most OB-R (endogenously expressed and transfected) are in intracellular membranes (16–21) and are fully functional in terms of ligand binding (19, 20). However, they are unable to participate in the functional response, as leptin does not penetrate the cell. Thus, increasing the number of OB-Rs exposed on the cell surface is an attractive therapeutic strategy to improve the leptin sensitivity of cells in obese patients.

We report here that OB-RGRP negatively regulates OB-R cell surface expression and OB-R-associated signaling in cell culture. Importantly, silencing of OB-RGRP in the hypothalamic arcuate nucleus (ARC) prevents the development of diet-induced obesity (DIO) in mice fed a high-fat diet (HFD).

## Results

**OB-RGRP Overexpression Decreases OB-R Cell Surface Expression.** Immunofluorescence studies showed that OB-RGRP localizes in the Golgi complex and in endosomes of HeLa cells [supporting information (SI) Fig. 5*A*]. Similar localizations were observed with GFP- or HA-tagged proteins. This localization is compatible with the hypothetic role for OB-RGRP in OB-R trafficking. To determine whether OB-RGRP is involved in the regulation of OB-R cell surface expression, HeLa cells were transduced with a fixed dose of an adenoviral vector

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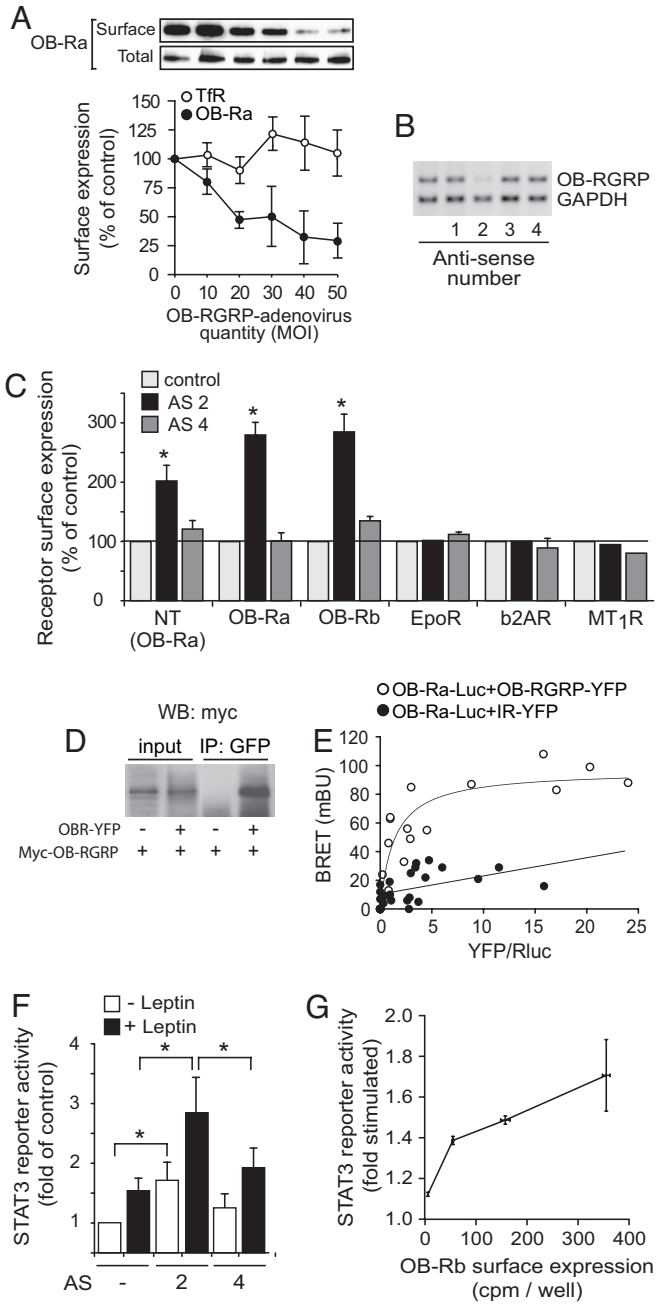


**Fig. 1.** OB-RGRP interacts with OB-R and regulates its cell surface expression. (*A*) HeLa cells were transduced with Ad:OB-Ra and increasing doses of Ad:OB-RGRP. Cell surface proteins were biotinylated and isolated with streptavidin-Sepharose beads. Surface (biotinylated) over total OB-R and transferrin receptor were plotted as a function of Ad:OB-RGRP dose. (*B*) Effect of OB-RGRP-specific antisense (AS1, AS2) or control (AS3, AS4) oligonucleotides on endogenous OB-RGRP mRNA levels in HeLa cells monitored by semiquantitative RT-PCR. GAPDH was used as an internal control. (*C*) Effect of AS2 (OB-RGRP-specific) or AS4 (control) on the cell surface expression of the indicated receptors. NT, nontransfected HeLa cells expressing lower levels of endogenous OB-Ra. (*D*) Coimmunoprecipitation of OB-Ra and OB-RGRP. Cells expressing the indicated proteins were lysed (input) and subjected to immunoprecipitation (IP) and Western blotting (WB) with the indicated antibodies. (*E*) Interaction of OB-Ra and OB-RGRP studied by BRET in intact cells. BRET donor saturation curves were generated by expressing constant amounts of OB-Ra-Luc and increasing quantities of the indicated YFP-tagged proteins. The BRET, total luminescence, and total fluorescence were measured. Data obtained for the BRET acceptor OB-RGRP-YFP were best-fitted with a nonlinear regression equation assuming a single binding site, those obtained for insulin receptor (IR)-YFP were best-fitted with a linear regression equation. (*F*) Effect of AS2 or AS4 on the activation of a STAT3 reporter gene construct by leptin in HeLa cells. (*G*) Quantification of STAT3 reporter gene activity in HeLa cells expressing increasing amounts of surface expressed OB-Rb. Results are means ± SE of three independent experiments performed in duplicate (*, $P < 0.05$).

expressing HA-tagged OB-Ra and increasing doses of an adenoviral vector expressing OB-RGRP. Overexpression of OB-RGRP decreased the amount of OB-R on the cell surface in a dose-dependent fashion, without modifying the total amount of OB-R produced or cell surface expression of the transferrin and EGF receptors (Fig. 1*A* and SI Fig. 5*B*). Similar results were obtained for OB-Rb (SI Fig. 5*B*), which suggests that overexpression of OB-RGRP specifically down-regulates OB-R expression at the cell surface.

**OB-RGRP Silencing Increases OB-R Cell Surface Expression and Signaling.** We used OB-RGRP-specific antisense oligonucleotides (AS1, AS2) to test whether endogenously expressed OB-RGRP similarly regulates OB-R cell surface expression. Only AS2 decreased the expression of endogenous OB-RGRP in HeLa cells; AS1 and negative control oligonucleotides (AS3 and AS4) had no effect (Fig. 1*B*). In AS2-transfected cells, the cell surface expression of OB-Ra and OB-Rb was raised by ≈2.5-fold (Fig. 1*C*). In contrast, the transfection of AS4 had no significant effect. Similar results were obtained on endogenous OB-Ra expressed in HeLa cells. The total amount of OB-R in cells, as assayed by total $^{125}$I-leptin binding, did not vary under these conditions. In a control experiment, we demonstrated that the cell surface expression of the closely related erythropoietin receptor and two members of the G protein-coupled receptor superfamily, $\beta_2$-adrenergic ($\beta_2$AR) and $MT_1$ melatonin receptors, which are all internalized and directed to late endosomes as OB-R, was not modified by AS2 transfection (Fig. 1*C*). The interaction between OB-R and OB-RGRP was assessed by coimmunoprecipitation (Fig. 1*D*) and bioluminescence resonance energy transfer (BRET) studies in living cells (Fig. 1*E*). In both assays, results suggested that OB-RGRP interacts with OB-R. No interaction was seen with several other membrane receptors such as the insulin, $MT_1$, and $\beta_2$AR receptors (data not shown). These various findings show that OB-RGRP regulates OB-R cell surface expression in a specific manner.

OB-R signaling involves the JAK/STAT pathway. Consequently, an increase of OB-R cell surface expression should lead to increased signaling and greater activation of this pathway. To test this prediction, we monitored STAT3 reporter gene activity in OB-Rb-expressing HeLa cells in the presence of AS2 and of AS4. Cells treated with the OB-RGRP-specific AS2 showed higher reporter gene activity than controls (Fig. 1*F*). The amount of surface-exposed OB-R correlated with the signaling activity of OB-R via the JAK/STAT pathway (Fig. 1*G*). Taken together, these data suggest that OB-RGRP interacts with OB-R and negatively regulates its signaling and cell surface expression.

**OB-RGRP Silencing in the ARC Prevents the Development of DIO in Mice.** To determine the effect of *in vivo* silencing of OB-RGRP on OB-R function, we performed stereotactic injections of lentivirus to deliver shRNA molecules into ARC neurons of male C57/Bl6 mice. These neurons express both OB-R and OB-RGRP (8) and are the key site for leptin's effects on energy homeostasis (22, 23). In control experiments, the effectiveness of lentiviral vectors expressing OB-RGRP-specific shRNA was verified *in vitro* and *in vivo*. Endogenous OB-RGRP expression in murine Ltk cells was inhibited by treatment with vectors bearing the OB-RGRP-specific shRNA but not control shRNA (Fig. 2*A*). Lentiviral vectors carrying OB-RGRP-specific shRNA were then delivered to ARC neurons by stereotactic injections on both sides of the third ventricle. Three weeks after injection, animals were killed, and the ARC was isolated by punching brain slices to recover the appropriate regions. Despite the presence of injected and noninjected

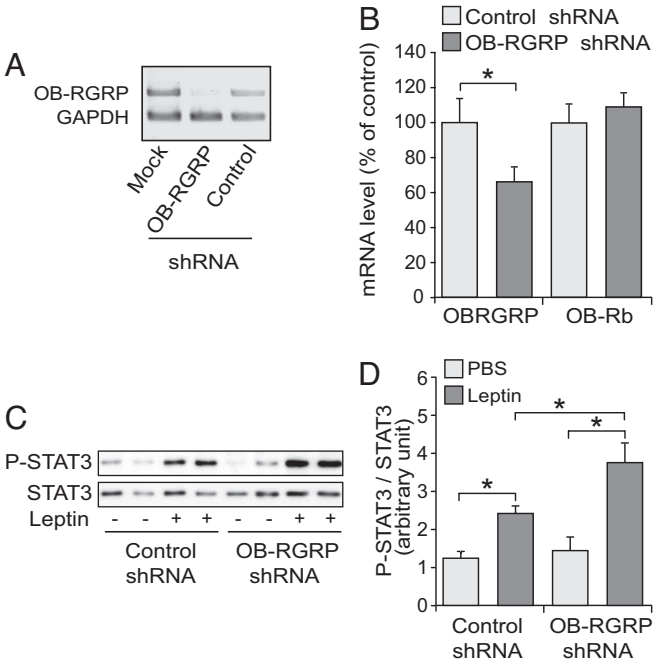


**Fig. 2.** OB-RGRP silencing in the ARC. (*A*) Infection of murine Ltk cells with control or OB-RGRP-specific shRNA expressing lentivirus. Endogenous OB-RGRP mRNA levels were monitored by semiquantitative RT-PCR 2 days after infection. GAPDH was used as an internal control. (*B*) Detection of OB-RGRP and OB-Rb mRNA levels in the ARC by real-time PCR (10 animals per condition). (*C* and *D*) Leptin-promoted STAT3 phosphorylation in the ARC from mice that have been injected with control or OB-RGRP shRNA expressing virus by stereotactic injection (*D*, means + SE of 12 animals per condition). *, $P < 0.05$.

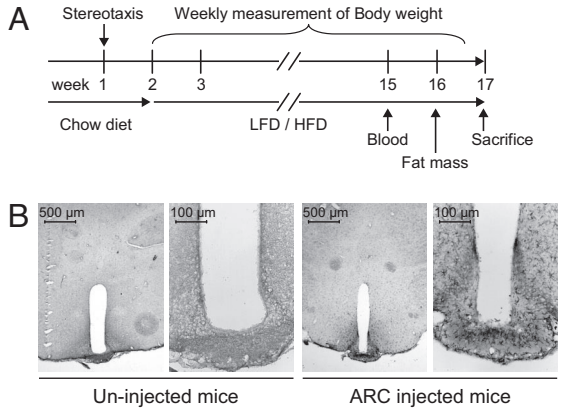


**Fig. 3.** Experimental groups and lentiviral targeting of the ARC. (*A*) Experimental protocol. (*B*) Immunohistological detection of the expression of the GFP reporter gene in the ARC.

brain regions in our sample, the overall expression of OB-RGRP in OB-RGRP shRNA animals was ≈40% lower than that in control shRNA-treated animals as determined by quantitative RT-PCR (Fig. 2*B*). In contrast, expression of the genetically linked OB-Rb was not affected, demonstrating the high specificity of this effect. STAT3 phosphorylation was significantly higher in OB-RGRP shRNA-treated animals than in control shRNA-treated animals (Fig. 2 *C* and *D*) Thus, the expected leptin-associated activation of the JAK/STAT pathway was confirmed. This finding demonstrates that specific OB-RGRP silencing in the ARC increases the OB-R signaling capacity *in vivo*.

We then investigated the therapeutic potential of OB-RGRP silencing in the DIO model by using the lentivirus-delivered-shRNA-based gene transfer approach. C57/Bl6 mice on a HFD develop obesity and hyperleptinaemia accompanied by a reduction in the leptin sensitivity of ARC neurons, thus establishing a state of leptin resistance (24, 25). Animals injected with lentiviral vectors expressing OB-RGRP-specific shRNA were fed a HFD or low-fat diet (LFD) for 15 weeks and weighed weekly (Fig. 3*A*). The injection success rate was determined by immunohistochemical detection of the lentivector-encoded GFP marker gene coexpressed in transduced brain regions (Fig. 3*B* and Table 1): the rate was ≈60%. The body weight of LFD-fed animals did not significantly differ between OB-RGRP shRNA and control shRNA groups, suggesting that the amount of surface-expressed OB-Rb is not decisive for body weight regulation under these conditions (Fig. 4*A*). Control shRNA animals showed the expected increase in body weight after week 3 on the HFD, whereas the body weight of OB-RGRP shRNA animals on the HFD was not significantly different from that of animals on the LFD. This result suggests that an increase of the number of functional OB-Rb can prevent the development of leptin resistance in animals fed with HFD. Importantly, animals (unintentionally) injected with OB-RGRP shRNA outside of the ARC and fed with HFD showed similar increases in body weight as HFD-fed control shRNA animals (Fig. 4*B*). Note that this weight increase was not observed in OB-RGRP shRNA-treated animals fed the LFD excluding nonspecific effects caused by virus delivery to non-ARC regions.

Induction of leptin resistance at the level of the STAT3 and the PI3K pathways was studied in HFD-fed animals by determining STAT3 and Akt phosphorylation in the ARC. HFD-treated control shRNA-injected animals were insensitive to leptin stimulation as expected (no activation of the Akt and STAT3). In contrast, leptin was able to activate STAT3 in animals injected with OB-RGP-specific shRNA but was unable to activate AKT. These results show that silencing of OB-RGRP has differential effects on OB-R signaling pathways (SI Fig. 6).

We measured body fat mass and plasma leptin levels to confirm that OB-RGRP silencing in the ARC protected animals from the development of DIO. The HFD did not lead to a significant increase in fat mass and leptin levels in OB-RGRP shRNA-treated animals (Fig. 4 *C* and *D*). In contrast, fat mass and leptin levels were higher in control shRNA-treated animals fed the HFD than those fed the LFD as expected.

The phenotype of injected animals was further investigated to test whether OB-RGRP silencing in the ARC affected glucose homeostasis. Plasma insulin and glucose levels were determined. Consistent with observations in the DIO model, insulin and glucose levels in control shRNA-treated animals increased while on the HFD (15th week on diet). Glucose and insulin levels in OB-RGRP shRNA-treated animals were not different from those in control shRNA-treated animals (Fig. 4 *E* and *F*). Specific silencing of OB-RGRP in the ARC thus prevents the development of obesity, hyperleptinaemia, and leptin resistance without interfering with the development of HFD-induced hyperglycemia or hyperinsulinemia.

**Table 1. Repartition of mice that have been injected in the ARC and outside of the ARC**

| | Control shRNA, *n* | | OB-RGRP shRNA, *n* | |
|---|---|---|---|---|
| Condition | LFD | HFD | LFD | HFD |
| Total | 10 | 10 | 20 | 20 |
| Lost or not analyzable | 3 | 2 | 3 | 1 |
| ARC injected | 6 | 6 | 8 | 11 |
| Outside ARC injected | 1 | 2 | 9 | 8 |

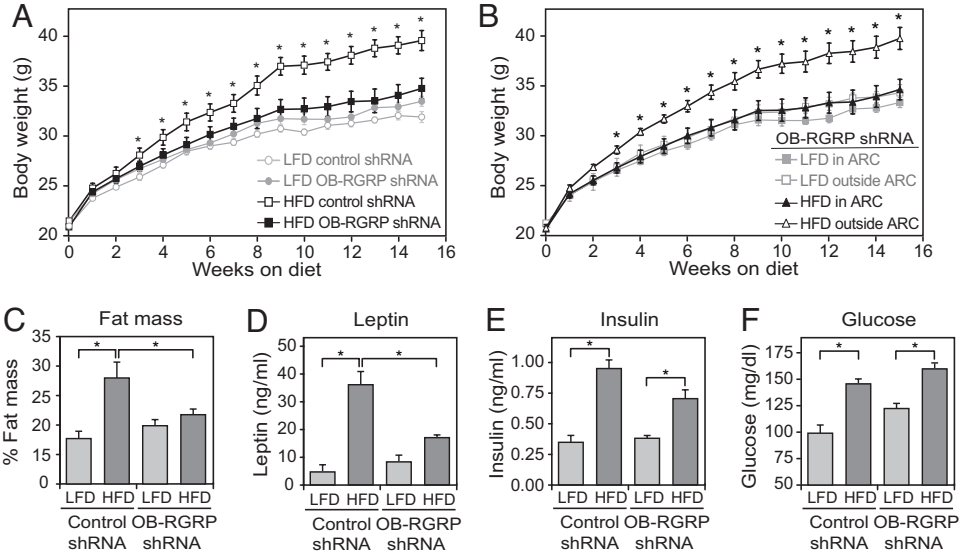


**Fig. 4.** Effect of OB-RGRP silencing in the ARC on DIO in mice. (*A*) Body weight evolution of mice expressing control shRNA or OB-RGRP shRNA in the ARC and fed a LFD or HFD. (*B*) Comparison of body weight of mice expressing OB-RGRP shRNA outside and in the ARC and fed a LFD or HFD. (*C–F*) Determination of fat mass (*C*), plasma leptin (*D*), insulin (*E*), and glucose (*F*) levels for the indicated experimental groups. *, $P < 0.05$.

## Discussion

We show that the OB-RGRP transcript codes for a protein that negatively regulates cell surface expression of OB-R. Silencing OB-RGRP specifically increases the number of surface-exposed OB-R and associated STAT3 signaling. Silencing of OB-RGRP in the hypothalamic ARC also prevents the development of HFD-induced obesity in mice. Our study provides interesting insights on regulatory mechanisms of OB-R signaling and shows that OB-RGRP is a promising therapeutic target for the treatment of obesity.

**Regulation of OB-R Trafficking by OB-RGRP.** Little is known about the intracellular trafficking of OB-R (19, 21, 26, 27). We recently showed that OB-R internalizes via a clathrin-mediated pathway and is transported to lysosomes where it is degraded. Most OB-Rs (endogenously expressed or transfected) are localized in intracellular membranes (16–21, 28). Importantly, these receptors are fully functional in terms of ligand binding (19, 20). Modulation of OB-RGRP expression (overexpression or down-regulation) affects the number of surface-exposed OB-R without altering the total receptor number, suggesting that OB-RGRP participates in the distribution of functional OB-R between the plasma membrane and intracellular compartments. OB-RGRP is unlikely to be a general regulator of cellular trafficking in mammalian cells, as several receptors are not affected by variations in OB-RGRP expression levels. Preliminary data show that OB-RGRP does not affect early endocytotic stages (unpublished results), which suggests rather a role in late endocytotic steps, a hypothesis, which is consistent with the function of Vps55p, a OB-RGRP homologue in yeast. Taken together, OB-RGRP appears to be involved in the sorting of OB-R (and maybe a limited number of other proteins) at a yet-to-be-defined step of the endocytotic pathway.

**Evolutionary Conservation of the Coexpression of OB-R and Its Negative Regulator OB-RGRP.** OB-R and OB-RGRP are cotranscribed in humans from the same promoter (7). The observation that OB-RGRP regulates the cell surface expression of OB-R may explain why this coexpression has been conserved through evolution. A mechanism that limits the number of surface-exposed OB-R may provide a selective advantage by restricting energy loss under conditions of food deprivation. As the activation of OB-R negatively regulates body fat mass by increasing energy expenditure and decreasing the food intake, limiting the maximal activation level of this pathway might be an advantage under these conditions. Clearly, more has to be learned about the regulation of OB-R cell surface expression by OB-RGRP in diverse metabolic situations to fully understand the evolutionary importance of regulated OB-R cell surface expression.

**Consequences of OB-RGRP Silencing on Glucose Homeostasis.** Traditionally, hyperglycemia and hyperinsulinemia have been considered to be secondary consequences of obesity. However, recent observations indicate that the diabetes and obesity phenotypes can be separated (29, 30). Our results are consistent with those observations as silencing of OB-RGRP in the ARC prevented the development of DIO but not of hyperglycemia or hyperinsulinemia. Leptin's effects on energy homeostasis and glucose metabolism have been suggested to be mediated by two separate signaling pathways, namely the STAT3 and PI3K pathways, respectively (31, 32). Maintenance of the responsiveness of the STAT3 pathway and desensitization of the PI3K pathway in OB-RGRP shRNA animals fed with HFD is consistent with the prevention of obesity but not of hyperglycemia and hyperinsulinemia in our animal model. Increased peripheral free fatty acid levels may also participate in the latter (33).

**Therapeutic Potential of OB-RGRP Silencing.** Regulation of OB-R trafficking, in particular by OB-RGRP, is a possible strategy for improving the leptin sensitivity of cells in situations of impaired OB-R function and, in particular, leptin resistance in obese people. OB-RGRP down-regulation is expected to be beneficial in at least two ways. First, increased cell surface expression of OB-R at the blood–brain barrier might increase leptin transport to the brain, which partially depends on the ubiquitously expressed OB-Ra isoform. Second, increased cell surface expression of the OB-Rb isoform in ARC neurons, central to energy homeostasis, would enhance OB-R-dependent signaling. Our results with the DIO mice model clearly support this possibility. The potential of OB-RGRP silencing as a therapeutic strategy will largely depend on the specificity of the effect. According to our results, OB-RGRP regulates the cell surface expression of OB-R but not that of several

other receptors. This observation excludes a general role of OB-RGRP in membrane trafficking in mammalian cells and indicates a certain degree of specificity for OB-R. Inducing RNAi through viral transduction has been successfully used in a tissue-specific manner in various mammalian systems (34). Nonintegrative lentiviral vectors have potential for gene therapy in nondividing cells, including neurons: they have all of the advantageous features of lentiviral vectors but do not present the mutagenic risks associated with insertion, making them suited to clinical applications (35). Alternatively, disruption of the OB-R/OB-RGRP interaction by small drugs might be used assuming that this interaction is involved in the targeting of OB-R to the cell surface. The BRET-based assay for the OB-R/OB-RGRP interaction would be a useful assay for screening for such compounds.

**General Perspectives.** Regulating receptor density at the cell surface may be relevant for many other receptors and is a promising approach for the development of new therapeutic strategies for various diseases. For example, the intracellular retention of the vasopressin V2 receptor accounts for ≈90% of patients diagnosed with nephrogenic diabetes insipidus, and cystic fibrosis transmembrane conductance regulator mutants accounts for 70% of cases of cystic fibrosis (36). The regulation of receptor trafficking seems to be determinant in other rare inherited diseases including cortisol resistance (37), warts, hypogammglobulinemia, immunodeficiency, and myelokathexis syndromes (38), and Glanzmann thrombasthenia (39).

The progress in systems biology will provide powerful means for the identification of specific regulators of many gene products involved in pathological processes. The concomitant development of RNAi technologies that allow the specific knockdown of almost any RNA target, combined with the refinement of gene transfer tools (40), should allow the rapid translation of such approaches to clinical applications. The current repertoire of molecular targets is limited mainly to receptors, ion channels, and transporters. However, targeting regulatory proteins by shRNA-mediated gene therapy would considerably broaden the spectrum of potential therapeutic targets (34).

## Materials and Methods

**Plasmids Constructions.** pcDNA3-OB-Ra, OB-Rb, OB-Ra-YFP, and OB-Ra-Luc have been described (20). The OB-RGRP coding region (7) was cloned into pcDNA3/CMV vector (Invitrogen). GFP and HA tags were cloned in continuation and the 6-myc in front of the OB-RGRP coding region by PCR. An U6-shRNA cassette targeting OB-RGRP or a mismatched sequence was inserted in the pTrip vector as described (34) to generate the pTripU6-OB-RGRP and pTripU6-control vectors. All sequences were verified and are available on request.

**Cell Culture and Transfection.** Murine Ltk and human HeLa cells were grown in DMEM supplemented with 10% (vol/vol) FBS, 4.5 g/liter glucose, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 1 mM glutamine (all from Life Technologies). Plasmid DNA was transfected by using the FuGene 6 reagent (Roche). Antisense oligonucleotides (AS) were transfected by using the Oligofectamine reagent (Invitrogen) (AS sequences are available on request). The transfection efficiency was routinely verified with a Cy3 fluorophore-labeled AS2 molecule.

**Adenovirus Vectors.** Recombinant defective adenovirus vectors expressing OB-RGRP were generated by homologous recombination in *Escherichia coli*. Viral stocks were generated, amplified, and tittered by the TCID50 method in N52.E36 cells. Adenoviruses expressing the HA-tagged OB-Ra or OB-Rb have been described (21).

**Cell Surface Biotinylation Assays.** HeLa cells were transduced with the adenoviral vectors and incubated with cleavable EZ-Link Sulfo-NHS-SS-Biotin, and biotinylated proteins were recovered by using streptavidin-agarose beads (GE Healthcare). Beads were resuspended in Laemmli buffer, and bound proteins were separated by SDS/PAGE and detected by immunoblotting using rat anti-HA 3F10 (Roche) or mouse anti-transferrin receptor (Zymed Laboratories) mAb or rabbit anti-EGFR (Santa Cruz Biotechnology).

**Semiquantitative RT-PCR.** Total RNA was extracted from HeLa or Ltk cells, and RT-PCRs were performed by standard methods. Analysis of mRNA levels was performed during the exponential phase of the amplification as described by Couturier *et al.* (41) for OB-RGRP and GADPH primer pairs (primers and conditions available on request).

**Radioligand Binding Assays.** Surface and total leptin binding experiments were performed as described (19) with $^{125}$I-leptin (GE Healthcare). The amount of surface-expressed erythropoietin, $\beta_2$-adrenergic, and $MT_1$ melatonin receptors was determined as described (42, 43).

**Coimmunoprecipitation.** HeLa cells were transfected with 6myc-OB-RGRP and OB-Ra-YFP. Total cell membranes were solubilized in 75 mM Tris, 2 mM EDTA, 12 mM $MgCl_2$, antiproteases (Complete, Roche Diagnostics), and 0.5% digitonin (Sigma) for 3 h at 4°C. The soluble fraction was subjected to immunoprecipitation with an anti-GFP mAb (Roche Diagnostics), and denatured complexes were separated on a 4–12% gradient gel and transferred to nitrocellulose. Immunoblotting was carried out with 9E10 anti-c-Myc mAb (Santa Cruz Biotechnology).

**BRET Assay, Luminescence, and Fluorescence Measurements.** BRET experiments, luminescence, and fluorescence measurements were performed as described (42, 44) with lumino/fluorometers Fusion (Packard Instrument) and Mithras (Berthold Technologies). Results were expressed in milliBRET units (mBU), with 1 mBU corresponding to the BRET ratio values multiplied by 1,000.

**Reporter Gene Activation Assay.** HeLa cells were cotransfected with a STAT3 firefly luciferase reporter gene plasmid, a Renilla lucifease plasmid, and the different OB-R constructs. Luciferase activities were measured by using the Dual Luciferase Assay System (Promega) with a Berthold Luminometer (Lumat LB 9507) as described (20). Results were expressed as ratios of activities for firefly luciferase over Renilla luciferase.

**Lentivirus Production.** The viral particles were produced by transient transfection of 293T cells by using the previously described p8.9 and pMD-G plasmids (45) and either pTripU6-OB-RGRP or pTripU6-control vector. Supernatants were collected 48 h after transfection, and high titer stocks were prepared as described (45). The stocks were titrated and normalized for the p24 antigen assayed by ELISA.

**Intracerebral Injection.** C57Bl6/N male mice (5 weeks old, body weight 19 g) were obtained from Janvier and housed in specific pathogen-free biosafety level 2 animal facility in a standard 12-hr on/off light cycle, according to institutional guidelines. Animals were anesthetized, and 40 ng of p24 of the vector in a volume of 1 $\mu$l was delivered (0.25 $\mu$l/min) in two injections into the ARC (either pTripU6-OB-RGRP or pTripU6-control vector). Stereotactic coordinates were taken relative to the bregma [anteroposteriority (AP − 1.3 mm], laterality (ML ± 0.3 mm), and the dorsoventrality relative to the skull (DV − 6 mm). The cannula was left in place for another 4 min and then withdrawn.

**RNA Extraction from the ARC and Real-Time PCR.** After intracerebral injection of mice with pTripU6-OB-RGRP vector ($n = 10$) or pTripU6-control vector ($n = 10$), animals were fed a standard diet for 3 weeks and then killed. Brains were then sectioned serially on a microtome cryostat (100-$\mu$m brain sections), and the ARC was recovered by micropunches. Total RNA was extracted according to Chomczynski and Sacchi (46), and the cDNA was synthesized with a cDNA archive kit (Applied Biosystem). GAPDH, OB-Rb, and OB-RGRP expression were quantified by using Taqman assays: Mm99999915, Mm01265583, Mm00838516.

**Hypothalamic STAT3 Activation.** After intracerebral injection of mice with pTripU6-OB-RGRP vector ($n = 12$) or pTripU6-control vector ($n = 12$), animals were fed a standard diet for 3 weeks. Mice were fasted overnight, then injected i.p. either with leptin (1 $\mu$g/g body weight) or PBS and killed 45 min later. Brains were dissected and immediately frozen in liquid nitrogen. Brains were then sectioned serially on a microtome cryostat (200-$\mu$m brain sections), and the ARC was recovered by micropunches. Tissue samples were denatured, and proteins were separated by SDS/PAGE, transferred to nitrocellulose membranes and immunoblotted with an antiphosphotyrosine (Tyr-705) STAT3 antibody (Cell Signaling) and then with anti-STAT3 antibodies (Cell Signaling). Results were quantified by densitometry.

**HFD Studies.** After intracerebral injection of mice with pTripU6-OB-RGRP vector ($n = 30$) or pTripU6-control vector ($n = 30$), animals were fed a standard diet for recovery and then fed a HFD (D12451; Research Diets) or LFD (D12450B; Research Diets) for the whole duration of the experiment. Animals were weighed every week. At week 15 on diet, blood glucose, leptin, and insulin levels were determined by tail bleeding of mice fasted for 12–16 h. Glucose levels were determined with a glucometer (A. Menarini Diagnostics), and leptin and insulin levels were determined by ELISA (CrystalChem). At week 16 on diet fat mass was measured with the LUNAR Pixi (Lunar).

**Histology.** Immunochemistry was performed on brain sections to detect the transgene expression (GFP) as described (45) by using a 1:3,000 dilution of a rabbit polyclonal anti-GFP antibody (Abcam).

**Statistical Analysis.** Data are expressed as means + SEM. Comparisons between groups were made by unpaired two-tailed Student *t* test or ANOVA followed by Fisher's test with Statview software.

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