Gene Therapy (2014) **21,** 638–644
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www.nature.com/gt

ORIGINAL ARTICLE

Endospanin 1 silencing in the hypothalamic arcuate nucleus contributes to sustained weight loss of high fat diet obese mice

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Leptin targets specific receptors (OB-R) expressed in the hypothalamus to regulate energy balance. Leptin decreases food intake in normal weight individuals, but this effect is blunted in obese subjects who are characterized by a state of leptin resistance. The prevention of leptin resistance is one of the major goals of obesity research. Recently, we identified endospanin 1 as a negative regulator of OB-R, which by interacting with OB-R retains the receptor inside the cell. We show here that in obese mice endospanin 1 is upregulated in the hypothalamic arcuate nucleus (ARC), the major brain structure involved in body weight regulation, suggesting that endospanin 1 is implicated in obesity development and/or the installation of leptin resistance. In contrast, silencing of endospanin 1 with lentiviral vectors in the ARC of obese mice fully restores leptin responsiveness when combined with a switch to *ad libitum* fed chow diet. The recovery of central leptin sensitivity is accompanied by sustained body weight loss and amelioration of blood lipid parameters and steatosis. Collectively, our results define endospanin 1 as a novel therapeutic target against obesity.

Gene Therapy (2014) **21,** 638–644; doi:10.1038/gt.2014.36; published online 1 May 2014

INTRODUCTION

The adiposity signal, leptin, is essential in the regulation of energy homeostasis. Mainly secreted by the adipose tissue, leptin reflects and controls the amount of body fat in a negative feedback mechanism between the periphery and the central nervous system. Leptin exerts its effects by activating the leptin receptor (OB-R) highly expressed in the brain and particularly in the hypothalamic arcuate nucleus (ARC), a central nervous structure known to regulate energy homeostasis. OB-R mediates leptin effects via the activation of multiple intracellular signaling pathways including the janus kinase 2/signal transducer and activator of transcription 3 (STAT3) pathway.[1,2] Deficiencies of leptin (*ob/ob* mice) or of leptin receptor (*db/db* mice, *fa/fa* rats) produce obesity and diabetes in both humans and rodents due to hyperphagia, decreased energy expenditure and insulin resistance.[3,4] However, most obese humans have high levels of blood leptin proportional to their excessive fat mass, and are not responsive to the hormone, characterizing a state of 'leptin resistance'.[5–7] Several deleterious mechanisms, such as endoplasmic reticulum stress[8,9] or impaired transport through the blood–brain barrier[10,11] contribute to leptin resistance. One other possible mechanism underlying leptin resistance could involve diminished exposure of OB-R at the neuronal cell surface leading to lower leptin signaling and sensitivity. The level of OB-R at the cell surface is of importance as only a small fraction of OB-R (5–20%) is expressed at the plasma membrane, where it can be activated by leptin.[12]

We identified endospanin 1, initially named leptin receptor gene-related protein (OB-RGRP), as a negative regulator of OB-R function.[14,15] Human endospanin 1 and OB-R are genetically linked as both corresponding transcripts are generated from the same *db* gene.[16,17] However, no sequence similarity is observed at the protein level. We had previously shown that endospanin 1 overexpression depletes OB-R cell surface exposure, whereas endospanin 1 silencing increases the number of OB-R at the cell surface, concomitant with enhanced leptin-induced STAT3 phosphorylation.[15] Correlated with these results, we recently showed that endospanin 1 interacts with OB-R and targets OB-R from endosomes to lysosomes increasing its degradation.[14] Hence, endospanin 1 silencing in the ARC of young and lean mice is sufficient to prevent the development of obesity in a high fat diet (HFD)-induced obesity (DIO) mouse model.[15]

In the present study, we show that endospanin 1 expression levels are increased in HFD-fed mice, indicating that elevated endospanin 1 expression levels might contribute to the development of obesity. To verify this hypothesis and to evaluate the curative potential of endospanin 1 silencing on obesity development, we silenced this time endospanin 1 in the hypothalamic ARC of fully obese mice. We observed that endospanin 1 depletion in the ARC of obese mice is beneficial at several levels: normalizing body weight (BW), restoring leptin sensitivity and ameliorating lipid blood parameters. This suggests that endospanin 1 silencing could constitute a therapeutic target against obesity.

RESULTS

Endospanin 1 expression is increased in obese mice and its downregulation in the ARC is sufficient to reverse the obese phenotype

We previously showed that silencing of endospanin 1 in the ARC, a major control center of energy homeostasis expressing high levels of OB-R, of young adult lean mice prevents DIO.[15] This implies that increased endospanin 1 abundance could participate in leptin resistance at the level of the ARC. To verify this hypothesis more

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Received 24 September 2013; revised 19 February 2014; accepted 21 March 2014; published online 1 May 2014

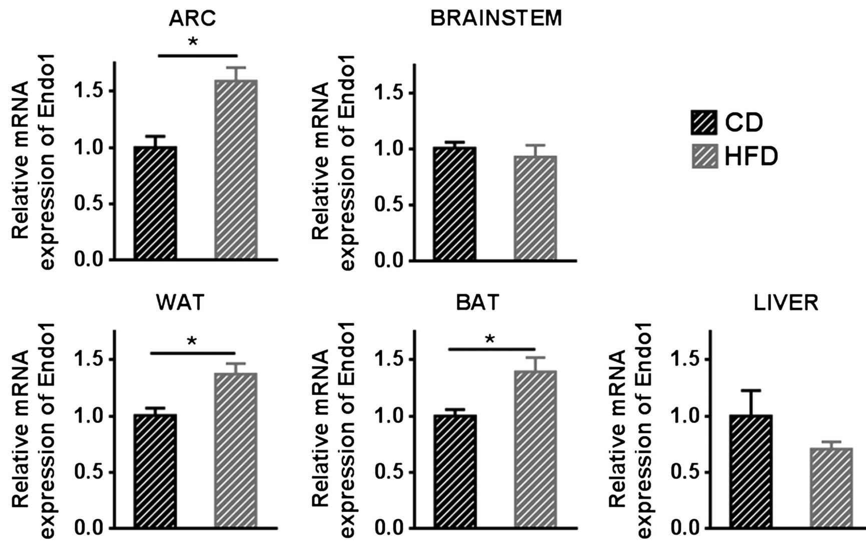

Figure 1. HFD increases endospanin 1 transcript. Endospanin 1 mRNA expression in the ARC, brainstem, epigonadal white adipose tissue (WAT), brown adipose tissue (BAT) and liver of C57Bl6J male mice fed a CD (black hatched bars) or HFD (gray hatched bars). $*P < 0.05$.

directly, we determined endospanin 1 expression levels in mice fed with HFD for 3 months. Interestingly, a significant increase of endospanin 1 mRNA levels was observed in the ARC, white adipose tissue (WAT) and brown adipose tissue, confirming the possible contribution of endospanin 1 in the development of obesity (Figure 1). This increase in endospanin 1 during HFD feeding is not observed in the liver and brainstem (Figure 1), suggesting that the phenomenon is not similarly regulated in all tissues. To further elaborate the importance of endospanin 1 in the development of obesity, we asked the question whether decreasing endospanin 1 expression in the ARC would be sufficient to reverse and cure DIO of obese mice. To answer this, we used endospanin 1 short hairpin RNA (shRNA)-expressing lentiviral vectors, which decreased endospanin 1 expression by 80% in the N46 hypothalamic cell line (Figure 2a). In a DIO mouse model, after 3 months on HFD, *in vivo* endospanin 1 silencing was achieved by stereotactic bilateral injection of endospanin 1 shRNA-expressing lentiviral vectors in the ARC of male C57/Bl6 mice with fully developed obesity, leading to a downregulation of endospanin 1 of at least 30% (Supplementary Figure 1) as achieved previously.[15] As shown previously, the level of silencing in ARC punches is judged to be underestimated as ARC punches are enriched in the ARC but also include surrounding nuclei of the hypothalamus, which did not express the endospanin 1 shRNA.

Obese animals either treated with control or endospanin 1 shRNA were then kept on HFD (Ctrl-HFD-HFD or Endo 1-HFD-HFD) or returned to regular chow diet (CD) (Ctrl-HFD-CD or Endo1-HFD-CD) (Figure 2b). Endospanin 1 silencing in Endo1-HFD-HFD animals maintained on HFD showed reduced BW gain compared with control Ctrl-HFD-HFD mice during the first 3 months after endospanin 1 silencing but not in the 4th month (Figure 3a), indicating a transient but not sustained BW improvement after endospanin 1 silencing under these conditions. In control animals, switching from HFD to CD was sufficient to stop BW increase and to reduce BW of obese Ctrl-HFD-CD mice beginning 2 weeks after diet switch (−5%), as expected. However, since animals were fed *ad libitum*, after stabilizing their BW, control Ctrl-HFD-CD animals regained all the weight they initially lost. Conversely, endospanin 1 silencing led to a greater weight loss and prevented weight regain. Thus, the combination of diet switch from HFD to CD with endospanin 1 silencing (Endo1-HFD-CD) was able both to reduce BW to 20% of initial levels and to maintain this BW loss even in the long term after 4 months of diet switch. Weight loss of silenced mice was accompanied with lower

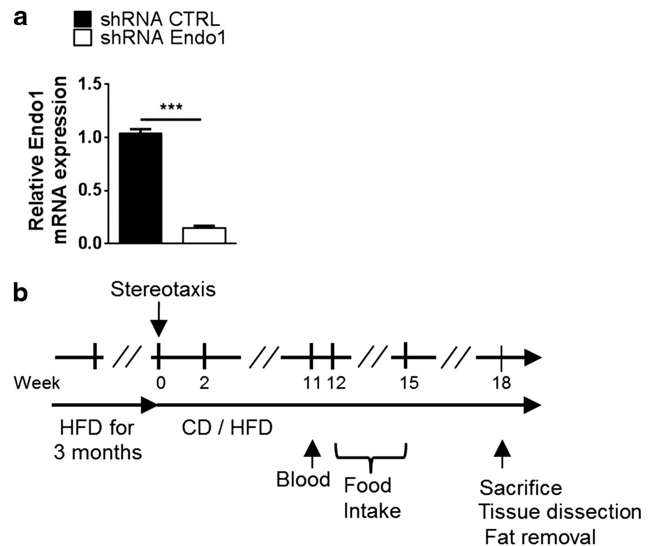

Figure 2. Silencing of endospanin 1. (**a**) Silencing of endospanin 1 in N46 hypothalamic cell line with lentiviral vectors expressing control (Ctrl) shRNA or shRNA Endo1. $***P < 0.001$. (**b**) Experimental protocol. After 3 months on HFD, C57Bl6 obese mice were submitted (at day 0) to stereotactic injection of lentiviral vectors expressing Ctrl shRNA or endospanin 1 shRNA in the ARC and kept on HFD or switched to CD. Time points of blood withdrawal for the measurement of lipid parameters and the day they were killed are indicated by an arrow.

food intake (Figure 3b) and decreased fat mass, down to levels of age-matched animals continuously fed with CD (Figure 3c). The decrease of fat-pad weight was expectedly accompanied with smaller adipocyte size in Endo1-HFD-CD-silenced animals compared with controls (Figure 3d). Endospanin 1-silenced animals during continuation of HFD feeding as well as switching to CD exhibited an unexpected trend (for most of them, without reaching statistical significance) of increased central orexigenic neuropeptides and decreased anorexigenic peptides relative to respective controls. This profile is consistent with periods of caloric restriction,[18] despite animals being fed *ad libitum* (Supplementary Figure 2).

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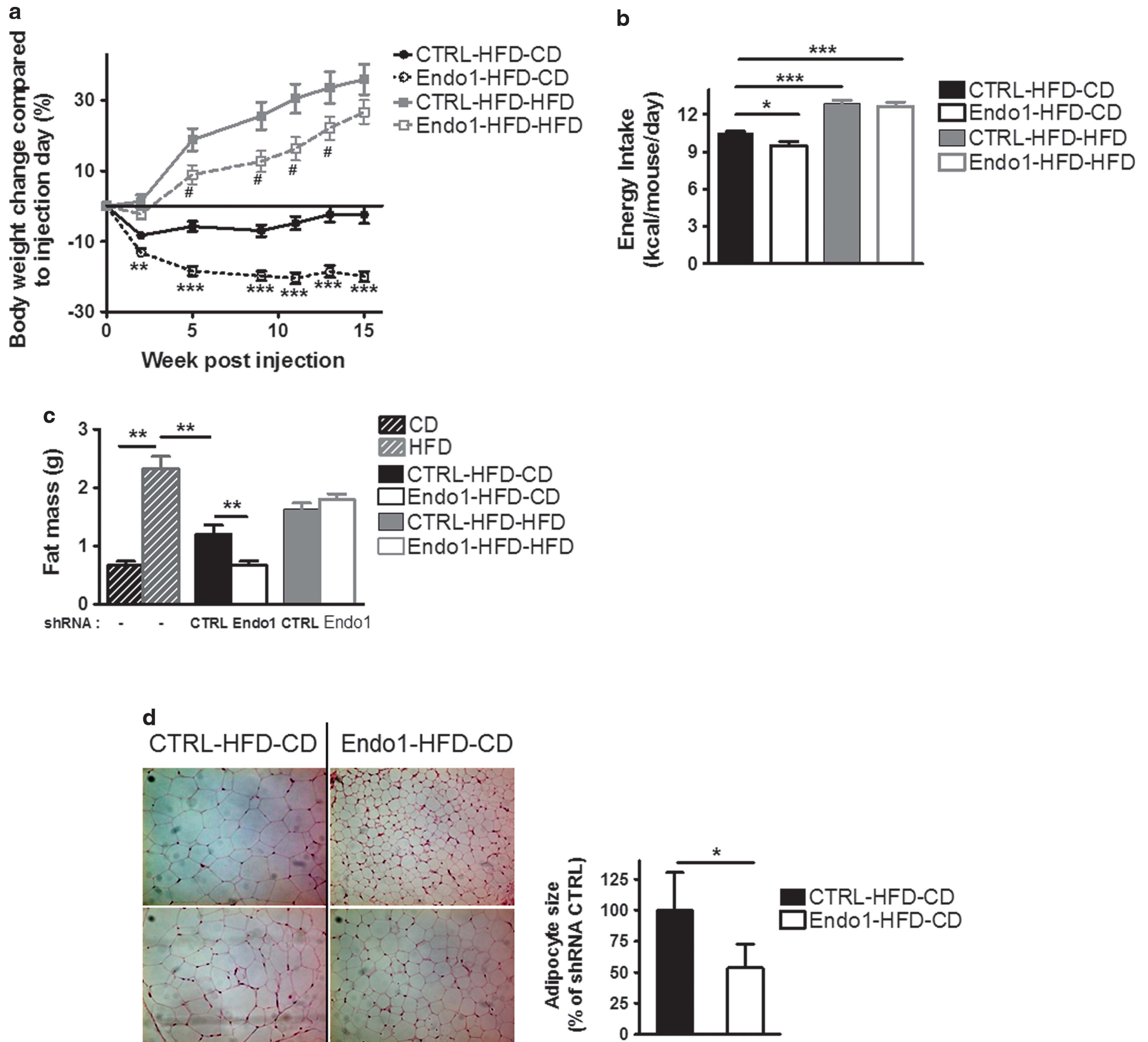

Figure 3. Endospanin 1 downregulation in the ARC of obese mice decreases BW, food intake and fat mass. (**a**) BW of obese mice after stereotactic injection of lentiviral vectors expressing control (Ctrl) shRNA or endospanin 1 shRNA in the ARC and kept on HFD (gray lines) or switched to CD (black lines); $n = 20$ per group, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to CTRL-HFD-CD; $^{\#}P < 0.05$ as compared to CTRL-HFD-HFD. (**b**) Caloric intake from weeks 12 to 15 (reported as mean per day). (**c**) Epigonadal fat-pad mass of naive mice on CD or HFD (hatched bars) and of obese mice treated with Ctrl (plain bars) or endospanin 1 shRNA (empty bars) kept on HFD (gray bars) or switched to CD (black bars). (**d**) Left panel: hematoxylin and eosin stain of paraffin-embedded adipose tissue of Ctrl-HFD-CD and Endo1-HFD-CD. Two representative pictures of WAT sections from different mice of the same group are shown. Right panel: Mean size of adipocytes estimated from hematoxylin/eosin stained paraffin-embedded adipose tissue. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Taken together, diminishing endospanin 1 levels specifically in the ARC of obese mice clearly facilitates the recovery from DIO by inducing sustained weight loss in combination with CD.

Endospanin 1 silencing in the ARC of obese mice switched to CD improves steatosis and lipid parameters

The DIO model is accompanied by hyperlipidemia. To verify the effect of endospanin 1 silencing on metabolic parameters, we determined the plasma levels of several lipids and the degree of hepatic steatosis. Endospanin 1 silencing in mice switched to CD was accompanied with lowered lipid parameters compared with controls as illustrated by plasma levels of glycerol (Figure 4a), triglycerides (Figure 4d), non-high-density lipoprotein-cholesterol (Figure 4e) and total cholesterol (Figure 4f) without changes in nonesterified fatty acids (Figure 4b) and ketones (Figure 4c). Total-to-high-density lipoprotein-cholesterol ratio is decreased in endospanin 1-silenced mice (Figure 4g), suggesting lower risk for coronary heart disease. Whereas the liver of HFD mice were steatotic as assessed by Oil Red-O staining in liver sections, hepatic lipid accumulation was diminished upon switching to CD, consistent with previous findings[19] (Figure 4g). Importantly a further decrease of lipid accumulation was observed in endospanin 1-silenced mice compared with controls switched to CD (Figures 4h–j). Taken together, these data indicate that endospanin 1 silencing in the ARC of obese mice switched to CD not

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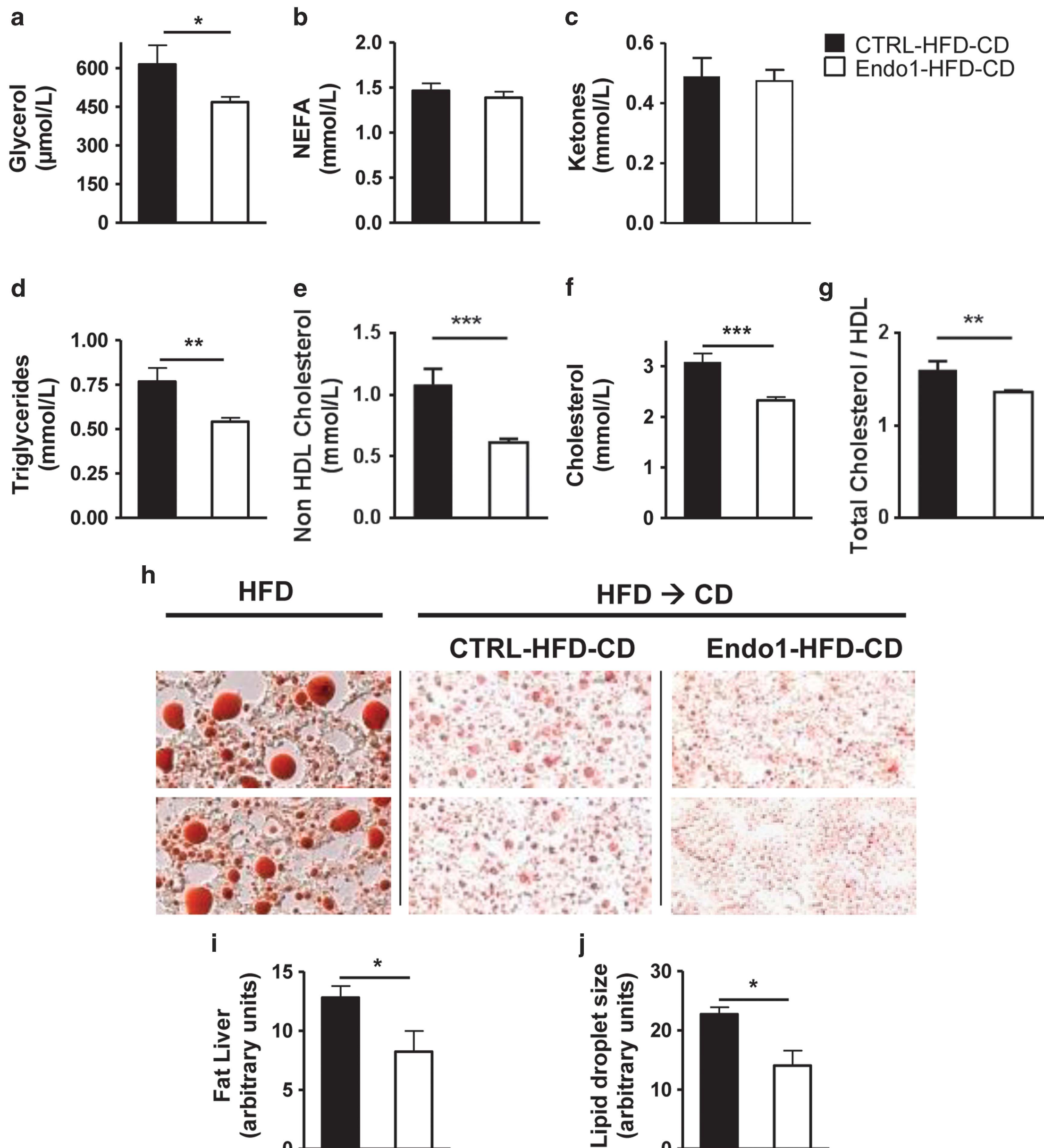

Figure 4. Endospanin 1 downregulation in the ARC of obese mice decreases plasma lipid parameters and steatosis. (**a**–**g**) Plasma concentrations of lipid parameters from blood collected at week 11 after silencing. (**h**) Oil Red-O staining of fat on frozen section of liver tissue of naive mice fed an HFD or treated mice. Two representative pictures of liver sections from different mice of the same group are shown. (**i**) Quantification of fat droplets with ImageJ software. (**j**) Quantification of fat droplet size with ImageJ software. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

only normalizes BW but also improves blood lipid parameters and liver steatosis.

Endospanin 1 silencing in the ARC of obese mice switched to CD significantly improves recovery of leptin-stimulated STAT3 phosphorylation in the ARC

The ARC neurons such as proopiomelanocortin and Agouti-related peptide (AgRP) neurons are able to sense and integrate information from plasma leptin to regulate food intake and BW. Phosphorylation of STAT3, which regulates the expression of target genes responsible for the anorectic effect of leptin is increased upon leptin activation. This effect is largely blunted in obese mice.[20] We hypothesized that the effect of silencing of endospanin 1 on BW was associated with an improved capacity of leptin to activate STAT3. We therefore assessed leptin-induced STAT3 phosphorylation in the ARC of control and silenced mice. Mice (Ctrl-HFD-HFD) challenged with HFD for 4 months exhibited no leptin-induced STAT3 phosphorylation in the ARC upon intraperitoneal injection of leptin revealing the well-known central resistance to leptin (Figure 5a). Similarly, endospanin 1 silencing was unable to recover the leptin response in obese mice kept on HFD for a long time (Figures 5a and c), consistent with the absence of any BW difference between silenced and control animals after 4 months on HFD (see Figure 3a). Importantly, after switching to CD, control animals (Ctrl-HFD-CD) only partially recovered from DIO-induced inhibition of STAT3 activation in the ARC, whereas endospanin 1-silenced mice (Endo1-HFD-CD) exhibited an important increase in leptin-induced STAT3 activation (Figures 5b and c).

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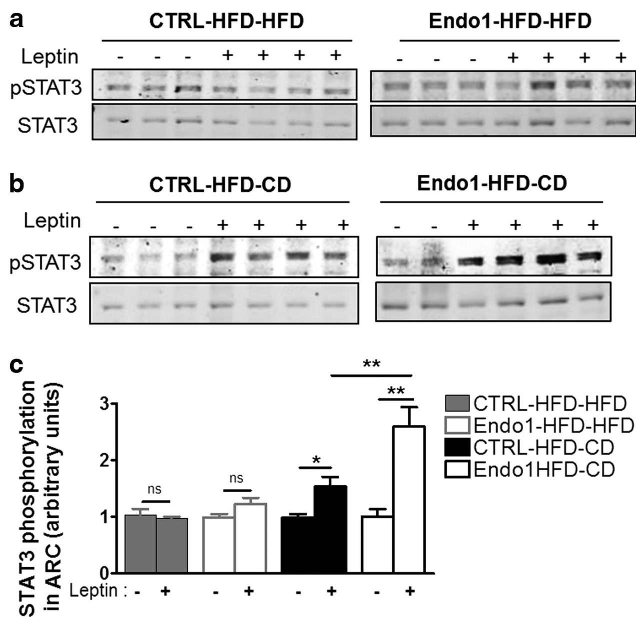

Figure 5. Effect of endospanin 1 downregulation in the ARC of obese mice on leptin-induced STAT3 phosphorylation. Leptin-promoted STAT3 phosphorylation in the ARC of obese mice injected with control or endospanin 1 shRNA-expressing lentiviral vectors kept on HFD (**a**) or switched to CD (**b**) (*$P < 0.05$; **$P < 0.01$) and quantified with ImageJ software (**c**). Every lane represents one mouse ARC. The selection of the mice injected either with leptin or saline before being killed and ARC punches was carried out randomly.

Taken together, reducing elevated endospanin 1 levels specifically in the ARC of obese mice clearly facilitates the recovery from DIO by inducing sustained weight loss in combination with CD, via an improved leptin responsiveness of the STAT3 pathway.

DISCUSSION

In our modern society, ingestion of highly caloric meals enriched in fat is a major contributor to human obesity. In the present study, we investigated the therapeutic properties of silencing endospanin 1 in mice fed with high fat diet and showed that endospanin 1 has a curative potential. The central role of endospanin 1 in the regulation of BW is suggested by the observation that endospanin 1 is upregulated in obese mice and that its downregulation in the ARC is sufficient to reverse DIO when associated with a standard diet.

Endospanin 1 downregulation in the ARC affects BW control

In obese control animals (Ctrl-HFD-CD), the switch to *ad libitum* CD showed only a transient and minor effect on BW loss. Similar observations were reported by Guo *et al.*,[21] who found that returning mice to a non-obesigenic diet, on *ad libitum* feeding, after exposure to HFD resulted in sustained elevation of body fat mass. Efficient weight loss, after switching HFD-fed mice to CD, was observed in another study, but only under conditions of caloric restriction matching the food intake of normal mice,[22] suggesting that inefficient weight loss is characterized by unrestrained higher food intake. Maintaining sustained weight loss over a long period of time is also a challenge not easily achieved in humans by mean of specific diet. Indeed, caloric restriction is a potential mean to reduce weight, but as in rodents, weight reduction cannot be preserved in the long term because of increased hunger, which promotes regain of weight.[23–25] One suggested explanation for this is the defense of body fat levels and energy homeostasis, in which goal is to accomplish exceptionally accurate balance between energy intake and energy expenditure, making voluntary weight loss difficult to maintain.[26,27]

As shown in the present study, endospanin 1 downregulation, when combined with a switch to CD, is able to reverse fully developed obesity with improved beneficial effects on circulating lipid parameters and steatosis. Whereas the switch to *ad libitum* CD alone is not successful, simultaneous silencing of endospanin 1 in the ARC decreased both food intake and fat mass, leading to a significant and sustained weight loss, most likely by enhancing leptin sensitivity of the ARC neurons. It has been proposed that weight loss associated with loss of fat mass is perceived like a state of 'leptin deficiency', which promotes increased appetite and overeating driving to weight regains.[25] In endospanin 1-silenced mice, this defense mechanism is compensated by higher leptin sensitivity in the hypothalamic ARC, likely resulting in the maintenance of weight loss.

On the other hand, on HFD the prevention of obesity development is possible when endospanin 1 silencing occurs in naive young mice, but the reversal becomes more difficult when the silencing happens in the ARC of older and already obese mice, for which only a transient but not sustained BW decrease was observed. Possibly, alterations such as leptin resistance, which are already installed in obese mice cannot be reversed solely by endospanin 1 downregulation in the ARC, in conditions where a high energy diet continues to be provided. This lack of effect seen in the endospanin 1-HFD-HFD mice should not be a consequence of poor endospanin 1 knockdown in HFD conditions as HFD-induced upregulation of endospanin 1 mRNA is also decreased in silenced mice to the same extent as in CD-silenced mice.

Endospanin 1-silenced mice in the ARC are characterized with increased AgRP mRNA expression in the ARC compared with WT mice. Higher AgRP levels in leptin-sensitive rodents are consistent with previous works showing also trends toward higher AgRP in the ARC of leptin-sensitive mice on CD compared with DIO mice, which are leptin resistant.[22] Moreover, Bouret *et al.* observed that AgRP projections from the ARC into the paraventricular nucleus were significantly higher in leptin-sensitive and DIO-resistant rats compared with DIO rats.[28] Elevated levels of AgRP in a context of higher leptin sensitivity in contrast with the fact that leptin administration is known to decrease AgRP mRNA expression warrant further investigations. In this regard, the degree of AgRP projections into the paraventricular nucleus of endospanin 1-silenced mice would also deserve a closer examination.

Most campaigns for the identification of potential target for obesity study the involvement of a specific gene in the prevention of obesity development (i.e. knockdown or knockout of a specific gene before the obesity is installed), whereas therapeutic aspects are often not addressed (i.e. reversal of obesity in obese mice). Our study attempted to tackle the latter aspect as there is an obvious urge to investigate molecular targets in a therapeutic setting that would better account for the actual need in human obesity.

Bariatric surgery is the only actual obesity treatment that is efficient and can reduce the mortality of patients with morbid obesity.[29,30] However, mortality during surgery and postoperative complications are not negligible and often encountered.[31–33] Moreover, surgery is usually prescribed for patients with morbid obesity. Hence, there is an obvious requirement for effective and safe non-surgical treatments. Treatment of obesity with the mean of single drug administration shows limited efficacy[34] and can provoke adverse effects.[35–37] Multitherapies using simultaneous combination of multiple medications appears to be a promising approach to reverse obesity with sufficient safety and efficacy.[38] Therefore, identifying good targets and developing several drugs that would affect different mechanisms controlling BW regulation in an integrative manner is needed. Increasing leptin sensitivity of

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the cell by reversing leptin resistance via endospanin 1 inhibition could be part of these solutions.

Leptin resistance has been suggested to arise from several concomitant mechanisms such as impaired leptin transport across the blood–brain barrier,[10,11] a lack of connection between neurons,[28] endoplasmic reticulum stress[8,9] or overexpression of OBR-negative regulators.[39] Here, we suggest that low amounts of leptin receptor at the cell surface of neurons, concomitant with an increased expression of endospanin 1, can also participate in the development of leptin resistance. The importance of the cell surface pool of OBR in neurons in the development of leptin resistance in humans has not been addressed and would need further investigation. In agreement with this hypothesis, a therapeutic treatment leading to decreased endospanin 1 expression would ameliorate leptin resistance in obese individuals. Thus, in a therapeutic setting, copharmacotherapy treatments, such as the administration of leptin with GLP-1[40] or amylin,[41] would further benefit from increased leptin sensitivity during endospanin 1 silencing even in a context of high fat feeding.

Proof of concept: therapeutic perspectives

We previously showed that endospanin 1 regulates the post-internalization steps of OBR trafficking, degradation, and cell surface expression:[14,15] endospanin 1 retains the OBR inside the cell and thus limiting the sensitivity of the cell to this hormone. Interestingly, in the present work, we show that silencing of endospanin 1 by lentiviral vectors in the ARC reverses the development of obesity in mice fed with HFD. These results indicate that restoring leptin sensitivity in obese patients and induction of weight loss by increasing the number of OBR at the cell surface could be a feasible therapeutic approach to treat obesity in humans.

A crucial concern in therapy is the need to examine the question of specificity and possible side effects. The specificity of endospanin 1 for OBR is suggested by the fact that endospanin 1 and OBR are genetically linked in humans and are encoded by the same gene. Moreover, our recent study on a human deletion of part of *LEPR* gene leading to the absence of endospanin 1 and OBR expression suggested that the absence of endospanin 1 had no major phenotypic consequences in humans different from a context of functional OBR deficiency.[17] This observation suggests a certain degree of specificity of endospanin 1 function for OBR in humans.

We show here that targeted delivery of shRNA molecules expressed from lentiviral vectors is feasible and could be the basis for future gene therapeutic strategies using secure viral vectors. Interestingly, two recent studies reported promising results from lentivial-driven gene therapy trials in humans.[42,43] As an extension, a more classical approach based on small molecular weight compounds interfering with the OBR/endospanin 1 interaction could also lead to an increase in OB-R cell surface exposure and beneficial therapeutic effects on BW.[44] More generally, targeting of specific interacting proteins, such as endospanin 1, instead of receptors itself could constitute an interesting alternative for novel and efficient therapeutic approaches.

In conclusion, this work shows that endospanin 1 is upregulated in the ARC of obese mice contributing to leptin resistance, and its silencing in the ARC of fully obese mice associated with a cessation of the HFD can achieve a sustained reversal of obesity.

MATERIALS AND METHODS

Animals

C57BL/6J male mice (Charles River, L'Arbresle, France) were housed in specific pathogen-free biosafety level 2 animal facility in a standard 12-h on/off light cycle, according to institutional guidelines. Animals were fed a standard rodent diet (CD) or HFD (D12451; Research Diets) and were provided with water and food *ad libitum*.

RNA extraction and quantitative real-time RT-PCR

RNA was extracted from tissues with RNeasy Mini Kit (Qiagen, Courtaboeuf, France). Retrotranscription was performed using reverse transcriptase (Invitrogen, Cergy Pontoise, France), followed by qPCR with LightCycler SYBR Green (Roche, Meylan, France). Primers for endospanin 1 (5'-GGA CTTCCTGTTGTTCTTGC-3' and 5'-AAGACGAGGAAGAAGCCTTG-3'), RPLP0 (5'-GGACCCGAGAAGACCTCCTT-3' and 5'-GCACATCACTCAGAATTTCAATGG-3') was used as internal controls.

RNA from ARC punches was extracted with RNeasy Micro Kit (Qiagen), and reverse transcription was performed using Maxima First Strand cDNA Synthesis for RT-PCR (Thermo Fisher, Villebon-sur-Yvette, France). Taqman inventoried gene expression assays (Life Technology, Saint Aubin, France) were used to examine ARC levels for AgRP (Mm00475829_g1), CART (Mm04210469_m1), endospanin 1 (Mm00838516_g1), NPY (Mm03048253_m1), proopiomelanocortin (Mm00435874_m1) and RPLP0 (Mm00725448_s1).

Lentivirus production and intracerebral stereotactic injection

Lentiviral production and stereotactic injection were performed as described previously.[15] Stereotactic injection of endospanin 1 or control (1 mismatch on endospanin 1 sequence) shRNA was performed on 4 month old obese mice ($n = 80$). Two weeks after surgery, half of control or silenced mice were either fed a CD or an HFD.

BW and food intake

BW was monitored weekly for 32 weeks. Food intake was measured at weeks 12–15 after silencing. Perigonadal WAT was weighted to estimate the body fat mass after being killed.

Histological staining

Adipose tissue was incubated in fixation solution containing 4% paraformaldehyde at 4 °C overnight and embedded in paraffin according to standard protocols. Tissue blocks were cut into 7 mm sections on a sliding microtome and mounted onto gelatin-coated slides. Following deparaffinization (20 min xylol, 2 min isopropanol, 2 min 96% ethanol, 2 min 75% ethanol), slides were washed in phosphate-buffered saline and subsequently hematoxylin and eosin stained. Relative adipocyte size was estimated by counting the number of adipocytes on a 100 μm section. Frozen liver sections of 7 μm thick were stained for neutral fat with freshly prepared Oil red-O staining (0.5%) and visualized by light microscopy.

Plasma assays

Plasma was obtained from whole blood collected from tail vein on heparin capillaries and centrifuged at 5000 *g* for 5 min. Plasma was analyzed with commercial kits for triglyceride concentrations (Beckman Coulter, Villepinte, France), nonesterified fatty acids (Randox, Roissy-en-France, France), cholesterol (Beckman Coulter), high-density lipoprotein (Beckman Coulter), glycerol (Randox) and hydroxybutyrate (Beckman Coulter).

Leptin signaling in hypothalamic ARC

At week 32, 16 h-fasted Ctrl-HFD-CD, Endo1-HFD-CD, Ctrl-HFD-HFD and Endo1-HFD-HFD mice were intraperitoneal injected either with 1 mg kg^{-1} murin leptin (PLR Ltd, Rehovot, Israel) or saline and killed 60 min later. Hypothalamic ARC, recovered by micropunches of 200 μm frozen brain sections, were denatured in Laemmli buffer containing phosphatase inhibitors (10 mM sodium fluoride and 2 mM orthovanadate) and analyzed in western blot analysis.

Western blot analysis

Tissue lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and immunoblotted with anti-phosphotyrosine (Tyr-705) STAT3 and anti-STAT3 (Cell Signaling, Danvers, MA, USA) antibodies. Western blot analysis were scanned on the Odyssey Infrared Imaging System (Licor, Lincoln, NE, USA) and quantified with the ImageJ software (Bethesda, MD, USA).

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Statistics
Data were analyzed either by one-way analysis of variance followed by Dunnett's or Tukey's multiple comparison test using GraphPad software (La Jolla, CA, USA). Results are presented as means ± s.e.m., and differences were considered significant if $P>0.05$.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGEMENTS
This work was supported by grants from the European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 241592 (to RJ), the Agence Nationale de la Recherche ANR-12-JSV1-0011 (to JD), Institut National de la Santé et de la Recherche Médicale (INSERM), Centre National de la Recherche Scientifique (CNRS), a research fellowship of the Fondation pour la Recherche Médicale (to VV), a PhD fellowship from the Region Ile de France (CODDIM) (to MP) and a PhD fellowship from the University Paris-Sud (to CR). We thank the Cochin Institute's *Morphology and Histology* facility for technical assistance.

AUTHOR CONTRIBUTIONS
JD and RJ wrote the manuscript; JD and RJ managed the project; VV, PC, TDS, MP, CR, JD, CS performed research and analyzed data.

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