Obesity hormone leptin induces growth and interferes with the cytotoxic effects of 5-fluorouracil in colorectal tumor stem cells

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Abstract
The incidence of colon cancer has increased in developed countries, possibly due to sedentary lifestyle and high caloric diet. Experimental and epidemiological evidence suggests a link between colon cancer development and adipose tissue-derived circulating hormones. Leptin, a pluripotent cytokine secreted by adipocytes, is a key regulator of appetite and energy balance acting in the brain. On the other hand, leptin also controls many physiological and pathological processes in peripheral organs. Recent studies in colon cancer cell lines and human tumors suggested that leptin and its receptor (ObR) are implicated in colon carcinogenesis, and may serve as new biomarkers and pharmacological targets. Here, we explored, for the first time, whether leptin can affect the biology of colorectal tumor stem cells (CTSCs). We found that our previously established and characterized CTSC clones express ObR and respond to leptin with cell proliferation, activation of the extracellular signal-related kinase (ERK)1/2 and AKT signaling pathways, enhanced growth in soft agar, and improved sphere formation associated with E-cadherin overexpression. Moreover, leptin counteracted cytotoxic effects of 5-fluorouracil, a common colon cancer therapeutic agent. These results suggest that obesity and increased leptin levels might promote colorectal cancer by increasing growth and survival of CTSCs.

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Introduction
Colorectal cancer is one of the most common neoplasms in the western world (Wilmink 1997, Jemal et al. 2006). A large body of epidemiological evidence suggests that obesity increases colorectal cancer risk by 0.4- to 1.0-fold in men and up to 2.0-fold in premenopausal women (Calle & Thun 2004, Renehan et al. 2008). Although molecular mechanisms underlying this association are still unclear, in vitro data suggested direct involvement of fat tissue in colon cancer development. Indeed, adipocytes and preadipocytes can stimulate the growth of colon cancer cells (Amemori et al. 2007). Furthermore, biologically active substances produced by adipocytes, such as cytokines (especially pro-inflammatory), growth factors, steroid hormones can exert oncogenic effects in the large bowel (Karagiannides & Pothoulakis 2007, Pischon et al. 2008, Pais et al. 2009).

The principal hormone synthesized by adipocytes is leptin. In humans, circulating leptin levels correlate with body mass index, and are significantly elevated in obese individuals (Wauters et al. 2000, Zhang et al. 2005). Leptin was originally discovered as a neurohormone controlling feeding behavior and energy homeostasis. However, later studies proved that leptin is a pleiotropic cytokine involved in a great variety of
physiological and pathological processes in peripheral organs (Wauters et al. 2000). Recent data clearly indicate that leptin, due to its mitogenic, antiapoptotic, pro-inflammatory, and angiogenic properties can promote development and progression of different cancer types (Garofalo & Surrmacz 2006).


New evidence suggests that leptin can be implicated in colorectal cancer. Increased leptin levels, at least in some studies, have been associated with greater risk of colorectal cancer development, especially in males (Stattin et al. 2003, 2004). In human colorectal cancer tissues, but not in normal mucosa or adenomas, leptin and its receptor (ObR) are significantly overexpressed, and correlate with the proneoplastic transcriptional regulator, hypoxia-inducible factor 1, and a more advanced tumor phenotype (G2 grade; Koda et al. 2007a,b, Paik et al. 2009).

In colon epithelial cells, leptin can induce chemokine production associated with macrophage activation similar to that observed in an adenomatous polyposis coli genotype (Fenton et al. 2007, 2008). In vitro, leptin can stimulate DNA synthesis, enhance proliferation and survival (mostly through the ERK1/2 and phosphatidylinositol 3-kinase (PI3K)/AKT pathways) as well as promote migration and invasion in colorectal cancer cells (Attoub et al. 2000, Hardwick et al. 2001, Rouet-Benzinb et al. 2004, Jaffe & Schwartz 2008, Ratke et al. 2010).

Colorectal cancer originates from epithelial cells lining the gastrointestinal tract that undergo sequential and specific DNA mutations disrupting normal mechanisms of proliferation. Stem cells of the gastrointestinal tract might be a principal target of tumorigenic mutations, due to their naturally long lifespan and capacity for self-renewal. The concept of stem cell-driven tumorigenesis in colorectal cancer is supported by the identification and phenotypic characterization of a subpopulation of colon cancer cells capable of initiation and faithful reproduction of human colon carcinomas in immunocompromised mice (tumor-initiating cells or colorectal tumor stem cells, CTSCs). Important screening characteristics of CTSC include the presence of cluster of differentiation (CD)133, CD166, and Lgr5 antigens (labeling undifferentiated cells) and the lack of cytokeratin 20 (CK20), which is expressed only by differentiated progeny (Barker et al. 2007, 2009, Dalerba et al. 2007, O’Brien et al. 2007, Ricci-Vitiani et al. 2007, 2009). Previously, we developed tumor-derived clones that possess features of CTSCs, namely, they express CD133, lack CK20, and readily reproduce the original tumor in immunodeficient mice (Ricci-Vitiani et al. 2007). Here, we investigated whether our CTSCs express functional ObR and whether the obesity hormone leptin might affect their growth, survival, and tumorigenic properties in vitro.

Materials and methods

Reagents

Leptin was purchased from R&D Systems, Inc. (Minneapolis, MN, USA), 5-fluorouracil (5-FU), and LY294002 from Sigma–Aldrich and U0126 from Promega.

Cell culture of CTSCs

The CTSC1.1, CTSC1.2, CTSC18, CTSC36, and CTSC85 clones were obtained from tumor samples of patients who underwent surgical resection of colon tumors, as described by us previously (Ricci-Vitiani et al. 2007). In brief, surgical specimens were washed several times and left overnight in DMEM:F12 medium supplemented with 500 Units/ml penicillin, 500 µg/ml streptomycin, and 5 µg/ml amphotericin B. Tissue dissociation was carried out by enzymatic digestion with 1.5 mg/ml collagenase II (Gibco-Invitrogen) for 2 h at 37 °C. Recovered cells were cultured at clonal density in a growth medium specifically adapted for CTSCs. The complete serum-free medium (SFM) for CTSCs consists of DMEM:F12 (Gibco-Invitrogen), supplemented with 50 µg/ml insulin, 100 µg/ml apo-transferrin, 10 µg/ml putrescine, 0.03 mM sodium selenite, 2 µM progesterone, 0.6% glucose, 5 mM HEPES, 0.1% sodium bicarbonate, 0.4% BSA, 2 mM L-glutamine, 100 Units/ml penicillin, 100 µg/ml streptomycin, 20 ng/ml epidermal growth factor (EGF), and 10 ng/ml basic fibroblast growth factor (bFGF). Nontreated, sterile polysyrene flasks for suspension cell cultures (Nunc, Thermo Fischer Scientific, Rochester, NY, USA) were used to reduce cell adherence and support growth as undifferentiated tumor spheres (Ricci-Vitiani et al. 2007). The medium was replaced or supplemented with fresh growth factors (20 ng/ml EGF and 10 ng/ml bFGF) twice a week, until cells started to grow as floating...
aggregates. Cultures were expanded by mechanical dissociation of spheres, followed by replating of single cells and residual small aggregates in complete fresh SFM. Flow cytometry was used to analyze the immunophenotype of the subpopulations. The CTSCs used in this study were clearly positive for CD133 and negative for CK20 (Ricci-Vitiani et al. 2007). The colorectal adenocarcinoma cell lines SW 480 and HT-29 (purchased from AATC, LGC Standards s.r.l., Milan, Italy) were cultured in RPMI 1640 medium (PAA Laboratories, Pasching, Austria).

**Cytofluorimetric cell staining**

Colon tumor spheres were dissociated, and cells were counted. A total of 10^5 single cells were labeled with CD133/1-phycoerythrin (PE) antibody (Ab; Miltenyi Biotec, Bergisch Gladbach, Germany) and/or CD166-PE Ab (BD Bioscience, Franklin Lakes, NJ, USA). For Lgr5 staining, a total of 2 × 10^5 single cells were fixed with 2% paraformaldehyde for 20 min room temperature, and then permeabilized with 0.1% Triton X-100/PBS for 5 min at 4°C before incubation with Lgr5–FITC Ab (Gene Tex Inc., Irvine, CA, USA). Expression profiles were acquired with a FACS Canto instrument using FACS Diva software (Becton Dickinson, Milan, Italy). IgG mouse-PE Ab (Beckman Coulter Inc., Brea, CA, USA) was used as a negative control for CD133 and CD166, and Alexa Fluor 488 goat anti-rabbit IgG was used as negative control for Lgr5. Data were analyzed with Flowjo software (www.flowjo.com, Flowjo, Ashland, OR, USA).

**Cell growth and viability**

CTSCs were seeded at 50 000 cells/ml in duplicates in a 12-well plate and treated with leptin (300 ng/ml) for 96 h, with readdition of leptin (300 ng/ml) after 48 h. In viability experiments, leptin treatment was followed by a 72-h exposure to 60 µg/ml 5-FU. For AKT inhibition experiments, cells were pretreated with leptin (300 ng/ml) for 24 h, then treated with LY294002 (50 µM) for 2 h, before being exposed to 5-FU (60 µg/ml) for 48 h. U0126, a specific inhibitor of mitogen-activated protein kinase kinase (MEK)1/2, was used to block ERK1/2 activity. Cells were pretreated with U0126 (5 µM) for 1 h, and then treated with leptin (300 ng/ml) for 48 h. Cell proliferation in control and treated cells was evaluated by collecting, dissociating, and counting cells with Trypan blue exclusion method or using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All data are a mean of three independent experiments.

**Western blot**

CTSCs were treated with leptin (300 ng/ml) for 96 h, with readdition of leptin (300 ng/ml) after 48 h. In some experiments, leptin treatment was followed by a 72-h exposure to 60 µg/ml 5-FU. Whole cell lysates from treated cells were prepared in 1% NP40, 20 mM Tris (pH 7.2), 200 mM NaCl, Phosphatase Inhibitor Cocktail 1 (used 1:100, P2850, Sigma–Aldrich), Phosphatase Inhibitor Cocktail 2 (used 1:100, P5726, Sigma–Aldrich), and Protease Inhibitor Cocktail (used 1:100, P8340, Sigma–Aldrich). The expression of proteins was assessed in 20 µg of protein extracts with specific Abs: for ObR, H300 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), for total ERK1/2, K-23 (Santa Cruz Biotechnology), for phosphorylated ERK1/2, E-4 (Santa Cruz Biotechnology), for phosphorylated AKT (Ser473), 193H12 (Cell Signaling Technology, Danvers, MA, USA), and for E-cadherin, 36/E-Cad (BD Bioscience, San Jose, CA, USA). Protein loading was assessed by measuring the expression of β-actin, using AC-15 Ab (Sigma–Aldrich), or β-tubulin, using TUB 2.1 Ab (Sigma–Aldrich).

**Apoptosis assay (TUNEL staining)**

This assay utilized terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Following treatments, CTSC spheroids and single cells were attached to poly-1-lysine coated coverslips by sedimentation, fixed with 2% paraformaldehyde, and permeabilized with 0.5% Triton X-100 overnight at 4°C. Apoptosis was determined with the In Situ Cell Death Detection kit, Fluorescein, following the manufacturer’s instructions (Roche). Cells containing DNA strand breaks were stained with fluorescein-dUTP, and 4’,6-diamidino-2-phenylindole was used to visualize cell nuclei. Slides were analyzed using Olympus FV-1000 confocal microscope with the Olympus objective Ultraplan Apochromatic 60X N.A.1.35 and the software Olympus Fluoview (Olympus, Center Valley, PA, USA).

**Migration/chemoattraction assay**

Migration of CTSCs in response to leptin was evaluated in 24-well transwell chambers with upper and lower culture compartments separated by polycarbonate membranes with 8-µm sized pores (3422, Corning Inc., Lowell, MA, USA). Single CTSCs suspended in 200 µl of complete SFM were placed into upper chambers (10 000 cells/chamber), while complete SFM supplemented with 300 ng/ml leptin was placed into bottom chambers to act as a
chemoattractant. After 48 or 96 h of incubation at 37 °C, the cells remaining on the upper surface of the membrane were removed with a cotton swab. The cells that migrated through pores and adhered to the lower surface of the membrane were stained with Coomassie Brilliant Blue and counted under the microscope.

Soft agar assay
CTSCs (1000 cells/well) were plated in 24-well plates containing a soft agar bilayer (0.3% top and 0.4% bottom layer, SeaPlaque Agarose, Cambrex, East Rutherford, NJ, USA), plain or supplemented with leptin (300 ng/ml). Cultures were incubated at 37 °C for 21 days. Colonies were stained with crystal violet (0.01% in 10% MetOH) and counted under the microscope. Average size of the colonies was calculated using ImageJ Software (http://rsb.info.nih.gov/ij). The experiments were done in triplicate.

Statistical analysis
The statistical significance of the results for leptin on cell growth, intracellular signaling, migration, and colony formation was evaluated by r-test. The statistical significance of the results for leptin interference with chemotherapeutics and signaling inhibitors was evaluated by ANOVA and Bonferroni post-tests. A P value <0.05 was represented by a single asterisk, while three asterisks indicate P<0.001. All statistical analyses were performed using GraphPad Prism 4 (GraphPad Software Inc., La Jolla, CA, USA, www.graphpad.com).

Results
Expression of stem cell markers on CTSCs
CTSCs used in this study were extensively described by us previously and proved to express CD133 and lack the differentiation marker CK20 (Ricci-Vitiani et al. 2007). However, to further validate the undifferentiated status of all our CTSCs, we assessed the expression of CD133 as well as of other known markers of CTSCs: CD166 (Dalerba et al. 2007) and Lgr5 (Barker et al. 2007, Sato et al. 2009). Flow cytometry analysis with specific Abs proved that all our stem cell lines are positive for the expression of CD166, Lgr5, and CD133 (Fig. 1A).

Human CTSCs express ObR
Leptin exerts its biological functions through binding to ObR. The long form of ObR (130–190 kDa) mediates downstream signals by activating multiple signaling pathways, while short forms of ObR have limited or no signaling capabilities (Ahima & Osei 2004). To study the possible effects of leptin on CTSCs, we first examined the expression of ObR in five different clones of colon tumor stem cells. Western blot analysis demonstrated that all our cell lines expressed the long form of ObR. The highest levels of ObR were found in CTSC36 and CTSC1.2, while lower levels were noted in CTSC1.1, CTSC18, and CTSC85 (Fig. 1B). The colon cell lines SW 480 and HT-29, known to express ObR (Rouet-Benzineb et al. 2004, Aparicio et al. 2005b), were included as positive controls.

Leptin stimulates proliferation of CTSCs through the activation of the ERK1/2 signaling pathway
We tested leptin effects on the growth of CTSC1.1, CTSC1.2, CTSC18, and CTSC36 cells. The results indicated that leptin can stimulate proliferation of CTSCs expressing higher levels of ObR, but does not exert significant mitogenic activity in cells with low ObR expression. Leptin effects in CTSCS36 and CTSC1.2 cells were evident at 96 h post treatment (Fig. 1C), and noticeable at 48 h, but not at 24 h (data not shown).

To determine the intracellular signaling mechanisms responsible for leptin growth effects, we examined the activation of major postreceptor signaling pathways that are known to mediate ObR proliferation and survival effects in various cancer cell types, such as the ERK1/2 and AKT pathways (Fruhbeck 2006, Garofalo & Surmacz 2006). An increased phosphorylation of ERK1/2 was observed in clones CTSC36 and CTSC1.2, and these effects coincided with elevated cell proliferation (Fig. 1C). To confirm the involvement of ERK1/2 signaling in leptin-induced cell growth, we inhibited ERK1/2 activity using U0126, a specific inhibitor of MEK1/2 (Favata et al. 1998). As demonstrated in Fig. 1D, blocking ERK1/2 completely abrogated leptin-enhanced cell growth, suggesting that the ERK1/2 pathway is responsible for the increased CTSC proliferation induced by leptin.

Leptin activates PI3K/AKT signaling pathway and counteracts 5-FU-induced apoptosis in CTSCs
Stimulation with leptin induced a moderate increase in phosphorylation of AKT in CTSC1.2 and CTSC36 expressing higher ObR levels, but had no effects in CTSC18 (Fig. 2A), characterized by low ObR expression. Activation of AKT signaling is known to promote cell survival and protection from apoptosis (Yang et al. 1997, Ozes et al. 1999). We investigated whether leptin has the ability to interfere with
apoptotic effects of 5-FU in CTSCs. To this end, CTSC1.2, CTSC18, and CTSC36 were first exposed to leptin and thereafter treated with 5-FU. Cell viability assays demonstrated that leptin inhibited 5-FU-induced apoptosis in CTSCs expressing high levels of ObR, but was ineffective in CTSC18 containing low ObR levels (Fig. 2B). These observations were confirmed with the TUNEL assay, where leptin addition significantly reduced the occurrence of apoptotic cells in cultures treated with 5-FU (Fig. 2C).
Figure 2 Leptin activates the PI3K/AKT signaling pathway and counteracts 5-FU-induced apoptosis in CTSCs. (A) Leptin-mediated activation of AKT was evaluated by western blotting, as described in Materials and methods. β-Actin levels were assessed as loading control. (B) Cell viability upon 5-FU treatment in the presence or in the absence of leptin was evaluated by direct cell counting, as described in Materials and methods. All data are a mean of three independent experiments. (C) Apoptosis in CTSCs treated with 5-FU in the presence or in the absence of leptin was assessed by TUNEL assay, as described in Materials and methods. Cells containing DNA strand breaks were stained with fluorescein-dUTP (red), and DAPI was used to visualize cell nuclei (blue). (D) Activation of AKT in CTSCs was evaluated by western blotting, as described in Materials and methods. β-Actin levels were assessed as loading control. (E) Involvement of AKT in leptin-mediated protection from apoptosis was assessed using a PI3K inhibitor, LY294002. Cell viability and AKT expression and activation were determined as described in Materials and methods. β-Actin levels were assessed as loading control.
Next experiments demonstrated that leptin counteracted the effects of 5-FU on a molecular level. Specifically, while 5-FU treatment reduced AKT activation in CTSCs, leptin partially or totally reversed this effect, suggesting that survival activity of leptin is mediated by AKT signaling (Fig. 2D). This possibility was addressed with the PI3K inhibitor LY294002 (Favata et al. 1998), which is known to block leptin-induced AKT activation (Burguera et al. 2006, Sharma et al. 2006). Indeed, LY294002, at least in part, blocked leptin-dependent anti-apoptotic activity in 5-FU-treated CTSCs (Fig. 2E). However, leptin did not modulate ERK1/2 activity in the presence of 5-FU (data not shown), suggesting that this pathway is not involved in survival effects of ObR in our model.

Leptin does not affect migration of CTSCs

Enhanced cell migration is a feature associated with highly aggressive tumors (Attoub et al. 2000, Yamaguchi et al. 2005). Since leptin has been shown to promote migration and invasion of normal and cancer colon cells (Attoub et al. 2000, Jaffe & Schwartz 2008, Ratke et al. 2010), we analyzed the possible effects of the hormone on CTSCs migration in vitro. We found that while leptin moderately induced CTSCs migration through the membranes, the effects were not statistically significant at all tested time points (24, 48, and 96 h; Fig. 3A and data not shown).

Leptin promotes cell proliferation and colony forming ability

Plasma leptin levels are proportional to body fat content, implicating that obese people are under chronic exposure to high circulating concentrations of this cytokine. In order to investigate the long-term impact of leptin on CTSCs, we carried out colony-forming assays in soft agar (Fig. 3B). We found that leptin significantly increased the number of colonies after 21 days of incubation. In addition, we observed a greater number of large aggregates and colonies in leptin-treated cultures relative to control. On the average, leptin exposure increased colony size from 210 to 250 μm in CTSC36 cells, and from 280 to 290 μm in CTSC1.2 cells, which might be related to enhanced cell–cell adhesion. Indeed, we observed upregulation of the cell adhesion protein E-cadherin in CTSC36, but not in CTSC1.2 (Fig. 3C). In addition, enhanced occurrence and larger size of aggregates and spheres could be observed when cells were cultured in normal medium supplemented with leptin (Fig. 3D).

Discussion

Cancer stem cells (CSCs) constitute a subpopulation of tumor cells that are selectively equipped with tumor initiation, self-renewal capacities, and the ability to give rise to bulk populations of nontumorigenic cancer cell through differentiation (Sabbath et al. 1985, Hanahan & Weinberg 2000, Reya et al. 2001, Clarke et al. 2006, Morrison & Kimble 2006). CSCs have been identified in several human malignancies, and their relative abundance in clinical cancer specimens has been correlated with disease progression (Arce et al. 2006, Schatton et al. 2008, Zhou et al. 2009). Understanding how CSCs interact with tumor environment, including circulating and locally produced cytokines and hormones, might impact clinical management of different cancer types and development of novel therapeutics.

The risk of colorectal cancer development is increased in obese individuals, possibly due to elevated activity of biologically active substances produced by the fat tissue (Pischon et al. 2008). The adipokine leptin has been implicated in colorectal cancer development and progression due to its mitogenic, anti-apoptotic, mitogenic, and pro-inflammatory activity (Garofalo & Surmacz 2006, Karagiannides & Pothoulakis 2007, Ratke et al. 2010). Here, we investigated, for the first time, the role of leptin in the biology of CTSCs. The characteristic stem cell features of CTSCs used in this study were described by us before (Ricci-Vitiani et al. 2007). Additionally, we confirmed in this paper that CTSC lines express several recognized markers of undifferentiated colorectal CSCs such as CD166 (Dalerba et al. 2007) and Lgr5 (Barker et al. 2007).

We found that all our CTSCs express different isoforms of ObR; some clones express ObR at levels comparable with that detected in immortalized colon cancer cell lines. In CTSCs expressing higher levels of the long (signaling) form of ObR, leptin was able to induce cell growth and survival. Furthermore, in these clones, leptin activated the known growth/survival pathways, i.e. ERK1/2 and AKT (Balmanno & Cook 2009, Engelman 2009, Huang & Chen 2009). In addition, we found that in CTSCs, leptin can interfere with the efficacy of 5-FU, a commonly used colorectal cancer chemotherapeutic agent (Chung & Saltz 2007, De Doosso et al. 2009), by ways of increasing cell viability and reducing 5-FU-inflicted DNA damage.

Experiments with specific inhibitors suggested that leptin-dependent growth in CTSCs relies principally on the ERK1/2 pathway, while leptin-mediated survival requires AKT signaling. Some of these effects
Figure 3  Leptin promotes colony forming ability and cell–cell adhesion of CTSCs. (A) Leptin effects on migration of CTSCs were evaluated as described in Materials and methods. (B) Effects of leptin on soft agar growth were determined in CTSCs as described in Materials and methods. The lower panel shows representative pictures of colonies. (C) Leptin-mediated effects on E-cadherin expression were determined by western blotting, as described in Materials and methods. β-Actin levels were assessed as loading control. (D) CTSCs were plated in normal culture medium with or without leptin, as described in Material and methods. CTSCs aggregation was documented using an Olympus IX81 inverted microscope equipped with an F-View monochrome camera, and representative pictures (magnification 10×) are shown for each condition.
might be related to increased expression of E-cadherin, a cell–cell adhesion protein that can be up-regulated by leptin in some cancer cells (Mauro et al. 2007) and promote anchorage-independent growth and survival (Steinberg & McNutt 1999, Reddy et al. 2005). In line with this hypothesis, we observed that leptin addition increased E-cadherin expression, colony forming ability of CTSCs as well as cell–cell adhesion, especially in one of the clones expressing high levels of ObR.

In different colon cancer cell lines, leptin has been shown to promote migration and invasion through multiple pathways (Jaffe & Schwartz 2008, Ratke et al. 2010). We, however, did not observe substantially increased migratory activity of CTSCs, even after long exposure to leptin (300 ng/ml). This discrepancy might result from the particulars of our experimental system. Namely, CTSCs naturally and readily form spheres in culture, which in turn prevents their dispersal and migration. The propensity of CTSCs to form aggregates could be additionally potentiated by E-cadherin overexpression in leptin-treated cultures.

In summary, our results suggest that leptin, a cytokine that is normally increased in overweight and obese individuals, can affect the growth and survival of CTSCs, promoting colorectal carcinogenesis and/or potentially interfere with chemotherapy. Since CSC-targeted approaches might represent translationally relevant strategies to improve clinical outcome, especially for those malignancies that are refractory to conventional anticancer agents directed at bulk tumor cell populations, interfering with leptin signaling by targeting ObR might become a novel attractive option for colorectal cancer treatment, especially in obese patients.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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