

Leptin deficiency suppresses MMTV-Wnt-1 mammary tumor growth in obese mice and abrogates tumor initiating cell survival

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Abstract

Obesity increases both the risk and mortality associated with many types of cancer including that of the breast. In mice, obesity increases both incidence of spontaneous tumors and burden of transplanted tumors. Our findings identify leptin, an adipose secreted cytokine, in promoting increased mammary tumor burden in obese mice and provide a link between this adipokine and cancer. Using a transplantable tumor that develops spontaneously in the murine mammary tumor virus-Wnt-1 transgenic mice, we show that tumors transplanted into obese leptin receptor (LepRb)-deficient (db/db) mice grow to eight times the volume of tumors transplanted into lean wild-type (WT) mice. However, tumor outgrowth and overall tumor burden is reduced in obese, leptin-deficient (ob/ob) mice. The residual tumors in ob/ob mice contain fewer undifferentiated tumor cells (keratin 6 immunopositive) compared with WT or db/db mice. Furthermore, tumors in ob/ob mice contain fewer cells expressing phosphorylated Akt, a growth promoting kinase activated by the LepRb, compared with WT and db/db mice. *In vivo* limiting dilution analysis of residual tumors from ob/ob mice indicated reduced tumor initiating activity suggesting fewer cancer stem cells (CSCs). The tumor cell populations reduced by leptin deficiency were identified by fluorescence-activated cell sorting and found to express LepRb. Finally, LepRb expressing tumor cells exhibit stem cell characteristics based on the ability to form tumorspheres *in vitro* and leptin promotes their survival. These studies provide critical new insight on the role of leptin in tumor growth and implicate LepRb as a CSC target.

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Introduction

Today, over 25% of the US population is obese, a condition of excess adipose tissue and fat (Flegal *et al.* 2002, 2004, Baskin *et al.* 2005, Haslam & James 2005, Ogden *et al.* 2006). Associated with obesity is a significantly increased risk in development of multiple diseases including diabetes, cardiovascular disease, and cancer (Calle *et al.* 2003). Obesity increases the mortality risk of over 12 different cancers including

breast, ovarian, prostate, and colon cancer (Calle *et al.* 2003). Furthermore, obese patients are at greater risk of tumor recurrence and metastasis resulting in poor overall survival (Loi *et al.* 2005, Huber *et al.* 2009).

Breast cancer is the second leading cause of cancer death in women in the US (American Cancer Society 2011) and obesity increases the mortality risk of luminal type A tumors in post-menopausal women and in basal-like tumors in both pre- and post-menopausal

women (Calle *et al.* 2003, Millikan *et al.* 2008). Human basal-like tumors are a subset of triple-negative breast tumors because they do not express the receptors for estrogen, progesterone, or human epidermal growth factor receptor 2 (HER2) and are highly aggressive (Cheang *et al.* 2008, Millikan *et al.* 2008).

While adipose tissue was thought to be strictly a fat-storing organ, in the past 10 years, it has emerged as an active organ, secreting cytokines (leptin and adiponectin) and inflammatory mediators (Halberg *et al.* 2008), many of which can influence various processes involved in tumorigenesis (Brakenhielm *et al.* 2004). Because adipokines influence growth of breast cancer cells *in vitro* (Hu *et al.* 2002), it is proposed that excess body fat alters breast tumors through the increased production of these factors.

Among the adipokines, leptin has received significant attention and a number of studies propose that it is a tumor promoter (Surmacz 2007). As such, leptin is thought to increase or stimulate tumor growth. The importance of leptin in cancer is strongly implicated by the observation that increased expression of leptin and its functional receptor (LepRb) in human grade-III invasive breast tumors are associated with shorter time to tumor recurrence and patient death (Ishikawa *et al.* 2004, Garofalo *et al.* 2006, Miyoshi *et al.* 2006, Maccio *et al.* 2010). Similarly, *in vitro* cellular studies indicate that leptin promotes breast cancer cell proliferation, migration, invasion, and induction of angiogenesis (Gonzalez *et al.* 2006, Fiorio *et al.* 2008, Saxena *et al.* 2008, Rene Gonzalez *et al.* 2009). Collectively, these data led us to hypothesize that tumors expressing functional leptin receptors (LepRb) would thrive in environments with excess leptin and fail to grow in leptin deficient environments.

To test this hypothesis, we used tumor cells derived from spontaneous tumors that develop in the murine mammary tumor virus (MMTV)-Wnt-1 proto-oncogene transgenic mice (Li *et al.* 2000). The tumors arise as a consequence of activation of Wnt/ β -catenin signaling in the mammary gland (Li *et al.* 2000, Brown 2001). The MMTV-Wnt-1 transgenic mouse mammary tumors exhibit molecular and pathological characteristics of human basal-like tumors (Herschkowitz *et al.* 2007). Recent studies indicate that Wnt/ β -catenin signaling pathway is activated in human basal-like breast cancer (Khrantsov *et al.* 2010). In this study, we validate this hypothesis and show that leptin deficiency leads to loss of tumor cells that express LepRb and the residual tumors in leptin-deficient mice exhibit reduced ability to form new tumors indicating reduced tumor initiating cells.

Methods

Antibodies and other reagents

Antibodies to: keratin 14 from Abcam (Cambridge, MA, USA), the keratin 8 (TROMA-1) antibody developed by Brulet and Kemler was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242, USA and keratin 6 from Covance (Princeton, NJ, USA). All secondary antibodies, including Alexa Fluor 488 and 568 goat anti-rabbit, goat or mouse antibodies, were purchased from Molecular Probes (Eugene, OR, USA).

Mice

All animals were maintained in microisolator units and provided free access to food and water. All mouse procedures were performed under strict adherence to protocols approved by the Institute Animal Care and Use Committee at the Lerner Research Institute, Cleveland Clinic Foundation. Leptin-deficient (B6.V-*Lep*^{ob/J}; ob/ob), LepRb-deficient (B6.BKS(D)-*Lep*^{db/J}; db/db), and control wild-type (WT, C57Bl/6J) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The MMTV-Wnt-1 mice, the source of the mammary tumor cells, were kindly provided by Dr Stephen Hursting, co-author on these studies (Nunez *et al.* 2008). The genetic defects in these mice have been extensively studied in mammary tumorigenesis (Li *et al.* 2000). MMTV-Wnt-1 transgenic mice are maintained on the C57Bl/6J strain, syngenic with the WT, ob/ob, and db/db mice.

Dissociation of Wnt-1 tumors

Tumors were minced with a scalpel and dissociated for 1–1.5 h at 37 °C on a rotary shaker in Medium 199 (Invitrogen) supplemented with 0.28 Wünsch units/ml liberase TH and TM (Roche, Indianapolis, IN, USA) and 100 Kunitz units/ml DNase I (Sigma-Aldrich, St. Louis, MO, USA). Samples were pipetted every 20 min while shaking. Cells were passed through 40 μ m filter and centrifuged at 300 g for 10 min. Cell pellets were resuspended in ACK buffer (Invitrogen, Carlsbad, CA, USA) and red blood cell lysed for 1 min at room temperature (RT). Cells were either stored in liquid nitrogen at a concentration of 5–10 million cells/ml until transplant or processed for flow cytometry.

Inoculation of Wnt-1 cells

Tumor cells from spontaneous MMTV-Wnt-1 tumors were orthotopically transplanted (50 000 cells/mouse)

into the right mammary fat pad #4 of female mice at 6 weeks of age ($n=6-10/\text{group}$). Initially, mice were monitored twice weekly, then daily once tumors were palpable. Tumor volume (cubic millimeters) was measured using an electronic caliper, applying the formula ($\text{volume}=0.52 \times (\text{width}) \times (\text{height}) \times (\text{length})$) for approximating the volume of a spheroid. Before tumors reached 1.5 cm^3 , the animals were euthanized and the tumors were collected for histological, immunofluorescence, and fluorescence-activated cell sorting (FACS) analyses. For serial transplantation analysis, tumors were isolated under sterile conditions, dissociated into single cell suspension, viability assessed by trypan blue exclusion, and 50 000, 20 000, 5000, or 500 tumor cells injected into recipient WT mice.

Flow cytometry

Cells were counted and resuspended in PBS containing 0.5% BSA and 2 mM EDTA (BSA solution) per 10^7 total cells. Cells were purified on CD45 and Ter119 by magnetic-activated cell sorting (MACS) on LD column microbeads (Miltenyi Biotec, Auburn, CA, USA). FACS analysis for CD45 and CD31 was used to confirm lineage negative (Lin^- , depleted of endothelial and hematopoietic cells, not shown). Lin^- cells were resuspended in PBS containing 2% FBS and labeled with allophycocyanin (APC)-conjugated rat antibody to mouse CD49f (eBioscience, San Diego, CA, USA), APC-rat anti-mouse CD29 (eBioscience), phycoerythrin (PE)-conjugated rat anti-mouse CD24 (eBioscience) at a concentration of 0.5 million cells/ml for 1 h on ice. Cells were subjected to FACS analysis and sorting on BD LSR II cytometer (BD Biosciences, San Jose, CA, USA). Data analysis was performed on the FlowJo version 8.8.6 software (Tree Star, Inc., Ashland, OR, USA). Isotype control FACS analysis indicated no nonspecific binding to the cells.

Immunofluorescence analysis

Sections were deparaffinized by HistoClear (National Diagnostics, Atlanta, GA, USA) and rehydrated in graded alcohols. After Antigen Retrieval, sections were blocked with 2% goat serum in PBS for 1 h at RT and then incubated overnight with antibody to keratin 14, 8, and 6. The next day, sections were washed and incubated for 2 h with fluorescent secondary antibody at RT. Images were acquired using an epifluorescent digital microscope (Leica Microsystems, Buffalo Grove, IL, USA).

Immunofluorescence analysis of flow sorted lin^- cells

Cells were plated on poly-L-lysine (0.01%, Sigma) coated glass cover slips for 1 h then fixed with 4%

paraformaldehyde, permeabilized with 0.05% Triton X-100, and blocked with 3% milk in PBS for 30 min at RT. Cells were incubated overnight at 4°C with antibody to keratin 14, 8, or 6 in 1% milk/PBS. The next day, cover slips were washed and incubated for 1 h with fluorescent secondary antibody at RT and mounted in permount containing DAPI. Images were obtained with an epifluorescent digital microscope (Leica Microsystems). For quantification, 6–10 fields were counted under a microscope for keratin 8, 14, and DAPI-fluorescent cells. At least 200 cells from each population were counted and data are expressed as percentage of DAPI-stained cells.

RT-PCR analysis

Total RNA was extracted with TRI reagent (ABI, Carlsbad, CA, USA) and cDNA was synthesized using high capacity cDNA reverse transcription kit with RNase inhibitor (ABI). Amplification of transcripts was performed with 200 ng cDNA. The primer sets were:

mouse GAPDH forward: 5'-GCACAGTCAAGGC-CGAGAAT-3';

mouse GAPDH reverse: 5'-GCCTTCTCCATGGT-GGTGAA-3';

mouse LepRb forward: 5'-GAAGATGTTCC-GAACCCCAAGAATTGTTCC-3' and

mouse LepRb reverse: 5'-GCACTTGGTGACT-GAACTATTTATAAGCCC-3'.

Tumorsphere assays

Distinct tumor subpopulations (10 000 cells/well) were cultured in six-well ultra-low binding plates in 2 ml serum-free DMEM/F12 medium supplemented with 20 ng/ml basic fibroblast growth factor (Invitrogen), 10 ng/ml epidermal growth factor (Biosource, Carlsbad, CA, USA), 2% B27 (Invitrogen), 10 $\mu\text{g}/\text{ml}$ insulin and 1 $\mu\text{g}/\text{ml}$ hydrochloride (Sigma). Cells were incubated without or with 400 ng/ml leptin that was supplemented in the media every other day for 5 days. On day 5, tumorspheres were harvested and counted under a Leica dissecting scope.

Statistical analysis

Data are expressed as mean \pm s.d. or s.e.m. as indicated in the figure legend. Statistical analysis was performed by a Student's *t*-test. Significance was set at $P \leq 0.05$. All microscopic images were prepared by Adobe Photoshop CS2 adjusted for brightness/contrast. Stem cell frequencies for each transplanted population and *P* values were calculated using Extreme Limiting

Dilution Analysis (ELDA) software based on the algorithm defined by Hu & Smyth (2009) (<http://bioinf.wehi.edu.au/software/elda/>). We confirmed that our data fits a single-hit linear model assumption by a likelihood ratio test to analyze goodness of fit.

Results

Reduced tumor burden in obese leptin-deficient mice transplanted with MMTV-Wnt-1 tumor cells

Leptin-deficient (ob/ob) and LepR-deficient (db/db) mice develop early onset obesity, that is, virtually identical with respect to metabolic, inflammatory, and growth characteristics (Table 1; Ingalls et al. 1950, Hummel et al. 1966, Spiegelman & Flier 2001). Both mice are morbidly obese with large adipocytes, but db/db mice have high circulating leptin and ob/ob mice have no circulating leptin (Coleman 2010). The virtually identical obesity syndrome of ob/ob and db/db mice provides a unique opportunity to discern the role of circulating leptin on obesity promoted tumor growth.

We chose a mouse mammary tumor model previously shown to exhibit increased tumor burden upon transplant into obese mice, MMTV-Wnt-1 (Nunez et al. 2008) and expresses the LepRb. Spontaneous mammary tumors from MMTV-Wnt-1 transgenic mice were dissociated into a single cell suspension and injected orthotopically into db/db and leptin-replete WT female mice. In WT mice, tumor outgrowth was detected as early as 36 days post-injection (Fig. 1A). In obese db/db mice, tumor outgrowth was detected as early as day 32 post-injection (Fig. 1A). Furthermore, tumor growth rate and burden was significantly increased, with tumors reaching eight times the volume of WT mice at necropsy (2145 ± 320 vs 280 ± 63 mm³, Fig. 1A). These studies confirm previous findings showing that obesity promotes increased tumor burden in mice (Dogan et al. 2007, Nunez et al. 2008).

To assess the role of leptin in the tumor promotion, we injected tumor cells orthotopically into ob/ob mice. In contrast to the obese db/db mice, tumors ob/ob mice could not be palpated, though at necropsy we identified

tumors in 4/8 ob/ob mice (Fig. 1A and B). Tumors in ob/ob mice were smaller than in WT mice (58 ± 30 vs 280 ± 63 mm³) though the difference was not statistically significant. Tumor volume at necropsy (Fig. 1B) provides clear indication that obesity increases tumor growth (compare db/db and WT mice, $P < 0.05$), however, leptin deficiency is sufficient to suppress the obesity induced growth (compare obese ob/ob and db/db mice, $P < 0.01$). The reduced outgrowth and tumor burden in leptin-deficient mice indicates that leptin can promote increased Wnt-1 tumorigenesis in obesity.

Serial transplant of MMTV-Wnt-1 tumor cells from spontaneous tumors results in tumor outgrowths that recapitulate the histology and immunophenotype of the spontaneous tumor (Cho et al. 2008, Vaillant et al. 2008). The transplanted tumors in WT mice had histomorphologic findings consistent with those previously described in MMTV-Wnt-1 induced adenocarcinomas including cellular heterogeneity with both neoplastic epithelial and myoepithelial cells, foci of squamous metaplasia, and high mitotic rates. Furthermore, the majority of WT tumors (90%) had at least 50% or greater well-formed acinar/glandular formation (Fig. 1C, denoted by *). Tumors in db/db mice recapitulated the morphology of the tumors in WT mice, however, the accelerated growth resulted in large regions of central necrosis (Fig. 1C, denoted by long black arrows). On the contrary, the ob/ob mice developed adenocarcinomas that maintained some features of the spontaneous tumor but were more homogeneous with a solid architecture having marked areas of single cell apoptosis (Fig. 1C, arrows) and few well-formed acinar/glandular forms.

To examine the apparent histopathological differences, MMTV-Wnt-1 tumors from WT, ob/ob, and db/db mice were analyzed by immunofluorescence using lineage selective markers. Luminal epithelial (keratin 8) and myoepithelial (keratin 14) neoplastic cells were present throughout the tumors in WT, ob/ob, and db/db mice (Fig. 2A). The presence of these cells suggests the presence of bipotential progenitors. Strikingly, analysis of keratin 6, a marker of less differentiated tumor cells revealed a significant

Table 1 Circulating levels of leptin, insulin, and adiponectin in wild-type (WT), ob/ob, and db/db mice

	Body weight (g)	Adiponectin (mg/ml)	Insulin (ng/ml)	Leptin (ng/ml)
Wild-type	22.0 ± 1.99	44.9 ± 26.7	0.8 ± 0.7	0.9 ± 0.7
ob/ob	$57.3 \pm 3.04^*$	33.8 ± 13.6	$5.1 \pm 3.2^\dagger$	ND
db/db	$56.6 \pm 2.63^*$	30.4 ± 7.7	$15.0 \pm 9.1^{\dagger,\#}$	$24.4 \pm 12^\dagger$

* $P < 0.001$ (compared with WT); $^\dagger P < 0.0001$ (compared with WT); $^\# P > 0.05$ (compared with ob/ob); $n = 8$. ND, not detected.

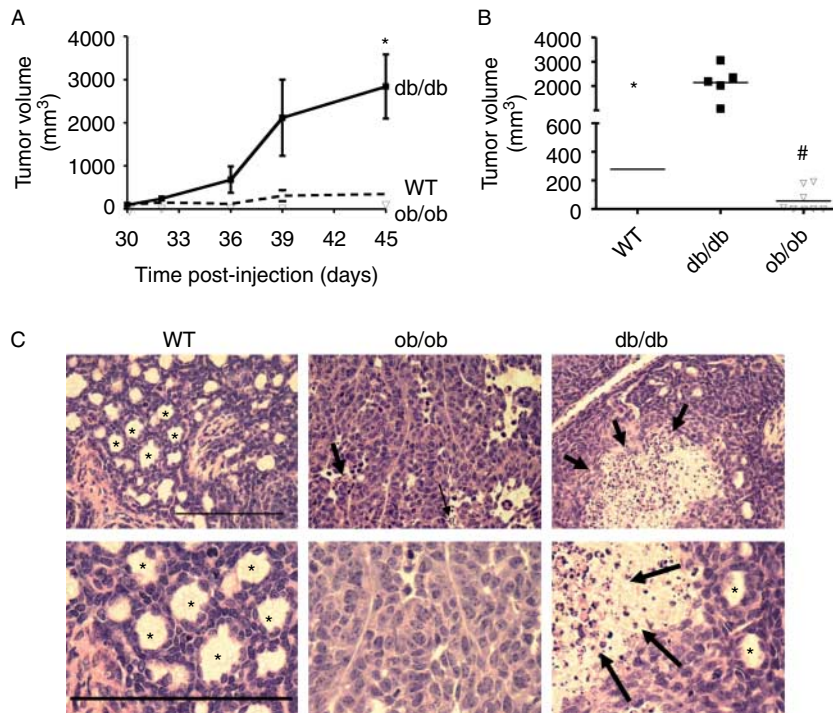


Figure 1 Effect of leptin on obesity induced tumor burden. Tumor cells (50 000) derived from spontaneous MMTV-Wnt-1 tumors were transplanted orthotopically within the 4th mammary fat pad of wild-type (WT), leptin receptor-deficient (db/db) mice, and leptin-deficient (ob/ob) mice. (A) Tumor growth was assessed in WT (open circle), ob/ob (open triangle), and db/db (filled square) mice by palpation. Data are presented as mean \pm s.e.m., $n=6-10$ /group. $*P<0.01$ db/db compared with either WT or ob/ob mice. (B) Tumors in ob/ob mice were not palpable but tumors were identified at necropsy and measured. Data presented as scatter dot plot and line indicates mean. $*P<0.05$ db/db compared with WT, $\#P<0.01$ db/db compared with ob/ob mice. (C) Pathological analysis of tumors from WT, ob/ob, and db/db mice. Hematoxylin and eosin stained sections were assessed where arrows indicate single cell apoptosis and asterisk indicate acini (bar = 50 μ m). Representative images shown at low and high magnification. Full colour version of this figure available via <http://dx.doi.org/10.1530/ERC-11-0102>.

reduction in keratin 6 immunopositive cells in tumors from ob/ob mice (Fig. 2A). Quantification of keratin 6 immunopositive cells indicated that tumors in ob/ob mice contained only one-third as many cells compared with WT tumors within an equivalent area (Fig. 2B). To control for the difference between the lean WT and obese ob/ob mice, we analyzed tumors from obese db/db mice for keratin 8, 14, and 6 (Fig. 2A). Quantification of the number of keratin 6 immunoreactive cells in tumors from db/db mice indicated they were not significantly different compared with WT mice, but were significantly different compared with the obese ob/ob mice. Because obesity in the ob/ob and db/db mice is virtually identical, these findings indicate that leptin is necessary for survival or maintenance of tumor cell populations that express keratin 6.

To query this further, we analyzed the expression and phosphorylation of Akt, an important kinase in the PI-3 kinase/mTOR growth-promoting signaling pathway, that is, activated by leptin (Myers *et al.*

2008), in tumors transplanted into WT, ob/ob, and db/db mice. We assessed total Akt in histological sections from all three tumors and determined that the kinase is ubiquitously expressed equivalently in tumors from all three mice (Fig. 2C, data for WT mice shown). On the contrary, activated/phosphorylated Akt (p-Akt) immunopositive cells exhibited leptin-dependent expression (Fig. 2C). Quantification of the sections indicated that fewer than 2% of the cells in tumors from ob/ob mice contained p-Akt immunopositive cells whereas 10–15% of the cells were immunopositive in tumors in WT and db/db mice (Fig. 2D). This suggests leptin-dependent activation of Akt in the MMTV-Wnt-1 tumors.

Tumors in leptin-deficient mice exhibit diminished tumor initiating activity

The decrease in p-Akt immunopositive cells combined with the decrease in undifferentiated keratin 6 immunopositive cells suggested tumors derived from ob/ob mice may be less tumorigenic (He *et al.* 2011).

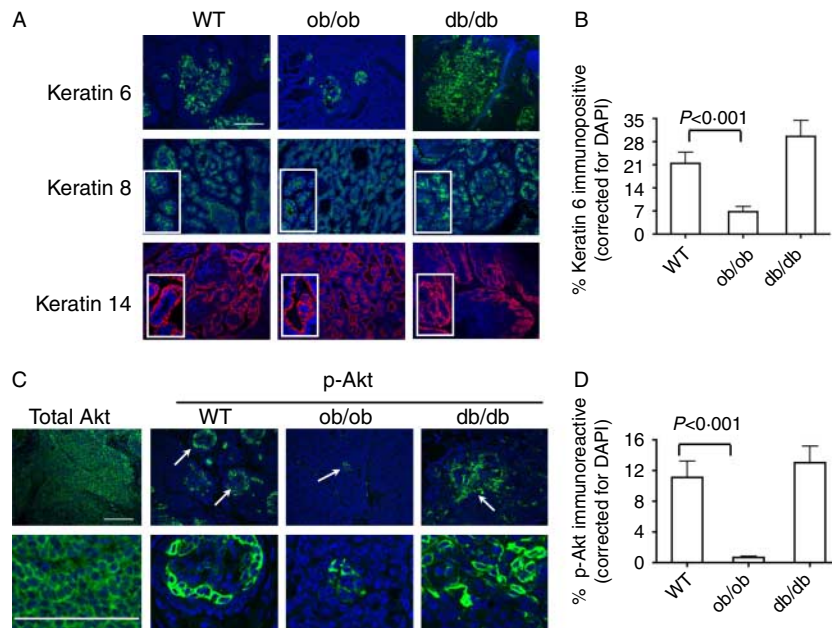


Figure 2 Leptin deficiency lead to reduced MMTV-Wnt-1 tumor cell populations and expression of leptin-dependent intracellular signaling protein Akt. (A) Immunofluorescence analysis of tumor cell markers in tumors from WT, ob/ob, and db/db mice. Formaldehyde-fixed tissues were sectioned and immunostained for expression of cellular markers keratin 6, 8, and 14. Representative images shown (bar = 100 μ m). Inset shows magnified view of acini in keratin 14 panels. (B) Quantification of keratin 6 immunoreactive cells in sections of tumors from WT, ob/ob, and db/db mice. Data are representative of an analysis repeated at least three times and presented as mean \pm S.E.M. (C) Immunofluorescence analysis of total and phosphorylated Akt in WT, ob/ob, and db/db mice. (D) Quantification of p-Akt immunopositive cells in tumors from WT, ob/ob, and db/db mice. Data are representative of an analysis repeated at least three times and presented as mean \pm S.E.M. Comparisons between the ob/ob and WT mice were performed on equivalent sized tumors. Full colour version of this figure available via <http://dx.doi.org/10.1530/ERC-11-0102>.

Thus, we assessed whether the residual tumors in leptin-deficient mice would yield reduced tumor outgrowth in an *in vivo* limiting dilution analysis. Dissociated cells from tumors that developed in WT (leptin replete) or ob/ob mice were transplanted in a limiting dilution series (50 000, 20 000, 5000, and 500 cells) into WT mice (Fig. 3A). Injection of 500 tumor cells was insufficient to generate secondary tumors, whether the cells arose from WT or leptin-deficient mice. The injection of 5000, 20 000, or 50 000 tumor cells from WT mice resulted in tumor outgrowth in 100% of tested mice (Table 2). At necropsy, the average tumor volume was 300–400 mm³ (Fig. 3B). On the contrary, the injection of 5000 tumor cells from ob/ob into WT mice resulted in no secondary tumors, and the injection of 20 000 or 50 000 cells from these mice resulted in tumors in 3/4 and 2/3 mice respectively (Table 2). At necropsy, the average tumor size was <50 mm³ (Fig. 3B). Extreme limiting dilution analysis (Hu & Smyth 2009) of the data in Table 2 indicated a stem cell frequency of 1/2100 for cells from WT mice and only 1/30 000 for cells from leptin-deficient mice, a highly significant ($P < 0.001$)

90% reduction in cancer stem cell (CSC) activity. Goodness of fit to a single-hit model was confirmed by a likelihood ratio test (Hu & Smyth 2009).

Identification of leptin sensitive tumor cells

To identify and quantify the populations of leptin-dependent tumor cells, we isolated cells from tumors derived from either WT or ob/ob mice and fluorescence activated cell sorting (FACS) analyzed them using integrin β 1 (CD29), α 6 (CD49f), and heat stable antigen (CD24) surface ligands/receptors for enrichment of cancer cell populations including lineage progenitors and CSCs (Mani et al. 2008, Charafe-Jauffret et al. 2009, Zhang et al. 2010). Cells were first depleted of CD45 and Ter119 cells by MACS and confirmed to be hematopoietic and endothelial cell lineage negative (lin^-) by FACS for Ter119, CD45, and CD31 (data not shown). Single and double staining controls for each antibody are shown in Fig. 4A and B respectively. In ob/ob mice, CD29⁺CD24⁻, CD29⁻CD24^{lo}, CD49f⁺CD24⁻, CD49f⁻CD24⁻CD29⁺CD49f^{Lo/Med}, and CD29⁻CD49f⁻ populations

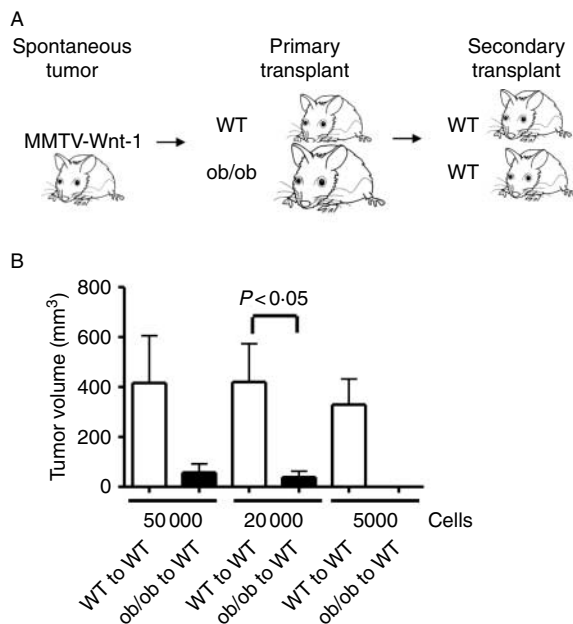


Figure 3 Leptin deficiency leads to reduced tumorigenesis. (A) Scheme for the evaluation tumors. 50 000 cells from spontaneous MMTV-Wnt-1 tumors were orthotopically transplanted into ob/ob and WT mice (primary transplant). Tumors, which developed in primary transplant mice were excised, cells isolated then orthotopically transplanted in a limiting dilution series into WT mice (secondary transplants) and monitored for tumor development. (B) Analysis of tumor volume of secondary transplant tumors at necropsy. Data are expressed as mean \pm S.E.M., $n=3-4$ /group. Statistical differences were determined by Student's *t*-test. Data are representative of an analysis that was repeated at least three times.

were significantly reduced compared with tumors from WT mice (circled, Fig. 4C). Table 3 shows the quantification of FACS dot plots from two independent experiments and shows a decrease in the leptin sensitive cancer cell populations derived from ob/ob mice. Collectively, these data indicate that leptin deficiency leads to selective reduction in cancer cell populations.

CD29⁺CD24⁻ population depleted by leptin deficiency express the full-length LepRb and exhibit stem cell behaviors

The reduction in tumor initiating potential in tumor cells derived from the leptin-deficient tumors suggested that the leptin-sensitive cells might exhibit CSC behavior *in vitro*. CSCs, also known as tumor initiating cells, found in blood and solid tumors (including breast), have emerged as a population of cancer cells in mice and humans that are highly tumorigenic (Rich & Eyley 2008, Rosen & Jordan 2009, Liu & Wicha 2010). We flow sorted tumors from WT mice and isolated the cells sensitive to leptin

deficiency, CD29⁺CD24⁻ and CD29⁻CD24⁺ (Fig. 4C and Table 3). The CD29⁺CD24⁻ cells are proposed to contain cells with CSC behavior (Mani *et al.* 2008, Zhang *et al.* 2010). On the contrary, the CD29⁻CD24⁺ cells, are considered to be differentiated cancer cells and are less tumorigenic (Zhang *et al.* 2010). We also isolated CD29^{Hi}CD24^{Hi} population because this population is considered enriched in CSCs, but were not sensitive to leptin depletion (Fig. 4C). CD29⁻CD24⁺, CD29⁺CD24⁻, and CD29^{Hi}CD24^{Hi} cells were sorted (Fig. 5A) plated on polylysine coated cover slips and immunostained for keratin 8 (luminal epithelial marker) and keratin 14 (basal/myoepithelial marker). We determined that CD29⁻CD24⁺ are primarily comprised tumor luminal epithelial cells with >80% of the DAPI-positive cells expressing keratin 8 (Fig. 5B and C). On the contrary, the CD29⁺CD24⁻ population contained an equal proportion of keratin 8 and 14 that totaled <20% of the total cancer cells (Fig. 5B and C). Importantly, nearly 50% of the CD29⁺CD24⁻ cells expressed keratin 6 (data not shown) that are reduced in tumor sections from leptin-deficient mice (Fig. 2A and B). The CD29^{Hi}CD24^{Hi} also contained an equal proportion of keratin 8 and 14 neoplastic cells and combined totaled about 60% of the population. This indicates that leptin deficiency leads to depletion of neoplastic cells both differentiated cells expressing keratin 8 and a more heterogeneous population with cells expressing keratin 8 and 14, as well as keratin 6.

Next, we determined whether the leptin-sensitive cells expressed the LepRb. RNA from each population was purified and RT-PCR performed to identify the functional LepRb (LepRb). We chose RT-PCR because the available antibodies are not suitable to differentiate functional and nonfunctional leptin receptor isoforms (Friedman 1998). RT-PCR analysis indicated that both CD29⁺CD24⁻ and CD29⁻CD24⁺ cells indeed expressed the functional LepRb, whereas the CD29^{Hi}CD24^{Hi} expressed low levels of LepRb mRNA (Fig. 5D). This indicates that the LepRb is expressed in subpopulations of cancer cells that require leptin for their survival or viability.

Table 2 Limiting dilution analysis of residual tumors in wild-type (WT) and ob/ob mice

	No. of cells transplanted				Stem cell freq.
	500	5000	20 000	50 000	
WT	0/3	3/3	4/4	3/3	1:2100
ob/ob	0/3	0/3	3/4	2/3	1:30 000

$P < 0.001$.

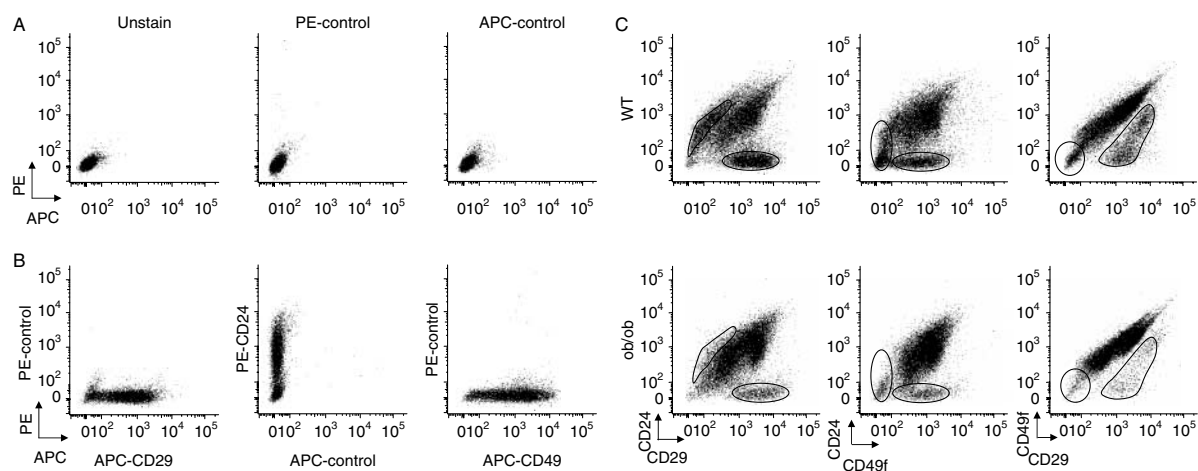


Figure 4 Leptin deficiency results in reduction in MMTV-Wnt-1 tumor cell subpopulations. Lineage negative (lin^{-}) tumor cells, depleted of immune and endothelial cells were isolated from WT and ob/ob mice. (A) FACS analysis of MMTV-Wnt-1 tumor lin^{-} cells unstained, or stained with APC- or PE control antibodies. Isotype control fluorescent labeled antibodies. (B) FACS analysis of MMTV-Wnt-1 tumor lin^{-} cells stained with APC-CD29 or APC-CD49f antibody with isotype control PE labeled antibody. lin^{-} cells were also stained for PE-CD24 with isotype control APC-labeled antibody. (C) lin^{-} cells from MMTV-Wnt-1 tumor outgrowth derived from WT and ob/ob mice were stained with APC-CD29, APC- or PE-49f, or PE-CD24 and analyzed by FACS. Cells exhibiting significant difference are boxed. Representative dot plots shown from an analysis that was repeated at least three times in mice injected independently. Panels show representative FACS dot plots (20 000 cells/panel).

Leptin increases the efficiency of tumorsphere formation in $CD29^{+}CD24^{-}$ cancer cells

To determine whether the LepRb in $CD29^{+}CD24^{-}$ enriched population is functional, we evaluated the effect of leptin on tumorsphere formation. Tumorsphere assays are a novel assay for prospective assessment of CSC behavior because under this harsh culture condition only the most tumorigenic cells are able to survive and develop into spheroids (Al-Hajj et al. 2003). $CD29^{+}CD24^{-}$, $CD29^{Hi}CD24^{Hi}$, and $CD29^{-}CD24^{+}$ populations were cultured in tumorsphere medium in the absence or presence of leptin. Both $CD29^{+}CD24^{-}$ and $CD29^{Hi}CD24^{Hi}$ population formed tumorspheres (Fig. 5E), however, only the $CD29^{+}CD24^{-}$ are leptin-sensitive exhibiting an increase in tumorsphere size (Fig. 5E) and number (Fig. 5F). We quantified the efficiency of tumorsphere generation in the $CD29^{+}CD24^{-}$ cell population and determined that leptin led to a twofold increase in tumorspheres compared with untreated cells (Fig. 5F). The $CD29^{-}CD24^{+}$ cells failed to form tumorspheres and died suggesting they are differentiated cancer cells.

Discussion

We report here that host-derived leptin, especially in hyperleptinemic db/db mice provides a survival and growth-promoting signal for cells capable of initiating

MMTV-Wnt-1 tumors. First, we show that leptin in obese mice promotes tumor growth in MMTV-Wnt-1 mice. Secondly, leptin deficiency leads to decreased tumor growth in obese mice. Thirdly, residual tumors have reduced p-Akt immunopositive cells and reduced populations of cells that express LepRb. Fourthly, leptin promotes an increase in efficiency of tumorsphere generation in CSC enriched populations. Finally, leptin deficiency results in functional depletion of CSCs leading to less tumor outgrowth in limiting dilution transplant studies suggesting therapeutic benefit of reducing leptin.

Leptin-deficient mice provide a powerful model to define the role of leptin on tumor growth (Thompson et al. 1983). Our findings provide compelling *in vivo* evidence that leptin deficiency suppresses mammary tumor growth in the MMTV-Wnt-1 transgenic model.

Table 3 Fluorescence-activated cell sorting analysis of residual tumors in wild-type (WT) and ob/ob mice

	Experiment 1		Experiment 2	
	WT (%)	ob/ob (%)	WT (%)	ob/ob (%)
$CD29^{+}CD24^{-}$	21	6	36	20
$CD29^{-}CD24^{lo}$	15	5	29	7
$CD49f^{+}CD24^{-}$	12	5	12	7
$CD49f^{-}CD24^{-}$	7	2	25	9
$CD29^{+}CD49f^{Lowmed}$	10	2	25	12
$CD29^{-}CD49f^{-}$	5	1	14	1

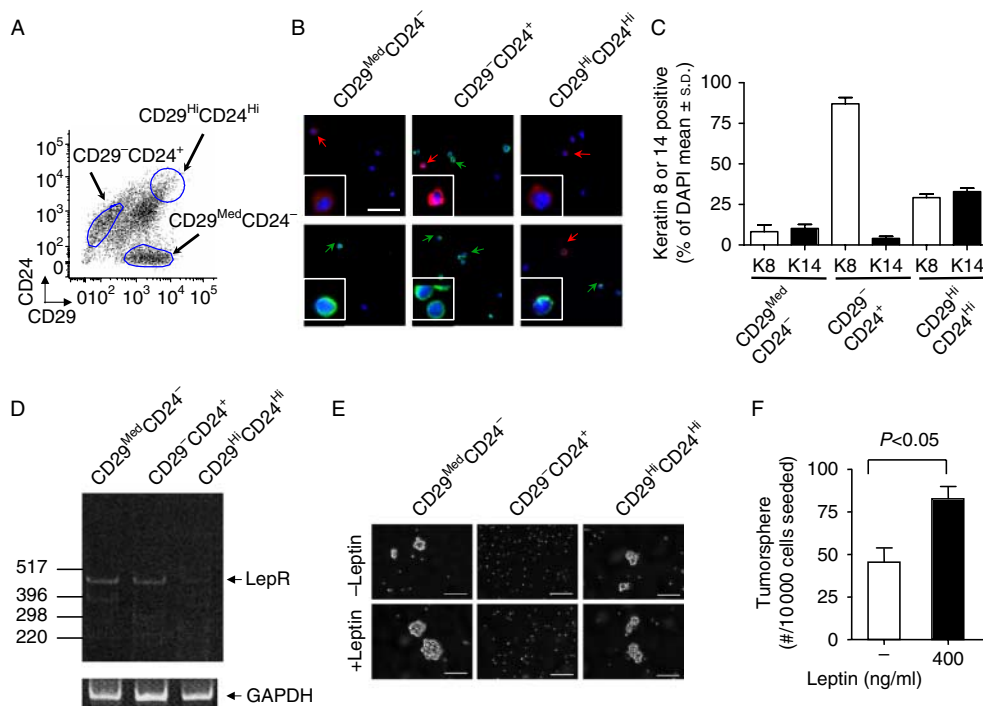


Figure 5 Leptin sensitive MMTV-Wnt-1 tumor cell populations express the leptin receptor and leptin promotes CSC survival *in vitro*. (A) Tumors from WT mice were isolated and lin^{-} cells sorted into the three distinct subpopulations based on expression of CD29 and CD24. Representative FACS dot plot identifying the three tumor cell populations is shown. The analysis was repeated at least three times. (B) Flow sorted cells were plated on polylysine coated cover slips, fixed, and immunostained for keratin 8 and 14, markers of luminal and basal/myoepithelial lineage respectively. Data shown are representative images from two fields of sorted cells, bar = 50 μ m. Green arrows denote keratin 8-positive cell and red arrows denote keratin 14-positive cell. Inset shows a 4 \times magnification of immunopositive cells. (C) Quantification of keratin 8 and 14 immunopositive cells in CD29⁺CD24⁺, CD29⁻CD24⁺, and CD29^{Hi}CD24^{Hi} lin^{-} cells. Ten microscopic fields were analyzed under epifluorescence microscope at 20 \times magnification. Data presented are % keratin 8 or 14 immunopositive cells corrected for total DAPI-positive nuclei (mean \pm s.d.). At least 200 DAPI-stained cells were counted from each population. (D) RT-PCR analysis for expression of the functional leptin receptor (LepRb) transcript from the flow sorted tumor cell populations. GAPDH mRNA was used as RNA control. (E) Effect of leptin on efficiency of tumorsphere formation from the CD29⁺CD24⁻, CD29^{Hi}CD24^{Hi}, and CD29⁻CD24⁺ enriched tumor cell populations. Cells were incubated in six-well low-binding plates in serum-free media containing EGF, FGF2, B27, hydrochloride, and insulin then supplemented without or with leptin (400 ng/ml) and monitored for tumorsphere generation. Images were obtained 5 days after tumorsphere cultures were initiated. Representative images shown. (F) Effect of leptin on the number of tumorspheres formed in CD29⁺CD24⁻ cells. Cells were seeded at 10 000 cells/well in low cell binding six-well plates and cultured in tumorsphere generating media either in the presence (400 ng/ml) or absence of leptin. Tumorspheres were quantified after 5 days of culture. Data represent mean \pm s.e.m. ($n=3$) from an experiment that was repeated at least three times. Student's *t*-test was used to test for significance.

Previous studies using the MMTV-TGF- α transgenic mice bred onto the leptin-deficient and LepRb-deficient genetic background suggested an *in vivo* link between leptin signaling and mammary tumors. In those studies, 30% of WT MMTV-TGF- α transgenic mice develop mammary tumors by 16 months of age, whereas double mutant TGF- α – leptin-deficient or – LepRb-deficient mice do not develop mammary tumors leading to the suggestion that leptin and its receptor are necessary for mammary tumorigenesis (Cleary *et al.* 2003, 2004). However, a potential problem in the interpretation of these findings is that LepRb- and leptin-deficient mice are infertile and fail to fully develop mammary glands (Hu *et al.* 2002), so

the mammary epithelial cells sensitive to oncogenic transformation never develop. Furthermore, because no tumors develop, these studies could not identify leptin's cellular targets or the *in vivo* mechanisms. Using an entirely different tumor model (MMTV-Wnt-1), we provide indisputable evidence for a role of circulating leptin in promoting mammary tumor growth as originally proposed by Cleary *et al.* (2003).

Our studies provide critical insights on leptin in regulating survival and maintenance of tumor cell populations *in vivo*. Leptin deficiency leads to depletion of select cell populations including cells that exhibit CSC behavior (Zhang *et al.* 2010). The CSCs were identified based on cell surface markers

used in the identification and enrichment of CSCs and nontumorigenic cells (Cho *et al.* 2008, Vaillant *et al.* 2008, Visvader & Smith 2010). In addition, we performed an *in vivo* serial dilution using the total tumor cell population from residual tumors in leptin-deficient and WT mice to determine the tumor initiating cell frequency. This assay is considered the ‘gold’ standard for assessing stem cells. Collectively, our studies provide the first definitive evidence that leptin is required for the viability of mammary CSCs *in vivo*.

The finding that leptin is required for CSC viability is highly significant because these cells have emerged as a population of highly tumorigenic cells in mice and humans (Rich & Eyster 2008, Rosen & Jordan 2009, Liu & Wicha 2010). CSCs are thought to underlie cancer initiation, recurrence, and metastasis in some but not all tumors (Al-Hajj *et al.* 2003, Rosen & Jordan 2009). In favorable microenvironments, the CSCs self-renew, proliferate, and can differentiate to cells that comprise the bulk of the tumor mass (Nusse *et al.* 2008, Charafe-Jauffret *et al.* 2009). These cells appear to evade immune surveillance, promote angiogenesis, and resist chemo- and radiotherapy (Chen *et al.* 2007, Diehn *et al.* 2009, Zhang *et al.* 2010). While current therapeutic approaches can eliminate or decrease the bulk of the tumor, it is proposed that CSCs can regrow and contribute to tumor resistance and progression (Liu & Wicha 2010). Thus, strategies are needed to identify targets and therapeutics for this specific population (Rich & Eyster 2008, Diehn *et al.* 2009).

Recent studies from the Scherer laboratory provide additional insights to the LepRb-breast cancer link. Their findings indicate that mice with LepRb deletion in the periphery with functional LepRb in the brain exhibit normal mammary gland development. However, MMTV-PyMT transgenic mice bred onto the peripheral LepRb-null mice leads to reduced tumor burden (Park *et al.* 2010). Their findings, like ours, indicate that peripheral LepRb are necessary to promote tumor progression.

These studies implicate leptin as a link between obesity and breast cancer. Despite the fact that obesity is likely to involve multiple factors such as insulin and IGF1 known to stimulate tumor growth, our paradigm provides clear evidence for an important role for leptin in tumorigenesis. In fact, our studies indicate that significant reduction in tumor growth can be achieved by reducing circulating leptin levels. This suggests that interference with the leptin–LepRb-breast cancer link may provide important therapeutic benefit

in controlling breast cancer. This complements the findings of Cao *et al.* (2010) showing that reduced circulating leptin leads to reduced tumor burden in mice. Our studies add critical insights by isolating and identifying the tumor cells sensitive to leptin deficiency. The identification and isolation of these LepRb tumor cells provides molecular and cellular targets for developing intervention strategies.

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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