Published in final edited form as:

Endocr Relat Cancer. 2011 August ; 18(4): 491–503. doi:10.1530/ERC-11-0102.

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

Qiao Zheng¹, Sarah M. Dunlap², Jinling Zhu¹, Erinn Downs-Kelly⁴, Jeremy N. Rich⁵, Stephen D. Hursting^{2,3}, Nathan A. Berger⁶, and Ofer Reizes¹

¹Department of Cell Biology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH

²Department of Nutritional Sciences, The University of Texas, Austin, TX

³Department of Carcinogenesis, The University of Texas MD Anderson Cancer Center, Smithville, TX

⁴Department of Anatomic Pathology, Cleveland Clinic Foundation, Cleveland, OH

⁵Department of Stem Cell Biology and Regenerative Medicine, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH

⁶Case Comprehensive Cancer Center, Case Western Reserve University School of Medicine, Cleveland, OH

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 (MMTV)-Wnt-1 transgenic mice, we show that tumors transplanted into obese leptin-receptor deficient (db/db) mice grow to 8-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 to WT or db/db mice. Further, tumors in ob/ob mice contain fewer cells expressing phosphorylated Akt, a growth promoting kinase activated by the leptin receptor (LepRb), compared to 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.

To whom correspondence should be addressed: Ofer Reizes, Ph.D., Department of Cell Biology, Lerner Research Institute, Cleveland Clinic Foundation, 9500 Euclid Avenue, NC10, Cleveland, OH 44195, Phone: 216-445-0880, FAX: 216-444-9404, reizeso@ccf.org.

Declaration of interest:

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Introduction

Today, over 25% of the US population is either obese or morbidly obese, a condition of excess adipose tissue and fat (Baskin et al. 2005, Flegal et al. 2004, Flegal et al. 2002, Haslam and 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). Further, obese patients are at greater risk of tumor recurrence and metastasis resulting in poor overall survival (Huber et al. 2009, Loi et al. 2005).

Breast cancer is the second leading cause of cancer death in women in the US (American 2009) and obesity increases the mortality risk of luminal type A 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 epidermal growth factor receptor 2 (HER-2), (Cheang et al. 2008, Millikan et al. 2008), and are highly invasive or metastatic (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, 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 X. 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, Vona-Davis and Rose 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 (Garofalo et al. 2006, Ishikawa et al. 2004, Maccio et al. 2010, Miyoshi et al. 2006). Likewise, *in vitro* cellular studies indicate that leptin promotes breast cancer cell proliferation, migration, invasion, and induction of angiogenesis (Fiorio et al. 2008, Gonzalez et al. 2006, Rene Gonzalez et al. 2009, Saxena et al. 2008). Collectively, these data led us to hypothesize that tumors expressing functional leptin receptors 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 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 (Brown 2001, Li et al. 2000). 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 (Khramtsov et al. 2010). Herein, we validate this hypothesis and show that leptin deficiency leads to loss of tumor cells that express leptin receptor 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), 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, and keratin 6 from Covance (Princeton, New Jersey). All secondary antibodies including Alexa fluor 488 and 568 goat anti-rabbit, goat or mouse antibodies, were purchased from Molecular Probes (Eugene, OR).

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), leptin receptor-deficient (B6.BKS(D)-*Lepr^{db}/J*; db/db) and control wild type (WT, C57Bl/6J) mice were purchased from the Jackson Laboratory. The Murine Mammary Tumor Virus (MMTV)-Wnt-1 mice, the source of the mammary tumor cells, were kindly provided by Dr. Stephen Hursting, coauthor on these studies (Nunez et al. 2008). The genetic defect 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, CA) supplemented with 0.28 Wünsch units/ml liberase TH and TM (Roche, Germany) and 100 Kunitz units/ml deoxyribonuclease I (Sigma). Samples were pipetted every 20 min while shaking. Cells were passed through 40 µm filter and centrifuged at 1000 rpm for 10 min. Cell pellets were resuspended in ACK buffer (Invitrogen) and red blood cell lysed for 1 min at room temperature. 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/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 [volume = $0.52 \times (\text{width}) \times (\text{height}) \times (\text{length})$] for approximating the volume of a spheroid. Prior to tumors reaching 1.5 cm³, 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, 5,000, or 500 tumor cells injected into recipient WT mice.

Flow cytometry

Cells were counted and resuspended in PBS containing 0.5% BSA and 2mM EDTA (BSA solution) per 10⁷ total cells. Cells were purified on CD45 and Ter119 by magnetic-activated cell sorting (MACS) on LD-column microbeads (Milteny Biotec). 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, CA), 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, CA). Data analysis was performed on the FlowJo version 8.8.6 software (Tree Star, Inc).

Immunofluorescence analysis

Sections were deparaffinized by Histoclear (National Diagnostics, Atlanta, Georgia) and rehydrated in graded alcohols. After Antigen Retrieval, sections were blocked with 2% goat serum in PBS for 1h at room temperature and then incubated overnight with antibody to keratin 14, keratin 8, and keratin 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).

Immunofluorescence analysis of flow sorted lin⁻ cells

Cells were plated on poly-L-Lysine (0.01 %, Sigma, MO) coated glass coverslips for 1 hr then fixed with 4% paraformaldehyde, permeabilized with 0.05% Triton X-100, and blocked with 3% milk in PBS for 30 min at room temperature (RT). Cells were incubated overnight at 4°C with antibody to keratin 14, keratin 8, or keratin 6 in 1% milk/PBS. The next day, coverslips were washed and incubated for 1 hr 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, keratin 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) and cDNA was synthesized using high capacity cDNA reverse transcription kit with RNase inhibitor (ABI). Amplification of transcripts was performed with 200 ng of cDNA. The primer sets were:

Mouse GAPDH forward: 5'-GCACAGTCAAGGCCGAGAAT-3'

Mouse GAPDH reverse: 5'-GCCTTCTCCATGGTGGTGAA-3'

Mouse Leptin receptor forward: 5'-GAAGATGTTCCGAACCCCAAGAATTGTTCC-3'

Mouse Leptin receptor reverse: 5'-GCACTTGGTGACTGAACTATTATAAGCCC-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), 2% B27 (Invitrogen), 10 µg/ml Insulin and 1 µg/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 ± SD or SEM as indicated in the figure legend. Statistical analysis was performed using a Student's *t*-test. Significance was set at *p* = 0.05. All microscopic images were prepared by using Adobe Photoshop CS2 adjusted for brightness/contrast. Stem cell frequencies for each transplanted population and *p* values were calculated

using ELDA: Extreme Limiting Dilution Analysis software based on the algorithm defined by Hu and Smyth (Hu Y. and Smyth 2009). We confirmed that our data fits a single-hit linear model assumption using 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 leptin receptor-deficient (db/db) mice develop early onset obesity that is virtually identical with respect to metabolic, inflammatory, and growth characteristics (Table 1) (Hummel et al. 1966, Ingalls et al. 1950, Spiegelman and 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) that expresses the leptin receptor. 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 wild type (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). Further, tumor growth rate and burden was significantly increased, with tumors reaching 8 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 in 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 mm³ 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. In contrast, 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 formation.

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 reduction of 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 to 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 to WT mice, but were significantly different compared to 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, in tumors transplanted into WT, ob/ob, and db/db mice (Myers et al. 2008). 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). In contrast, 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 phosphorylated 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). 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, 5,000, 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 5,000, 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 to 400 mm³ (Fig. 3B). In contrast, the injection of 5,000 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 less than 50 mm³ (Fig. 3B). Extreme limiting dilution analysis (ELDA) (Hu Y. and Smyth 2009) of the data in Table 2 indicated a stem cell frequency of 1/2,100 for cells from wild-type mice and only 1/30,000 for cells from leptin-deficient mice, a highly significant ($p < 0.001$) 90% reduction in CSC activity. Goodness of fit to a single hit model was confirmed using a likelihood ratio test (Hu Y. and Smyth 2009).

Identification of leptin sensitive tumor cells

To identify and quantify the populations of leptin-dependent tumor cells, we isolated cells from tumors either derived from WT or ob/ob mice and FACS analyzed them using integrin

$\beta 1$ (CD29), $\alpha 6$ (CD49f), and heat stable antigen (CD24) surface ligands/receptors for enrichment of cancer cell populations including lineage progenitors and CSCs (Charafe-Jauffret et al. 2009, Mani et al. 2008, Zhang et al. 2010). Cells were first depleted of CD45 and Ter119 cells by magnetic-activated cell sorting 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, $\text{CD29}^+\text{CD24}^-$, $\text{CD29}^-\text{CD24}^{\text{lo}}$, $\text{CD49f}^+\text{CD24}^-$, $\text{CD49f}^-\text{CD24}^-$, $\text{CD29}^+\text{CD49f}^{\text{Lo/Med}}$, and $\text{CD29}^-\text{CD49f}^-$ populations were significantly reduced compared to tumors from WT mice (circled, Fig. 4C). Table 3 shows the quantification of FACS dot plots from 2 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 leptin receptor 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 cancer stem cell (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 (Liu and Wicha 2010, Rich and Eyler 2008, Rosen and Jordan 2009). CSCs are thought to underlie cancer initiation, recurrence, and metastasis in some but not all tumors (Al-Hajj et al. 2003, Rosen and Jordan 2009). We flow sorted tumors from WT mice and isolated the cells sensitive to leptin deficiency, $\text{CD29}^+\text{CD24}^-$ and $\text{CD29}^-\text{CD24}^+$ (Fig. 4C, Table 3). The $\text{CD29}^+\text{CD24}^-$ cells are proposed to contain cells with CSC behavior (Mani et al. 2008, Zhang et al. 2010). In contrast, the $\text{CD29}^-\text{CD24}^+$ cells, are considered to be differentiated cancer cells and are less tumorigenic (Zhang et al. 2010). We also isolated $\text{CD29}^{\text{Hi}}\text{CD24}^{\text{Hi}}$ population because this population is considered enriched in CSCs, but were not sensitive to leptin depletion (Fig. 4C). $\text{CD29}^-\text{CD24}^+$, $\text{CD29}^+\text{CD24}^-$, and $\text{CD29}^{\text{Hi}}\text{CD24}^{\text{Hi}}$ cells were sorted (Fig. 5A) plated on poly-lysine coated coverslips and immunostained for keratin 8 (luminal epithelial marker) and keratin 14 (basal/myoepithelial marker). We determined that $\text{CD29}^-\text{CD24}^+$ are primarily comprised of tumor luminal epithelial cells with >80% of the DAPI positive cells expressing keratin 8 (Fig. 5B and C). In contrast, the $\text{CD29}^+\text{CD24}^-$ population contained an equal proportion of keratin 8 and keratin 14 that totaled less than 20% of the total cancer cells (Fig. 5B and C). Importantly, nearly 50% of the $\text{CD29}^+\text{CD24}^-$ cells expressed keratin 6 (data not shown) that are reduced in tumor sections from leptin-deficient mice (Fig. 2). The $\text{CD29}^{\text{Hi}}\text{CD24}^{\text{Hi}}$ 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 leptin receptor. RNA from each population was purified and RT-PCR performed to identify the functional leptin receptor (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 $\text{CD29}^+\text{CD24}^-$ and $\text{CD29}^-\text{CD24}^+$ cells indeed expressed the functional leptin receptor (LepRb), whereas the $\text{CD29}^{\text{Hi}}\text{CD24}^{\text{Hi}}$ 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.

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 formation is 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 2-fold increase in tumorspheres compared to 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. Second, leptin deficiency leads to decreased tumor growth in obese mice. Third, residual tumors have reduced p-Akt immunopositive cells and reduced populations of cells that express leptin receptors. Fourth, 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. Previous studies using the MMTV-TGF- α transgenic mice bred onto the leptin-deficient and leptin receptor-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 - leptin receptor-deficient mice do not develop mammary tumors leading to the suggestion that leptin and its receptor are necessary for mammary tumorigenesis (Cleary et al. 2004, Cleary et al. 2003). However, a potential problem in the interpretation of these findings is that leptin receptor- and leptin-deficient mice are infertile and fail to fully develop mammary glands (Hu X. et al. 2002), so the mammary epithelial cells sensitive to oncogenic transformation never develop. Further, 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 (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 CSC cells 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 and 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 (Liu and Wicha 2010, Rich and Eyler 2008, Rosen and Jordan 2009). CSCs are thought to underlie cancer initiation, recurrence, and metastasis in some but not all tumors (Al-Hajj et al. 2003, Rosen and Jordan 2009). In favorable microenvironments, the CSCs self-renew, proliferate, and can differentiate to cells that comprise the bulk of the tumor mass (Charafe-Jauffret et al. 2009, Nusse et al. 2008). 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 and Wicha 2010). Thus, strategies are needed to identify targets and therapeutics for this specific population (Diehn et al. 2009, Rich and Eyler 2008).

Recent studies from the Scherer laboratory provide additional insights to the LepRb-breast cancer link. Their findings indicate that mice with leptin-receptor deletion in the periphery with functional LepRb in the brain exhibit normal mammary gland development. However, MMTV-PyMT transgenic mice bred onto the peripheral leptin receptor null mice leads to reduced tumor burden (Park et al. 2010). Their findings like ours indicate that peripheral leptin receptors 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 IGF-1 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-leptin receptor-breast cancer link may provide important therapeutic benefit in controlling breast cancer.

Our studies suggest that inhibition of leptin signaling may provide therapeutic benefit particularly in targeting the CSC cells. This complements the findings of Cao et al. showing that reduced circulating leptin leads to reduced tumor burden in mice (Cao et al. 2010). Our studies add critical insights by isolating and identifying the tumor cells sensitive to leptin deficiency. The identification and isolation of these leptin receptor tumor cells provides molecular and cellular targets for developing intervention strategies.

Acknowledgments

Funding:

The research was supported by development grants from the NIH-NCI Transdisciplinary Research in Energetics and Cancer (U54 CA116867) and the Scott Hamilton CARES Foundation to OR. SH and SMP acknowledge the support of the Breast Cancer Research Foundation and DOD Post-doctoral Fellowship, respectively.

The authors would like to thank Dr. Lyuba Varticovski, National Cancer Institute, who developed the Wnt-1 tumor cell suspension, Lauren Malone (Hursting Laboratory) for technical assistance, and Drs. Thomas Egelhoff and Maria Febbraio at CCF and Dr. Ruth Keri at Case Western Reserve University School of Medicine for critical review of the manuscript.

References

- Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A*. 2003; 100:3983–3988. [PubMed: 12629218]

- American, CS. Cancer Facts and Figures 2009. Atlanta: American Cancer Society; 2009.
- Baskin ML, Ard J, Franklin F, Allison DB. Prevalence of obesity in the United States. *Obes Rev*. 2005; 6:5–7. [PubMed: 15655032]
- Brakenhielm E, Cao R, Gao B, Angelin B, Cannon B, Parini P, Cao Y. Angiogenesis inhibitor, TNP-470, prevents diet-induced and genetic obesity in mice. *Circ Res*. 2004; 94:1579–1588. [PubMed: 15155527]
- Brown AM. Wnt signaling in breast cancer: have we come full circle? *Breast Cancer Res*. 2001; 3:351–355. [PubMed: 11737884]
- Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med*. 2003; 348:1625–1638. [PubMed: 12711737]
- Cao L, Liu X, Lin EJ, Wang C, Choi EY, Riban V, Lin B, Duing MJ. Environmental and genetic activation of a brain-adipocyte BDNF/leptin axis causes cancer remission and inhibition. *Cell*. 2010; 142:52–64. [PubMed: 20603014]
- Charafe-Jauffret E, et al. Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature. *Cancer Res*. 2009; 69:1302–1313. [PubMed: 19190339]
- Cheang MC, Voduc D, Bajdik C, Leung S, McKinney S, Chia SK, Perou CM, Nielsen TO. Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype. *Clin Cancer Res*. 2008; 14:1368–1376. [PubMed: 18316557]
- Chen MS, Woodward WA, Behbod F, Peddibhotla S, Alfaro MP, Buchholz TA, Rosen JM. Wnt/beta-catenin mediates radiation resistance of Sc1+ progenitors in an immortalized mammary gland cell line. *J Cell Sci*. 2007; 120:468–477. [PubMed: 17227796]
- Cho RW, Wang X, Diehn M, Shedden K, Chen GY, Sherlock G, Gurney A, Lewicki J, Clarke MF. Isolation and molecular characterization of cancer stem cells in MMTV-Wnt-1 murine breast tumors. *Stem Cells*. 2008; 26:364–371. [PubMed: 17975224]
- Cleary MP, Juneja SC, Phillips FC, Hu X, Grande JP, Maihle NJ. Leptin receptor-deficient MMTV-TGF-alpha/Lepr(db)Lepr(db) female mice do not develop oncogene-induced mammary tumors. *Exp Biol Med* (Maywood). 2004; 229:182–193. [PubMed: 14734797]
- Cleary MP, Phillips FC, Getzin SC, Jacobson TL, Jacobson MK, Christensen TA, Juneja SC, Grande JP, Maihle NJ. Genetically obese MMTV-TGF-alpha/Lep(ob)Lep(ob) female mice do not develop mammary tumors. *Breast Cancer Res Treat*. 2003; 77:205–215. [PubMed: 12602920]
- Coleman DL. A historical perspective on leptin. *Nat Med*. 2010; 16:1097–1099. [PubMed: 20930752]
- Diehn M, Cho RW, Clarke MF. Therapeutic implications of the cancer stem cell hypothesis. *Semin Radiat Oncol*. 2009; 19:78–86. [PubMed: 19249645]
- Dogan S, Hu X, Zhang Y, Maihle NJ, Grande JP, Cleary MP. Effects of high-fat diet and/or body weight on mammary tumor leptin and apoptosis signaling pathways in MMTV-TGF-alpha mice. *Breast Cancer Res*. 2007; 9:R91. [PubMed: 18162139]
- Fiorio E, et al. Leptin/HER2 crosstalk in breast cancer: in vitro study and preliminary in vivo analysis. *BMC Cancer*. 2008; 8:305. [PubMed: 18945363]
- Flegal KM, Ogden CL, Carroll MD. Prevalence and trends in overweight in Mexican-american adults and children. *Nutr Rev*. 2004; 62:S144–S148. [PubMed: 15387481]
- Flegal KM, Carroll MD, Ogden CL, Johnson CL. Prevalence and trends in obesity among US adults, 1999–2000. *Jama*. 2002; 288:1723–1727. [PubMed: 12365955]
- Friedman JM. Leptin, leptin receptors, and the control of body weight. *Nutr Rev*. 1998; 56:s38–s46. discussion s54–75. [PubMed: 9564176]
- Garofalo C, Koda M, Cascio S, Sulkowska M, Kanczuga-Koda L, Golaszewska J, Russo A, Sulkowski S, Surmacz E. Increased expression of leptin and the leptin receptor as a marker of breast cancer progression: possible role of obesity-related stimuli. *Clin Cancer Res*. 2006; 12:1447–1453. [PubMed: 16533767]
- Gonzalez RR, et al. Leptin signaling promotes the growth of mammary tumors and increases the expression of vascular endothelial growth factor (VEGF) and its receptor type two (VEGF-R2). *J Biol Chem*. 2006; 281:26320–26328. [PubMed: 16825198]
- Halberg N, Wernstedt-Asterholm I, Scherer PE. The adipocyte as an endocrine cell. *Endocrinol Metab Clin North Am*. 2008; 37:753–768. x–xi. [PubMed: 18775362]

- Haslam DW, James WP. Obesity. *Lancet*. 2005; 366:1197–1209. [PubMed: 16198769]
- He X, Wang Y, Zhu J, Orloff M, Eng C. Resveratrol enhances the anti-tumor activity of the mTOR inhibitor rapamycin in multiple breast cancer cell lines mainly by suppressing rapamycin-induced AKT signaling. *Cancer Lett*. 2011; 301:168–176. [PubMed: 21168265]
- Herschkowitz JI, et al. Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. *Genome Biol*. 2007; 8:R76. [PubMed: 17493263]
- Hu X, Juneja SC, Maihle NJ, Cleary MP. Leptin--a growth factor in normal and malignant breast cells and for normal mammary gland development. *J Natl Cancer Inst*. 2002; 94:1704–1711. [PubMed: 12441326]
- Hu Y, Smyth GK. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. *J Immunol Methods*. 2009; 347:70–78. [PubMed: 19567251]
- Huber KE, Carey LA, Wazer DE. Breast cancer molecular subtypes in patients with locally advanced disease: impact on prognosis, patterns of recurrence, and response to therapy. *Semin Radiat Oncol*. 2009; 19:204–210. [PubMed: 19732684]
- Hummel KP, Dickie MM, Coleman DL. Diabetes, a new mutation in the mouse. *Science*. 1966; 153:1127–1128. [PubMed: 5918576]
- Ingalls AM, Dickie MM, Snell GD. Obese, a new mutation in the house mouse. *J Hered*. 1950; 41:317–318. [PubMed: 14824537]
- Ishikawa M, Kitayama J, Nagawa H. Enhanced expression of leptin and leptin receptor (OB-R) in human breast cancer. *Clin Cancer Res*. 2004; 10:4325–4331. [PubMed: 15240518]
- Khrantsov AI, Khrantsova GF, Tretiakova M, Huo D, Olopade OI, Goss KH. Wnt/beta-catenin pathway activation is enriched in basal-like breast cancers and predicts poor outcome. *Am J Pathol*. 2010; 176:2911–2920. [PubMed: 20395444]
- Li Y, Hively WP, Varmus HE. Use of MMTV-Wnt-1 transgenic mice for studying the genetic basis of breast cancer. *Oncogene*. 2000; 19:1002–1009. [PubMed: 10713683]
- Liu S, Wicha MS. Targeting Breast Cancer Stem Cells. *J Clin Oncol*. 2010
- Loi S, Milne RL, Friedlander ML, McCredie MR, Giles GG, Hopper JL, Phillips KA. Obesity and outcomes in premenopausal and postmenopausal breast cancer. *Cancer Epidemiol Biomarkers Prev*. 2005; 14:1686–1691. [PubMed: 16030102]
- Maccio A, Madeddu C, Gramignano G, Mulas C, Floris C, Massa D, Astara G, Chessa P, Mantovani G. Correlation of body mass index and leptin with tumor size and stage of disease in hormone-dependent postmenopausal breast cancer: preliminary results and therapeutic implications. *J Mol Med*. 2010
- Mani SA, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell*. 2008; 133:704–715. [PubMed: 18485877]
- Millikan RC, et al. Epidemiology of basal-like breast cancer. *Breast Cancer Res Treat*. 2008; 109:123–139. [PubMed: 17578664]
- Miyoshi Y, Funahashi T, Tanaka S, Taguchi T, Tamaki Y, Shimomura I, Noguchi S. High expression of leptin receptor mRNA in breast cancer tissue predicts poor prognosis for patients with high, but not low, serum leptin levels. *Int J Cancer*. 2006; 118:1414–1419. [PubMed: 16206269]
- Myers MG, Cowley MA, Munzberg H. Mechanisms of leptin action and leptin resistance. *Annu Rev Physiol*. 2008; 70:537–556. [PubMed: 17937601]
- Nunez NP, Perkins SN, Smith NC, Berrigan D, Berendes DM, Varticovski L, Barrett JC, Hursting SD. Obesity accelerates mouse mammary tumor growth in the absence of ovarian hormones. *Nutr Cancer*. 2008; 60:534–541. [PubMed: 18584488]
- Nusse R, Fuerer C, Ching W, Harnish K, Logan C, Zeng A, ten Berge D, Kalani Y. Wnt signaling and stem cell control. *Cold Spring Harb Symp Quant Biol*. 2008; 73:59–66. [PubMed: 19028988]
- Ogden CL, Carroll MD, Curtin LR, McDowell MA, Tabak CJ, Flegal KM. Prevalence of overweight and obesity in the United States, 1999–2004. *Jama*. 2006; 295:1549–1555. [PubMed: 16595758]
- Park J, Kusminski CM, Chua SC, Scherer PE. Leptin receptor signaling supports cancer cell metabolism through suppression of mitochondrial respiration in vivo. *Am J Pathol*. 2010; 177:3133–3144. [PubMed: 21056997]

- Rene Gonzalez R, Watters A, Xu Y, Singh UP, Mann DR, Rueda BR, Penichet ML. Leptin-signaling inhibition results in efficient anti-tumor activity in estrogen receptor positive or negative breast cancer. *Breast Cancer Res.* 2009; 11:R36. [PubMed: 19531256]
- Rich JN, Eyler CE. Cancer stem cells in brain tumor biology. *Cold Spring Harb Symp Quant Biol.* 2008; 73:411–420. [PubMed: 19329578]
- Rosen JM, Jordan CT. The increasing complexity of the cancer stem cell paradigm. *Science.* 2009; 324:1670–1673. [PubMed: 19556499]
- Saxena NK, Taliaferro-Smith L, Knight BB, Merlin D, Anania FA, O'Regan RM, Sharma D. Bidirectional Crosstalk between Leptin and Insulin-like Growth Factor-I Signaling Promotes Invasion and Migration of Breast Cancer Cells via Transactivation of Epidermal Growth Factor Receptor. *Cancer Res.* 2008; 68:9712–9722. [PubMed: 19047149]
- Spiegelman BM, Flier JS. Obesity and the regulation of energy balance. *Cell.* 2001; 104:531–543. [PubMed: 11239410]
- Surmacz E. Obesity hormone leptin: a new target in breast cancer? *Breast Cancer Res.* 2007; 9:301. [PubMed: 17274833]
- Thompson CI, Kreider JW, Black PL, Schmidt TJ, Margules DL. Genetically obese mice: resistance to metastasis of B16 melanoma and enhanced T-lymphocyte mitogenic responses. *Science.* 1983; 220:1183–1185. [PubMed: 6602379]
- Vaillant F, Asselin-Labat ML, Shackleton M, Forrest NC, Lindeman GJ, Visvader JE. The mammary progenitor marker CD61/beta3 integrin identifies cancer stem cells in mouse models of mammary tumorigenesis. *Cancer Res.* 2008; 68:7711–7717. [PubMed: 18829523]
- Visvader JE, Smith GH. Murine Mammary Epithelial Stem Cells: Discovery, Function, and Current Status. *Cold Spring Harb Perspect Biol.* 2010
- Vona-Davis L, Rose DP. Adipokines as endocrine, paracrine, and autocrine factors in breast cancer risk and progression. *Endocr Relat Cancer.* 2007; 14:189–206. [PubMed: 17639037]
- Zhang M, Atkinson RL, Rosen JM. Selective targeting of radiation-resistant tumor-initiating cells. *Proc Natl Acad Sci U S A.* 2010; 107:3522–3527. [PubMed: 20133717]

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 mice (ob/ob). A. Tumor growth was assessed in WT (open circle), ob/ob (open triangle), and db/db (filled square) mice by palpation. Data presented are mean $\pm$ SEM. n = 6–10/group. * p < 0.01 db/db compared to 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 to WT, # p < 0.01 db/db compared to ob/ob mice. C. Pathological analysis of tumors from WT, ob/ob, and db/db mice. Representative hematoxylin and eosin sections were assessed. Arrows indicate single cell apoptosis and asterisk indicate acini (Bar = 50 μ m).

Figure 2. Leptin-deficiency leads 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, db/db mice. Data are representative of an analysis repeated at least three times and presented as mean $\pm$ SEM. 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 $\pm$ SEM. Comparisons between the ob/ob and WT mice were performed on equivalent sized tumors.

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 mean $\pm$ S.E.M., $n = 3 - 4$ /group. Statistical differences were determined using Student's *t*-test. Data are representative of an analysis that was repeated at least three times.

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 db/db mice were stained with APC-CD29, APC- or PE-49f, or PE-CD24 and analyzed by FACS. Cells exhibiting significant difference are boxed. Representative dotplots shown from an analysis that was repeated at least 3 times in

mice injected independently. Panels show representative FACS dot plots (20,000 cells/panel).

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 3 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 poly-lysine coated coverslips, 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 μm . Green arrows denote keratin 8 positive cell and red arrows denote keratin 14 positive cell. Inset shows a 4X magnification of immunopositive cells. C. Quantification of keratin 8 and 14 immunopositive cells in CD29^{Med}CD24⁺, CD29^{Med}CD24⁻, and CD29^{Hi}CD24^{Hi} lin^- cells. 10 microscopic fields were analyzed under epifluorescence microscope at 20X magnification. Data presented are % keratin 8 or keratin 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^{Med}CD24⁻, CD29^{Hi}CD24^{Hi}, and CD29^{Med}CD24⁺ enriched tumor cell populations. Cells were incubated in 6-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 6-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 $\pm$ SEM (n = 3) from an experiment that was repeated at least three times. Student's *t*-test was used to test for significance.

Table 1

Circulating levels of leptin, insulin and adiponectin in 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±3.04 *	33.8±13.6	5.1±3.2 **	n.d.
db/db	56.6±2.63 *	30.4±7.7	15.0±9.1 ***#	24.4±12 **

* $p < 0.001$ (compared to WT);** $p < 0.0001$ (compared to WT);# $p > 0.05$ (compared to ob/ob);

n = 8;

n.d.: not detected

Table 2

Limiting dilution analysis of residual tumors in WT and ob/ob mice

	No. of cells transplanted				Stem Cell Freq.
	500	5,000	20,000	50,000	
WT	0/3	3/3	4/4	3/3	1:2,100
ob/ob	0/3	0/3	3/4	2/3	1:30,000

$p < 0.001$

Table 3

FACS analysis of residual tumors in 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%