Leptin receptor maintains cancer stem-like properties in triple negative breast cancer cells

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Abstract

Despite new therapies, breast cancer continues to be the second leading cause of cancer mortality in women, a consequence of recurrence and metastasis. In recent years, a population of cancer cells has been identified, called cancer stem cells (CSCs) with self-renewal capacity, proposed to underlie tumor recurrence and metastasis. We previously showed that the adipose tissue cytokine LEPTIN, increased in obesity, promotes the survival of CSCs in vivo. Here, we tested the hypothesis that the leptin receptor (LEPR), expressed in mammary cancer cells, is necessary for maintaining CSC-like and metastatic properties. We silenced LEPR via shRNA lentivirus transduction and determined that the expression of stem cell self-renewal transcription factors NANOG, SOX2, and OCT4 (POU5F1) is inhibited. LEPR-NANOG signaling pathway is conserved between species because we can rescue NANOG expression in human LEPR-silenced cells with the mouse LepR. Using a NANOG promoter GFP reporter, we showed that LEPR is enriched in NANOG promoter active (GFP+) cells. In lineage tracing studies, we showed that the GFP+ cells divide in a symmetric and asymmetric manner. LEPR-silenced MDA-MB-231 cells exhibit a mesenchymal to epithelial transition morphologically, increased E-CADHERIN and decreased VIMENTIN expression compared with control cells. Finally, LEPR-silenced cells exhibit reduced cell proliferation, self-renewal in tumor sphere assays, and tumor outgrowth in xenotransplant studies. Given the emergence of NANOG as a pro-carcinogenic protein in multiple cancers, these studies suggest that inhibition of LEPR may be a promising therapeutic approach to inhibit NANOG and thereby neutralize CSC functions.
Introduction

Breast cancer is the most common noncutaneous cancer among women in US, and while early detection and therapeutic advances have improved outcome, nearly 25% of diagnosed patients go on to develop secondary tumors at either primary or distant sites (Rakha et al. 2007, Stingl & Caldas 2007, Vargo-Gogola & Rosen 2007, Abdulkarim et al. 2011, Carey 2011, Hudis & Gianni 2011). These secondary tumors are the cause of the poor outcomes and reduced survival in these patients (Sorlie et al. 2001). Obesity is an established breast cancer risk factor and leads to poorer breast cancer outcomes in both pre- and postmenopausal women (Calle et al. 2003).

The mechanisms underlying the connection between obesity and tumorigenesis remain poorly understood (Rose & Vona-Davis 2009, Park et al. 2011). Adipose tissue has long been thought to be an inert lipid-storing tissue (Vona-Davis & Rose 2007, Halberg et al. 2008, Park et al. 2011). However adipose tissue is now recognized as an endocrine organ secreting cytokines, hormones, and inflammatory mediators (Halberg et al. 2008), many of which can influence tumor growth, including leptin (LEP; Brakenhielm et al. 2004).

LEP is a metabolic hormone primarily secreted from fat cells and necessary for the regulation of body weight (Zhang et al. 1994). LEP binds to LEP receptor (LEPR) and stimulates the associated JAK2 and activates STAT3, STAT5, ERK, and PI3K/Akt (Friedman 1999, Myers et al. 2008).

LEPR is also detected in the peripheral tissues and is highly expressed in multiple tumors including breast, colon, prostate, and brain (Somasundar et al. 2004, Garofalo & Surmacz 2006, Ando & Catalano 2011, Park & Scher 2011). In human breast cancer, LEPR expression is directly correlated with poor overall prognosis (Ishikawa et al. 2004, Garofalo et al. 2006, Miyoshi et al. 2006). Moreover, LEPR is expressed in 92% of triple negative breast cancers (TNBCs; Otvos et al. 2011), so called because these tumors do not express the estrogen and progesterone receptors or the HER2 oncogene and are refractory to current clinical strategies (Dent et al. 2007, Hudis & Gianni 2011).

At the root of these LEPR-associated processes is the ability of the LEPR to trigger JAK2/STAT signaling (Myers 2004), known pro-oncogenic pathways. JAK2/STAT3 signaling promotes cancer stem cell (CSC) survival and self-renewal thus LEPR may participate in stem cell signaling pathways. CSCs are recently discovered cancer cells that reside in the subsets of breast tumors including TNBCs. CSCs have the ability to self-renew and may be associated with recurrence and metastasis in breast cancer (Al-Hajj et al. 2003, Liu & Wicha 2010).

CSCs are molecularly characterized based on the expression of cell surface receptors including integrin α6 (CD49f), integrin β1 (CD29), hyaluronan receptor (CD44), and the stem cell self-renewal transcription factors NANOG, OCT4, and SOX2. NANOG has emerged as a pro-carcinogenic factor in cancer cell lines with CSC behaviors (Jeter et al. 2009). Compared with control cells, NANOG silencing in cancer cells leads to reduced proliferation, self-renewal based on tumor sphere assays, and tumors in xenograft transplant studies (Jeter et al. 2009, 2011). Thus, inhibition of NANOG expression may provide a novel therapeutic, though as a transcription factor, NANOG is a difficult drug target.

Research carried out in our laboratory and others has led to the proposal that LEPR maintains cancers in a stem cell-like state (Zheng et al. 2011, Feldman et al. 2012). To interrogate this hypothesis, we generated LEPR-silenced mammary cancer cells and assessed self-renewal, cell proliferation, and tumorigenicity in xenograft models. Moreover, because JAK2/STAT3 cytokine signaling is implicated in the expression of the stem cell transcription factors, we assessed whether LEPR is necessary for the expression of NANOG, OCT4, and SOX2 and in maintenance of cancer cells in an undifferentiated mesenchymal stem cell state. Our studies point to a necessary role of LEPR in maintenance of the stem cell and mesenchymal phenotype in TNBCs and suggest that silencing of LEPR may be used to inhibit cancer progression by blocking expression of stem cell transcription factors in CSCs.

Materials and methods

Cell culture

M-Wnt cells were derived from spontaneous tumors that develop in MMTV-Wnt-1 transgenic mice (Dunlap et al. 2012). Cells were maintained in RPMI with l-glutamine and 5% fetal bovine serum (FBS). MDA-MB-231 cells were purchased from American Tissue Culture Collection (ATCC, Manassas, VA, USA) and maintained in Leibovitz L-15 medium (Sigma) with 10% FBS.

Mice

WT C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were maintained in microisolator units and provided free access to...
food and water. All animal procedures were performed under strict adherence to the protocols approved by the Institute Animal Care and Use Committee at the Lerner Research Institute, Cleveland Clinic Foundation.

M-Wnt cells were orthotopically transplanted (200 000 cells/mouse) into the right mammary fat pad #4 of female mice at 6 weeks of age (n = 3). Mice were monitored twice weekly until tumors were palpable and then daily. Four weeks post-injection, mice were euthanized and the tumors collected for histological analysis. Tumor volume was measured using an electronic caliper, applying the formula (volume = 0.52 × (width) × (height) × (length)) for approximating the volume of a spheroid.

**Immunoblotting**

Cells were lysed in buffer containing 20 mM Tris, pH 7.4, 137 mM NaCl, 1% NP-40, 10% glycerol, 20 mM NaF, 1 mM Na orthovanadate, and 1 mM phenylmethylsulphonyl fluoride. Protein concentrations were measured using BCA protein assay (Thermo, Rockford, IL, USA). Membranes were incubated overnight at 4 °C with primary antibodies. NANOG, integrin α6, STAT3, P-STAT3, Akt, P-Akt, ERK, and P-ERK were purchased from Cell Signaling (Beverly, MA, USA) and actin from Sigma–Aldrich. Anti-rabbit IgG antibodies conjugated with HRP (Amersham) were used as secondary antibodies and visualized using the West Pico Chemiluminescent substrate from Pierce (Rockford, IL, USA).

**RT-PCR analysis**

Total RNA was isolated using TRI reagent (Ambion, Austin, TX, USA) and stored at −80 °C until use. RNA concentration was determined using NanoDrop 1000 Spectrophotometer (Thermo, Wilmington, DE, USA). Reverse transcriptase (RT) reactions were prepared using a high-capacity cDNA reverse transcription kit (Applied Biosystems). Two micrograms of total RNA was used as template for first strand cDNA. Amplification of transcripts was performed using Taq DNA polymerase kit (Qiagen) with 200 ng of total RNA. Semi-quantitative RT-PCR was quantified by scanning the gels digitally, followed by the analysis using ImageJ (NIH, Bethesda, MD, USA). Real-time PCR was performed on Steponeplus Real-Time PCR system from Applied Biosystems via SYBR-Green Mastermix (Applied Biosystems). The threshold cycle (CT) values for each gene were normalized to expression levels of GAPDH (Applied Biosystems). Primer sequences are provided in **Supplementary Table 2**, see section on supplementary data given at the end of this article.

**Flow cytometry**

Cells were resuspended in PBS containing 2% FBS and phycoerythrin (PE)-conjugated rat anti-mouse CD49f (1:100, eBioGoH3, eBioscience, San Diego, CA, USA), PE-conjugated rat anti-mouse CD24 (1:100, 30-F1, eBioscience), and APC-conjugated rat anti-mouse CD44 (1:100, Pgp1, eBioscience) at a concentration of 1 million cells/ml for 1 h on ice. After washing twice, cells were subjected to FACS analysis and sorting on a BD LSR II cytometer and an Aria II sorter, respectively (BD Biosciences, San Jose, CA, USA). Data analysis was performed on the FlowJo version 8.8.6 software (Tree Star, Inc., Ashland, OR, USA).

**MTS cell growth assay**

Five thousand cells per well were cultured in 96-well plates. Cell proliferation was evaluated using colorimetric MTS assay (Promega) that measures the restoration of 3-(4,5-dimethylthiazol-2-yl)-5-(3 carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) to formazan in metabolically active cells. Absorbance of the formazan at 490 nm was determined on a Spectramax 190 plate reader (Molecular Devices, Sunnyvale, CA, USA).

**Tumorsphere assays**

M-Wnt cells, 1 cell/well was cultured in ultra low 96-well plates (Corning, Tewkesbury, MA, USA) with 200 μl serum-free DMEM/F12 medium supplemented with 20 ng/ml basic fibroblast growth factor (Invitrogen), 10 ng/ml epidermal growth factor (Biosource, Grand Island, NY, USA), 2% B27 (Invitrogen), 10 μg/ml Insulin, and 1 μg/ml hydrochloride (Sigma). For MDA-MB-231 cells, 1000 cells/well were cultured in ultra low six-well plates. After 5–7 days, tumorspheres were counted under a Leica dissecting scope.

**Lentiviral production and transduction**

The lentiviral plasmid vector pLKO.1-puro-based shRNA clones and control shRNA vector were purchased from Sigma–Aldrich (Supplementary Table 1, see section on supplementary data given at the end of this article). The pLKO.1/LEPR shRNA and CONT lentiviruses were purchased from Sigma–Aldrich (Supplementary Table 2, see section on supplementary data given at the end of this article). The pLKO.1/LEPR shRNA and CONT lentiviruses were purchased from Sigma. Transductions were carried out in serum-free medium with either shRNA targeting LepR or control shRNA. After overnight incubation, the viral particle containing medium was removed and replaced with fresh complete medium. After 2 days, cells were treated.
with 2.5 μg/ml puromycin. Cells were harvested 3 days after puromycin selection for RT-PCR, western blotting, and flow cytometry. NANOG promoter GFP lentiviral construct was obtained from System Bioscience (Mountain View, CA, USA). Virus was transduced into MDA-MB-231 as described for shRNA viruses without puromycin selection.

### Lineage tracing analysis

Lineage tracing was performed as previously described (Lathia et al. 2011). Briefly, phase-contrast, time-lapse image stacks consisting of 288 frames (5 min intervals) and corresponding fluorescence image stacks (72 frames, 20 min intervals) were imported into Image-Pro Plus (v6.2, Media Cybernetics, Silver Spring, MD, USA). Lineage analysis was performed in a semiautomated manner using customized visual basic Image-Pro Plus macros. Briefly, intensity in each fluorescence stack was normalized across all time-points to account for photo-bleaching. Synchronized phase-contrast and time-lapse stacks were then displayed side by side and a user marked the center of a daughter cell, tracking the cell until the final frame was reached, another division event was encountered, or cell death occurred. For each click, a circular region of interest six pixels in diameter was placed at the click point on the corresponding fluorescence frame and mean intensity was recorded (automated process). This procedure was repeated for the corresponding daughter cell and any additional progeny for subsequent divisions of these daughter cells. Once the tracking of two daughter cells for a particular division event was completed, the mean fluorescence intensities, click coordinates, frame numbers, and cell health (live/dead) were exported to Excel for each tracking point for both daughter cells. Finally, the fluorescence image stacks were superimposed upon the corresponding phase-contrast images for validation and illustration.

### Results

#### Targeting of LEPR with short hairpin RNA

Tumor cells transplanted into LEP-deficient ob/ob mice exhibit reduced tumor initiating activity and reduced CSCs compared with WT mice (Zheng et al. 2011). To test the hypothesis that LEPR was necessary for survival, self-renewal, and tumorigenicity of mammary cancer cells, we silenced LEPR using shRNA lentivirus. As LEPR has not been silenced in mammary cancer cells, we screened shRNA lentiviral constructs for inhibition of mouse and human LEPR expression. Several shRNA constructs were identified, one mouse construct and two human constructs (Supplementary Table 1). LEPR was inhibited in both the mouse mammary cancer M-Wnt cells (Dunlap et al. 2012; Fig. 1A) and the human breast cancer MDA-MB-231 cells (Fig. 1B). LEP activates PI3K/Akt, ERK, and STAT3. LEP activation of these pathways is documented for MDA-MB-231 cells but not for M-Wnt cells. Thus, we confirmed that Lep stimulated phosphorylation of Akt, ERK, and STAT3.

**Figure 1**

shRNA silencing of leptin receptor (LEPR) inhibits basal and leptin-dependent signaling. (A and B) Semi-quantitative RT-PCR analysis of LEPR long form in M-Wnt (LEPR-L) and MDA-MB-231 (LEPR-L) cells transduced with either LEPR or control (CONT) shRNA. GAPDH was used as a loading control. (C and D) Analysis of LEP-stimulated STAT3 phosphorylation in M-Wnt and MDA-MB-231 cells. M-Wnt cells were treated with 400 ng/ml and MDA-MB-231 cells were treated with 200 ng/ml leptin and at indicated times cells were extracted and analyzed for STAT3 phosphorylation. Total STAT3 was used as loading control. Fold induction of p-STAT3 corrected for total STAT3 is shown below each lane. Quantification was performed using ImageJ (NIH). Data are representative of an experiment repeated three times.
To determine if reduced LEPR expression is sufficient to cause changes in downstream signaling pathways, we evaluated STAT3 phosphorylation. LEP (400 ng/ml) stimulated endogenous STAT3 phosphorylation (p-STAT3) by twofold in control transduced M-Wnt and MDA-MB-231 cells with normal LEPR. In contrast, LEPR-silenced M-Wnt and MDA-MB-231 cells, LEP did not stimulate phosphorylation of STAT3 (Fig. 1C and D). Moreover, basal p-STAT3 was inhibited in LEPR-silenced cells (Supplementary Figure 2, see section on supplementary data given at the end of this article). These studies provide evidence that LEPR silencing results in signaling alterations in mouse and human mammary cancer cells.

**LEPR regulates NANOG expression**

We next assessed whether silencing of LEPR altered the expression of stem cell transcription factors in mammary

![Supplementary Figure 1](http://erc.endocrinology-journals.org/)

![Figure 2](http://erc.endocrinology-journals.org/)

Silencing of LEPR in M-Wnt and MDA-MB-231 leads to inhibition of stem cell transcription factors. Analysis of M-Wnt cells (A) and MDA-MB-231 (B) for NANOG, SOX2, OCT4, and BMI1 by RT-PCR in LEPR or CONT shRNA-treated cells. GAPDH was used as an RNA loading control. Quantification of data from A and B is presented in Supplementary Figure 3. Data are representative of an experiment repeated four times. (C) Analysis of leptin-stimulated STAT3 and ERK in HEK 293 cells transfected with long form of the LepR. HEK293T cells were transfected with CONT or mLepR-L cDNA. Cells were incubated with leptin (100 ng/ml) for 0, 5, 30 min and pSTAT3 and pERK expression were analyzed by immunoblotting. Total STAT3 and ERK were assayed to control for loading. (D) Rescue of NANOG expression in LEPR silenced MDA-MB-231 cells by the long isoform of LepR. LEPR was silenced MDA-MB-231 cells followed by transfection with LepR-L cDNA. (E) Analysis of LEP stimulated STAT3 and ERK in LepR-L cells. HEK293T cells were transfected with empty vector or mLepR-L cDNA. Cells were incubated with LEP (100 ng/ml) for 0, 5, 30 min and pSTAT3 and pERK expressions were analyzed by immunoblotting. Total STAT3 and ERK were assayed to control for loading. (F) Lack of rescue of NANOG in LEPR-silenced MDA-MB-231 cells by the mouse LepR. LEPR-silenced or CONT MDA-MB-231 cells were transfected with LepR or empty vector cDNA and expression of NANOG was assayed by RT-PCR. Full colour version of this figure available via http://dx.doi.org/10.1530/ERC-13-0329.
cancer cells because LEP deficiency leads to reduced CSCs (Zheng et al. 2011). NANOG, SOX2, OCT4, and BMI1 were analyzed by RT-PCR. All of these transcription factors are expressed in M-Wnt and MDA-MB-231 cells (Fig. 2A and B), whereas LEPR-silenced cells do not express NANOG in either cell line (Fig. 2A and B). Further, LEPR-silenced MDA-MB-231 cells also do not express SOX2 and OCT4 (Fig. 2B). Relative expression corrected for GAPDH was quantified using NIH ImageJ (Supplementary Figure 3, see section on supplementary data given at the end of this article). Further, we determined that NANOG protein expression is inhibited in LEPR silenced compared with control MDA-MB-231 cells (Supplementary Figure 4).

There are two major LEPR isoforms, short (LEPR-S) and long (LEPR-L), expressed in cancer cells. LEPR-L is best studied, as it activates JAK2/STAT signaling pathways. LEPR-S contains a short cytoplasmic domain and does not activate JAK2-dependent signaling pathways and its function remains unknown (Bjorbaek et al. 2001). To exclude off-target effects of the LEPR shRNA and determine whether LEPR-L or LEPR-S isoforms are sufficient to rescue expression of the NANOG, OCT4, and SOX2, we transfected LEPR-silenced MDA-MB-231 cells with either the mouse LepR-L or LepR-S cDNA and assayed for NANOG expression. As a negative control, we used an empty pcDNA3.1 plasmid. Human embryonic kidney (HEK) 293 cells were used to assess LepR-L and LepR-S signaling, as they are validated cells to assay LEP-stimulated LEPR activity (Banks et al. 2000). Mouse LepR-L and LepR-S were transfected into HEK 293 cells and phosphorylation of STAT3 and ERK was assayed. As expected, LEP stimulates rapid phosphorylation of STAT3 and ERK in HEK 293 cells overexpressing LEPR-L (Fig. 2C), whereas LEP could not stimulate either STAT3 or ERK phosphorylation in cells overexpressing LEPR-S (Fig. 2E). Next, we transfected mouse LepR-L or LepR-S in LEPR silenced MDA-MB-231 and assayed NANOG expression (Fig. 2D and F). Only mouse LepR-L was sufficient to rescue NANOG. Collectively, the data indicate the LEPR-L expression is necessary to maintain the cells undifferentiated in a stem cell state.

Time-lapse lineage tracing of NANOG expressing MDA-MB-231 cells identifies symmetric and asymmetric cell division and death

Because NANOG is a stem cell transcription factor, we investigated whether it is expressed uniformly in MDA-MB-231 cells, and we assessed the fate of the NANOG expressing cells. We transduced MDA-MB-231 cells with a reporter in which the NANOG promoter drives GFP expression (Fig. 3A). Transduced cells were sorted to enrich for the GFP expressing (NANOG promoter active) population and cultured. Within two passages, the GFP-enriched cells showed mixed expression of GFP-positive (GFP+) and -negative (GFP−) cells (Fig. 3B). As the MDA-MB-231 cells were first sorted for >95% GFP+, this suggests that these cells are executing multiple modes of division (symmetric, asymmetric). We next assessed expression of LEPR-L and LEPR-S in the GFP+ and GFP− cells and determined that GFP+ express two- to threefold higher levels of both isoforms of LEPR compared with GFP− cells (Fig. 3C). We independently confirmed that GFP+ cells exhibit increased expression of NANOG compared with GFP− cells.

To evaluate the modes of cell division, cells were cultured, monitored, and tracked over a 48-h time period. We determined that the GFP+ MDA-MB-231 cells divide through symmetric division, leading to the generation of two daughter cells that both express GFP (Fig. 3D). Cells could also undergo asymmetric division leading to the production of one daughter cell that is GFP+ and second daughter cell, which is GFP− (Fig. 3D). Thus, GFP+ MDA-MB-231 cells generate both NANOG promoter active and inactive cells. In parallel, cell death was quantified. We determined that GFP− cells exhibited significantly (1.5 times) greater cell death compared with cells that are GFP+ (Fig. 3E). The data suggest that inhibiting NANOG expression may lead to differentiation, cell death, and reduced CSC behaviors.

LEPR is necessary for maintenance of MDA-MB-231 in a mesenchymal state

MDA-MB-231 cells are TNBCs with mesenchymal stem cell-like character (Lehmann et al. 2011). In contrast, LEPR-silenced cells exhibit a non-mesenchymal rounded epithelial-like morphology (Fig. 4A). Further, real-time PCR indicated that E-cadherin, a terminal differentiation epithelial marker, is significantly induced 300-fold in LEPR-silenced MDA-MB-231 cells compared with controls (Fig. 4B). In parallel, vimentin, a mesenchymal marker, was significantly inhibited by 60% in LEPR-silenced cells (Fig. 4C). This suggests that LEPR-silenced cells undergo a mesenchymal to epithelial transition.

LEPR-silenced mammary cancer cells exhibit reduced CSC frequency

These findings indicate that LEPR-silenced breast cancer cells were differentiated and indicated that LEP promotes
stem cell behaviors. We performed cell proliferation assays using an MTS assay and determined that LEPR-silenced cells exhibit reduced viability (Supplementary Figure 5, see section on supplementary data given at the end of this article). Thus, we assessed cell death using the DeadEnd Fluorometric TUNEL system (Promega), but we did not detect a significant increase in apoptosis in shLEPR compared with shCONT cells. We speculate that the inhibition in LEPR expression leads to a reduction in cell proliferation with limited increase in cell death.

Next, we evaluated the effect of LEP on CSC self-renewal in tumorsphere cultures. M-Wnt cells were plated in tumorsphere cultures in the absence or presence of LEP (400 ng/ml). Once tumorspheres were formed, spheroids were counted, collected, and recultured as tumorspheres and the procedure was repeated. The analysis revealed that...
LEP led to an increase in number of tumorspheres compared with cells cultured in the absence of LEP (Fig. 5A). In parallel, we assayed the effect of LEP on tumorsphere formation in MDA-MB-231 cells. LEP (200 ng/ml) increased tumorsphere formation by twofold compared with its absence (Fig. 5B). This indicates that LEP via LEPR is sufficient to increase the viability of CSCs in culture.

We next assessed whether LEPR silencing would inhibit tumorsphere formation in M-Wnt and MDA-MB-231 cells. To determine the stem cell frequency, we cultured single viable cells per well in a 96-well ultra-low binding plate. After 7–10 days, wells containing single tumorspheres were counted (Fig. 5C). The clonal sphere formation frequency in shRNA control transduced M-Wnt cells was $22.5 \pm 10$ and in LepR shRNA transduced cells was $6.8 \pm 5.4$ ($P < 0.05$). This indicates that LepR silencing leads to decreased CSCs. Likewise, we performed tumorsphere formation assays in LEP shRNA and control transduced MDA-MB-231 cells. LEP silencing led to a >80% reduction in sphere formation frequency (shCONT $1.1 \pm 0.1$, shLEPR1 $0.17 \pm 0.15$, and shLEPR2 no sphere formation frequency; Fig. 5D).

As further evidence that silencing LEPR reduces the percentage of CSCs, cells were analyzed by fluorescence-activated cell sorting (FACS) for the breast CSC markers CD44$^+$CD24$^-$ and CD49f. We determined that the proportion of CD44$^+$CD24$^-$ cells was reduced by >10% in LEPR silenced relative to control M-Wnt cells (Fig. 5E). In parallel, we observed a fourfold reduction in CD49f/integrin α6 immunoreactive cells in LepR silenced compared with control cells (Fig. 5F). Further, integrin α6 expression, based on immunoblotting, was significantly inhibited in LepR silenced compared with control M-Wnt cells (Fig. 5G). The data indicate that silencing of LEPR expression in mouse and human mammary cancer cells leads to fewer CSCs.

**LEPR-silenced mammary cancer cells exhibit inhibition in tumor outgrowth**

To assess the affect of LEPR silencing on tumor formation, control and LepR shRNA transduced M-Wnt cells were orthotopically transplanted (200 000 cells/mouse) into syngeneic C57Bl/6J female mice. Hundred percent of control transduced cells formed tumors that were >100 mm$^3$ within 4 weeks (Fig. 6). In contrast, no tumors were formed in mice transplanted with the LEPR-silenced cells (Fig. 6). This provides evidence that LEPR is necessary for maintenance of a tumorigenic phenotype.

**Discussion**

Our findings indicate that in both murine and human mammary cancer cells, LEPR is necessary for maintenance and self-renewal of undifferentiated cancer cells possessing CSC hallmarks. Inhibition of LEPR expression leads to inhibition of NANOG, a master regulator of self-renewal in normal stem cells, viability of CSCs, proliferation, and tumor-initiating activity. Importantly, LEPR-NANOG signaling is highly conserved because we showed that mouse LepR is able to rescue NANOG expression in LEPR-silenced human breast cancer cells. Further, mouse LepR rescues the inhibited cell proliferation in LEPR-silenced cells. Moreover, LEPR is necessary to maintain a mesenchymal and invasive state as evidenced by the transcriptional profiling of LEPR-silenced and control breast cancer cells.
LEPR is a cytokine receptor and LEP binding leads to activation of PI3K/Akt, ERK, and STAT3 signaling pathways. Previous studies have linked PI3K/Akt, ERK, and/or STAT3 signaling to cellular behaviors including cell proliferation, migration, and angiogenesis (Hu et al. 2002, Gonzalez & Leavis 2003, Sharma et al. 2006, Mauro et al. 2007). By silencing LEPR, the current studies reveal a necessary role for LEPR in CSC phenotypes including proliferation, invasion, self-renewal, and tumorigenicity.

The LEPR-silenced cancer cells provide unique evidence that LEPR is necessary for the expression of the core stem cell transcription factor NANOG in both mouse and humans. In humans, LEPR is also necessary for expression of SOX2 and OCT4. The differential regulation of SOX2 and OCT4 may indicate that the upstream regulation of these transcription factors is independent of JAK2/STAT3 signaling pathways in mouse CSCs. Indeed, in mouse embryonic stem cells, Sox2 is regulated by Stat3 whereas Nanog is regulated by Akt signaling (Niwa et al. 2009).

Figure 5
Inhibition of LEPR expression in mammary cancer cells results in reduced tumorsphere formation. (A) Effect of LEPE on tumorsphere formation in M-Wnt cells. Five thousand cells were plated in six-well plates in the absence or presence of 400 ng/ml leptin. After 10 days, spheres were counted from each well (P1). Spheres were dissociated and protocol repeated two times, P2 and P3. Data are presented as mean \( \pm \) S.E.M. (B) Effect of LEP on tumorsphere formation in MDA-MB-231 cells. Five thousand cells were plated in six-well plates in the absence or presence of 100 ng/ml leptin. After 10 days, spheres were counted from each well. Data are presented as mean \( \pm \) S.E.M. corrected for no leptin. Analysis was repeated at least three times. (C) Tumorsphere formation in LepR and CONT M-Wnt cells. Single M-Wnt cells from LepR or CONT transduced lentiviruses were cultured in ultra low 96-well plates. After 5 days, the tumorsphere containing wells were counted under a Leica dissecting scope. Representative tumorsphere shown in panel. (D) Tumorsphere formation in LEPR and CONT shRNA transduced MDA-MB-231 cells. LEPR1, LEPR2, and CONT shRNA transduced cells were cultured at a density of 1000 cells/well in six-well plates. After 5–7 days, tumorspheres were counted under a Leica dissection scope. Each bar represents the mean S.E.M., \(*P < 0.05\). (E) CD44+ CD24− population was quantified in LepR and CONT transduced M-Wnt cells. Cell were stained with APC-CD44 and PE-CD 24 and analyzed by fluorescence activated cell sorting (FACS). (F) CD49f/integrin α6 was quantified in LepR and CONT shRNA transduced M-Wnt cells. Data shown are a representative analysis of FACS experiment that was repeated three times. (G) Analysis of expression of Integrin α6 levels by immunoblotting LepR and CONT shRNA transduced M-Wnt cells. Full colour version of this figure available via http://dx.doi.org/10.1530/ERC-13-0329.
Thus, we speculate that in mouse CSCs LepR via Akt signaling regulates Nanog expression, whereas in human CSCs LEPR via STAT3 and Akt signaling pathways regulate Nanog, Sox2, and Oct4 expression.

LEPR-stem cell signaling is highly conserved between species because the mouse LepR can rescue the human silenced breast cancer cells. Further, we reveal that LEPR is necessary for maintaining breast cancer cells in an invasive mesenchymal state. Our approach is unprecedented in studying LEPR in cancer cells and we will use the same approach to tease apart the LEPR signaling pathways necessary for proliferation, migration/invasion, and angiogenesis.

Recent studies by Machida and colleagues indicates that LEPR is regulated by the self-renewal transcription factors Sox2 and Oct4 (Feldman et al. 2012). Further, their data suggest that LEP, in a STAT3-dependent manner, regulates Sox2 and Oct4. In their studies, Machida and colleagues present a LEPR-Sox2/Oct4 self-reinforcing loop. Our studies complement Machida, though there are several notable differences. Here we show that LEPR is necessary for regulation of Nanog expression in both mice and human cells and for cell proliferation and tumorigenesis. Moreover, LEPR-silenced cancer cells are not capable of initiating tumor outgrowth pointing to a necessary role for LEPR in tumorigenesis. Collectively, these studies are the first to show that LEPR has a necessary role, not just an accessory one, in breast cancer and specifically self-renewal of CSCs and tumorigenesis.

The MDA-MB-231 cells tagged with the GFP-Nanog reporter provide a unique model to track Nanog expressing breast cancer cells and their contribution to tumor progression and metastasis. Using these cells, we can determine whether Nanog promoter active (GFP+) cells (putative CSCs) home to specific niches in the tumor as well as target to other organs/tissues such as bone, lung, and brain, known sites of breast cancer metastasis (Aslakson & Miller 1992, Lin et al. 2008, Charafe-Jauffret et al. 2009). Because these cells coexpress the LEPR, we will determine whether inhibiting LEP or LEPR is sufficient to block survival, migration/invasion, and tumorigenicity in Nanog expressing CSCs. Finally, these reporter cells may prove to be a unique model to study the role of obesity in breast cancer progression, recurrence, and metastasis.

Obesity is an established risk factor for multiple cancers including those of the breast (Calle et al. 2003, Roberts et al. 2010). Leptin was an early candidate thought to link obesity and cancer, though LEPR antagonists have yet to progress to the clinic (Hu et al. 2002, Garofalo & Surmacz 2006, Surmacz 2007, Lautenbach et al. 2009, Ando & Catalano 2011). The studies of Goodwin et al. (2012) further suggest that LEP is a promoter of breast cancer recurrence and distant metastasis leading to overall poor patient survival, particularly in late stage breast cancer. This clinical observation is consistent with a role for LEP and LEPR in CSCs because CSCs have been implicated in recurrence and metastasis. This leads us to speculate that obesity and its associated increase level in LEP would exhibit an increase in maintenance of CSCs and thus increased recurrence, distant metastasis, and overall poor patient survival.

Our studies, as well as several recent reports in the literature in breast and other tumor cell models (Jeter et al. 2009, 2011), suggest that Nanog is a key regulator of CSC self-renewal, clonogenic growth, and tumorigenicity. However, Nanog is a particularly challenging therapeutic target given its role as a master transcription factor important in several cellular functions in CSC and non-CSCs, including adipose-derived and mammary gland stem cells (Dentelli et al. 2012, Kaimala et al. 2012). Our novel findings of the inhibitory effects of LEPR silencing on Nanog expression, CSC self-renewal, cell proliferation, and tumor outgrowth in murine and human breast cancer cell lines suggest that LEPR or components of its downstream signaling pathway may be promising as a

Figure 6
shRNA inhibition of LEPR leads to tumor outgrowth in vivo. Tumor outgrowth in mice injected with LEPR and CTRL shRNA transduced M-Wnt cells. WT mice were injected s.c. with 200 000 cells. After 4 weeks, mice were euthanized, tumors excised, and the tumor volume was measured. Data are presented as mean ± S.E.M. of three mice (*P < 0.001). Full colour version of this figure available via http://dx.doi.org/10.1530/ERC-13-0329.
druggable target to inhibit the pro-cancer effects associated with NANOG. This may be particularly important for treating breast and other cancers in the ever-increasing population of obese, hyperleptinemic women, who relative to normoweight women typically have a poorer prognosis.

**Supplementary data**

This is linked to the online version of the paper at [http://dx.doi.org/10.1530/ERC-13-0329](http://dx.doi.org/10.1530/ERC-13-0329).

**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.

**Funding**

Research was supported by funds to O Reizes from the Cleveland Clinic Foundation, NCI TREC development grant (US4CA116867, Berger (PI)), Case Comprehensive Cancer Center Pilot grant and Special Funds in Aging Cancer Energy Balance Research (P30 CA043703), the American Cancer Society (grant number IRG-91-022-15), and the Cleveland Clinical Translational Sciences Collaborative (UL1RR024989). This work was also supported by a K99/R00 Pathway to Independence Award CA157948 (J D Lathia), American Cancer Society (J D Lathia), and the Ohio Cancer Pathway to Independence Award CA157948 (J D Lathia) from the NIH, Collaborative (UL1RR024989). This work was also supported by a K99/R00 number IRG-91-022-15), and the Cleveland Clinical Translational Sciences Center (P30 CA043703), the American Cancer Society (grant number IRG-91-022-15), and the Ohio Cancer Research Associates (J D Lathia). S M Dunlap was funded by USMRC FY08 Breast Cancer Research Program Postdoctoral Fellowship (WB1XWH-09-1-0720). A Vasanji is an employee of Image IQ.

**Acknowledgements**

We would like to thank members of the Lerner Research Institute Flow Cytometry Core (Cathy Shemo and Sage O’Bryant) for assistance with the FACS and cell sorting.

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Received in final form 5 September 2013
Accepted 11 September 2013
Made available online as an Accepted Preprint 11 September 2013

Q Zheng et al. LEPR maintains breast cancer cell self-renewal.