Vitamin D Suppresses Leptin Stimulation of Cancer Growth through microRNA

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Abstract

Obesity is a pandemic and major risk factor for cancers. The reduction of obesity would have been an effective strategy for cancer prevention, but the reality is that worldwide obesity has kept increasing for decades, remaining a major avoidable cancer risk secondary only to smoke. The present studies suggest that vitamin D may be an effective agent to reduce obesity-associated cancer risks in women. Molecular analyses showed that leptin increased human telomerase reverse transcriptase (hTERT) mRNA expression and cell growth through estrogen receptor-α (ERα) activation in ovarian cancer cells, which was suppressed by 1α,25-dihydroxyvitamin D3 [1,25 (OH)₂D₃]. The suppression was compromised when miR-498 induction by the hormone was depleted with microRNA (miRNA) sponges. In mice, high-fat diet (HFD) stimulation of ovarian tumor growth was remarkably suppressed by 1,25(OH)₂D₃ analogue EB1089, which was also compromised by miR-498 sponges. EB1089 did not alter HFD-induced increase in serum leptin levels but increased miR-498 and decreased the diet-induced hTERT suppression. In tumors, Quantitative RT-PCR analyses revealed an inverse correlation between hTERT mRNA and miR-498 expression in tissues. The suppression was reversed by 1α,25-dihydroxyvitamin D3. The study suggests that miR-498-mediated hTERT downregulation is a key event mediating the anti-leptin activity of 1,25(OH)₂D₃ in estrogen-sensitive tumors in women. Cancer Res; 74(21); 6194–204. ©2014 AACR.

Introduction

Obesity is a major health problem in the United States and around the world, exceeding 30% in America in most gender and age groups. Its prevalence remained steady in the past decade, and in 2009–2010, the rate was estimated to be 35.5% and 35.8% among adults and women, respectively. Besides well-known metabolic and cardiovascular complications, obesity has been associated with increased incidence and mortality rates of various cancers and difficulties in treatments. About 20% of all cancers are estimated to be caused by excess body weight with obesity accounting for about 20% of cancer death in women and 14% in men, which is second only to smoking for the number of avoidable cancers (1). Agents capable of mitigating obesity-associated cancer risk will have a great potential to improve the outcomes of cancer intervention.

Among the complex biologic mechanisms linking obesity to cancer susceptibility, multiple lines of evidences support a connection between increased production of leptin and obesity-associated cancers, particularly estrogen-sensitive ones in postmenopausal women. Circulating levels of leptin, an adipocyte-derived adipokine that classically plays a crucial role in regulating appetite and energy balance, are strongly correlated with body fat content and markedly elevated in obese individuals (2,3) as well as in patients with breast, ovarian, and endometrial cancer (4,5). The Women’s Health Initiative studies have identified obesity as a major breast cancer risk factor for postmenopausal women who had never taken hormone replacement therapy (6). The Million Women Study has estimated that approximately half of endometrial cancers in postmenopausal women can be attributed to obesity (7). Similarly, some studies have suggested an association between obesity and the risk of ovarian cancer (8,9). In laboratory studies, leptin is capable of promoting an aggressive cancer phenotype by stimulating growth, migration, invasion, and angiogenesis (8,9). Particularly relevant to estrogen-sensitive cancers, leptin has been shown to amplify estrogen signaling by either increasing aromatase expression in stromal and cancer cells (10,11) or by activating estrogen receptor-α (ERα) through ERK and STAT3 signaling pathways (12–14), establishing a functional cross-talk between leptin and estrogen signaling pathways. The leptin-estrogen connection is further supported by studies showing that obesity is associated with an...
increase in systemic estrogen levels (15, 16) and that obese women exhibit higher levels of ERα expression than lean women in several types of cancers (17).

The active metabolite of vitamin D, 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] or calcitriol, has antiproliferative and proapoptotic properties and is widely recognized as an agent with great potential for cancer intervention (18, 19). Previously, we have shown that 1,25(OH)₂D₃ suppresses the growth of multiple human ovarian cancer cells and ovarian tumors in mice (20–25). It induces ovarian cancer cell death via telomerase suppression mediated through miR-498 (25, 26), a primate-specific microRNA (miRNA) of which the expression is normally restricted to placenta. In this study, we present experimental evidences showing that leptin and high-fat diet (HFD) stimulated the growth of estrogen-sensitive tumors through ERα activation and telomerase induction, which was suppressed by 1,25(OH)₂D₃ and its analogue EB1089 through miR-498. The studies suggest that 1,25(OH)₂D₃ or its precursors may be used to reduce obesity-associated risks for estrogen-sensitive cancers in women and that clinical trials with obese women may reveal the effectiveness of vitamin D in cancer intervention, a controversial issue debated in both the scientific community and public arena.

Materials and Methods

Materials and cell lines

1,25(OH)₂D₃, human recombinant leptin, 17β-estradiol (E2), and ICI 182,780 were purchased from Sigma. Anti-VD and anti-human telomerase reverse transcriptase (hTERT) antibodies were from Epitomics. Antibodies against ERα and β-actin were purchased from Santa Cruz Biotechnology. pLEN-hERα, hERβLuc, pCMV8βGal, and miR-498 sponges have been used in previous studies (26, 27). BG-1 cells (28) were cultured in DMEM/F-12 medium supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 5% fetal calf serum (FCS). MCF-7 and Ishikawa (29) cells were maintained in DMEM containing 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 5% FCS. PE-04 cells (30) were maintained in RPMI medium containing 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 5% FCS. PE-04 cells (30) were maintained in RPMI medium containing 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 5% FCS. All cells were maintained at 37°C in a humidified incubator with 5% CO₂.

Transfections and reporter assays

Cells were plated in medium containing 5% FCS at 1.5 × 10⁵ cells per well in 6-well plates. One day after plating, cells were transfected with Lipofectamine following the protocol from Invitrogen (Life Technologies) and were placed in phenol red–free DMEM/F-12 containing 2% charcoal-stripped FCS. Forty-eight hours after transfections, cells were treated with vehicle (EtOH), E2 or leptin in the presence or absence of 1,25(OH)₂D₃ for 3 days. For longer treatments, cells were re-fed with fresh culture medium every 2 days. Luciferase and β-galactosidase (β-gal) activities were determined as previously described (20).

To establish luciferase-marked cells that express miRNA sponges, BG-1 cells were cotransfected with control or miR-498 sponges together with pGL3 control plasmid as previously described (26). Stable clones were isolated after selection with puromycin (2 µg/mL) for about 5 weeks.

Quantitative RT-PCR assays

Total and small RNAs were extracted from cells using the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer’s instructions. cDNA was reverse-transcribed from 1 µg total cellular RNA with random hexamer primers and thermo-stable reverse transcriptase (Invitrogen). TaqMan assays (Life Technologies) were used to quantify the expression of hTERT mRNA and mature miR-498. GAPDH and U6 were used as controls for hTERT and miR-498 expression studies, respectively. PCR reactions were carried out in a 20-µL reaction mixture containing 1.33 µL of reverse transcribed product, 1.0 µL of corresponding TaqMan probe, and 10-µL Universal PCR master mix. Reactions were run in the ABI Prism 7900 Fast Real-Time PCR system in triplicate with incubation at 95°C for 10 minutes followed by 40 cycles of denaturation for 15 seconds at 95°C and annealing at 60°C for 1 minute. The target gene levels at each time point were normalized with cognate controls by subtracting the cycle threshold (Ct) value of controls from Ct of target genes to produce a ΔCt. The fold of induction over vehicle controls was calculated on the basis of the formula 2−ΔΔCt (treatment − vehicle). 

Immunoblotting analyses

Cells were estrogen-starved and treated with vehicle, E2 or leptin in the presence or absence of 1,25(OH)₂D₃ for 3 days and lysed on ice by incubating for 20 minutes with lysis buffer containing 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% (v/v) NP-40, 1 mmol/L PMSF, and protease inhibitor cocktail. After centrifugation, protein concentrations of the supernatant were determined using the Bio-Rad assays. Samples of equal amount proteins were separated in SDS-PAGE, transferred to a nitrocellulose membrane, and probed with cognate antibodies. Immunoreacted proteins were detected with enhanced chemiluminescence (ECL) plus.

MTT assays, apoptosis, and cell-cycle analyses

To quantify cell growth, the MTT assays were performed as described previously (31). Cells were estrogen-starved and plated at 5 × 10⁴ in 96-well plates and treated with vehicle, E2, or leptin in the presence or absence of 1,25(OH)₂D₃ for 6 days. The MTT reagents were added to each well to give a final concentration of 0.5 mg/mL and incubated with cells for 3 hours. After the removal of the media, 200-µL DMSO was added to the cells. The absorption at 595 nm was determined with a microplate reader (DYNEX Technologies).
To determine the apoptotic index, cells were treated with vehicle or leptin in the presence or absence of 1.25(OH)2D3 for 6 days, harvested, and washed with phosphate-buffered saline (PBS). Cell suspensions were incubated with FITC and propidium iodide (PI) according to the manufacturer’s protocol (FITC Annexin V Kit; BD Biosciences). Flow cytometry was performed in a FACScan (BD Biosciences). For each data point, triplicate samples were analyzed and the experiment was reproduced three times.

To determine cell-cycle distribution, cells were harvested, washed with PBS, and fixed with 70% ethanol for 2 hours. Fixed cells were washed again with PBS, resuspended in staining solution of 1-mL PBS containing 10-μg/mL DNase-free RNase and PI and incubated for 3 hours at 4°C. Cell populations distributed at different phases of the cell cycle were quantified by FACScan. For each data point, triplicate samples were analyzed and the experiment was repeated three times.

**HFD and ovarian tumor studies**

Female athymic nu/nu mice of 4- to 6-week-old were obtained from Harlan Sprague-Dawley. Mice were housed at the appropriate temperature and with a standard 12-hour light–dark cycle. All mouse studies were carried out according to procedures approved by the Institutional Animal Care and Use Committee at University of South Florida. For tumor studies, mice were divided into two diet groups (n = 20/group). Group I was fed with vitamin D–deficient diet (VDD) containing 22.9%-kcal fat supplemented with 0.47% calcium. Group II received the same calcium-supplemented VDD but containing HFD (55%-kcal). The diet was obtained from Harlan Teklad.

To establish ovarian tumors in mice, BG-1 cells stably transfected with pGL3-control (luciferase) and control or miR-498 sponges were harvested and resuspended at a concentration of 5 × 106 cells in 100 μL of DMEM/F-12 medium containing 5% FCS and ice-cold Matrigel (1:1) and injected i.p. into mice that have been on VDD or HFD diets for a week. Mice were randomized and treated every other day with either vehicle or EB1089 (Pure Chemistry Scientific Inc.) at 0.5 μg/kg body weight diluted in sesame oil in a volume of 50 μL by gavage. All mice were weighed once per week over the course of treatment to monitor changes in body weight. Tumor growths were measured with IVIS-200 (Xenogen) live imaging system at 30 and 60 days as described previously (26). All animals were euthanized after treatment for 60 days. Tumor weight and the number of nodules in the abdominal cavity were scored.

**Serum leptin measurements**

Serum was collected from mice after 60-day treatment. Serum leptin concentrations were measured using a mouse leptin ELISA kit (Crystal Chem Inc.). Serum calcium was measured as previously described (24).

![Figure 1. 1,25(OH)2D3 suppresses estrogen-induced hTERT expression and cell growth. BG-1 cells were treated with either ethanol as a vehicle control (Control), 10−7 mol/L 1,25(OH)2D3 (VD), 10−8 mol/L E2, or VD plus E2 for 3 or 6 (D only) days. A and B, small and total RNAs were isolated and the expression of miR-498 (A) and hTERT (B) was determined by qRT-PCR and normalized to U6 and GAPDH, respectively. The values are expressed as fold of the vehicle control. C, cellular extracts were subjected to immunoblotting analyses with indicated antibodies. Protein signals were quantified by ChemiDoc (Bio-Rad) and after being normalized to cognate β-actin signals, are presented as fold of control. D, cell growth was measured in the MTT assays. Data represent three independent experiments. Error bars are SD. Statistical analyses were performed with the Student t test (n = 3 for A and B; n = 10 for D).](image-url)
Statistical analysis
All values were presented as means ± SD of the indicated number of determinations (n). Significant differences were assessed with the Student t test. All categorical data used numbers, folds, and percentages.

Results
1,25(OH)2D3 dominates over estrogens in controlling miR-498 and hTERT expression and cell growth
Estrogens have been shown to stimulate hTERT gene expression and telomerase activity in breast (32), ovarian (33, 34), and endometrial (35) cancer cells, which involve estrogen response elements (ERE) present in the hTERT promoter. Our recent studies identified miR-498 as a primary target gene for 1,25(OH)2D3 that binds to hTERT 3’-untranslated region (UTR) and decreases telomerase activity in ovarian cancer cells (26), making it possible for 1,25(OH)2D3 to inhibit estrogen-induced telomerase activity and cell growth through miR-498. To test this idea, we examined whether 1,25(OH)2D3 induced miR-498 in estrogen-sensitive ovarian cancer cells and whether the induction occurred in the presence of E2. As shown in Fig. 1A, miR-498 was significantly induced by 1,25(OH)2D3 in BG-1 cells and that the induction occurred similarly in the absence or presence of E2. Consistent with the miR-498 data, 1,25(OH)2D3 suppressed E2-induced hTERT expression at mRNA (Fig. 1B) and protein (Fig. 1C) levels. These analyses show that 1,25(OH)2D3 antagonizes and dominates over estrogen actions in regulating miR-498 and hTERT expression in ovarian cancer cells. The 1,25(OH)2D3 dominance was further supported by the findings that 1,25(OH)2D3 suppressed E2 stimulation of BG-1 cell growth (Fig. 1D) and that 1,25(OH)2D3-induced increase in VDR protein expression was not affected by E2 (Fig. 1C). In these analyses, 1,25(OH)2D3 did not alter the ability of E2 to downregulate ERα (Fig. 1C), a process required for efficient ERα transactivation (36), indicating that the dominant effect of 1,25(OH)2D3 over E2 is likely to be exerted at steps downstream of ERα transactivation by its ligands.

Leptin increases hTERT expression and cell growth through ERα
Leptin has been shown to stimulate ERα transactivation (12–14), and as mentioned earlier, estrogens stimulate hTERT expression through ERα transactivation (36). Thus, it is possible for leptin to stimulate hTERT expression and cell growth through ERα in ovarian cancer cells. As shown in Fig. 2A, leptin significantly increased the transcriptional activity of ERα in BG-1 cells in reporter assays, which was blocked by pure ERα antagonist ICI 182,780. Similarly, qRT-PCR analyses and the MTT assays, respectively, revealed that leptin significantly stimulated hTERT expression (Fig. 2B) and cell growth (Fig. 2C), both of which were blocked by ICI 182,780. These analyses demonstrate that ERα mediates the leptin effect on telomerase expression and cell growth in ovarian cancer cells.

1,25(OH)2D3 suppresses leptin stimulation of hTERT expression and cell growth through miR-498
The ability of 1,25(OH)2D3 to induce miR-498 and its dominance over estrogen actions in BG-1 cells make it likely for the hormone to suppress leptin induction of hTERT
expression and ovarian cancer cell growth through the miRNA. To test this concept, we first assessed the ability of 1,25(OH)2D3 to induce miR-498 in the presence or absence of leptin in BG-1 cells. As shown in Fig. 3A, 1,25(OH)2D3 stimulated miR-498 expression to comparable levels in the presence or absence of leptin, showing a dominance of 1,25(OH)2D3 over leptin in miR-498 induction. Consistent with the dominance in controlling miR-498 expression, 1,25(OH)2D3 attenuated leptin-induced hTERT mRNA (Fig. 3B) and protein (Fig. 3C) expression as well as BG-1 cell growth (Fig. 3D). In these analyses, leptin decreased the ERα protein expression, likely due to ERα activation by the adipokine, and the decrease also occurred in cells cotreated with 1,25(OH)2D3 (Fig. 3E). On the other hand, 1,25(OH)2D3 increased and the decrease also occurred in cells cotreated with 1,25(OH)2D3 over leptin, control and miR-498 sponges (26) were transfected to leptin and 1,25(OH)2D3 was analyzed. As shown in Fig. 4, 1,25(OH)2D3 decreased leptin-induced hTERT expression (Fig. 4A) and cell growth (Fig 4B) in cells transfected with control sponges but had little effect in cells expressing miR-498 sponges, showing that the suppression of leptin actions was mediated through miR-498. Consistent with its known proliferative activity, leptin increased the percentage of BG-1 cells in S-phase, which was also suppressed by 1,25(OH)2D3 in cells transfected with control but not miR-498 sponges (Fig 4C). The studies demonstrate that leptin stimulates ovarian cancer cell growth through telomerase induction and that 1,25(OH)2D3 suppresses the leptin effect through miR-498. In the analyses, leptin had little effect on the ability of 1,25(OH)2D3 to induce cell death, whereas miR-498 sponges, as expected, significantly relieved the apoptotic induction by the hormone (Fig. 4D), showing that the role of miR-498 in the induction of ovarian cancer cell death initially established in OVCAR3 cells is applicable to estrogen-sensitive cells. In BG-1 cells, miR-498 sponges partially relieved the suppressive effect of 1,25(OH)2D3 on leptin stimulation of S-phase accumulation, suggesting that the role of miR-498 in 1,25(OH)2D3 actions in estrogen-sensitive ovarian cancer cells goes beyond cell death induction.

To assess the role of miR-498 in the dominant action of 1,25(OH)2D3 over leptin, control and miR-498 sponges (26) were stably transfected into BG-1 cells and the response of the stable transfectants to leptin and 1,25(OH)2D3 was analyzed. As shown in Fig. 4, 1,25(OH)2D3 decreased leptin-induced hTERT expression (Fig. 4A) and cell growth (Fig 4B) in cells transfected with control sponges but had little effect in cells expressing miR-498 sponges, showing that the suppression of leptin actions was mediated through miR-498. Consistent with its known proliferative activity, leptin increased the percentage of BG-1 cells in S-phase, which was also suppressed by 1,25(OH)2D3 in cells transfected with control but not miR-498 sponges (Fig 4C). The studies demonstrate that leptin stimulates ovarian cancer cell growth through telomerase induction and that 1,25(OH)2D3 suppresses the leptin effect through miR-498. In the analyses, leptin had little effect on the ability of 1,25(OH)2D3 to induce cell death, whereas miR-498 sponges, as expected, significantly relieved the apoptotic induction by the hormone (Fig. 4D), showing that the role of miR-498 in the induction of ovarian cancer cell death initially established in OVCAR3 cells is applicable to estrogen-sensitive cells. In BG-1 cells, miR-498 sponges partially relieved the suppressive effect of 1,25(OH)2D3 on leptin stimulation of S-phase accumulation, suggesting that the role of miR-498 in 1,25(OH)2D3 actions in estrogen-sensitive ovarian cancer cells goes beyond cell death induction.
miR-498 makes it likely for 1,25(OH)2D3 to suppress HFD-induced ovarian tumor growth. To test this idea, BG-1 cells were stably transfected with miR-498 (M-bSP) sponges were stably transfected into BG-1 cells. The stable clones were treated with EtOH (Control), 10⁻⁷ mol/L VD, leptin (100 ng/mL), or 10⁻⁷ mol/L VD plus leptin (100 ng/mL) for 3 days. Total RNAs were extracted and the expression of hTERT was determined by qRT-PCR and normalized to GAPDH. The values are expressed as fold of the control. B, cells were treated as in A but for 6 days. Cell growth was measured in the MTT assays. C, cells were treated as in B and stained with PI. Cell-cycle distribution was assessed by flow cytometry. D, cells were treated as in B. Apoptosis was measured by FITC Annexin-V analyses. Data represent three independent experiments and are presented as the mean ± SD. Statistical analyses were performed with the Student t test (n = 3 in A, C, and D; n = 10 in B).

Figure 4. 1,25(OH)2D3 works through miR-498 to suppress leptin-induced hTERT expression and cell proliferation. A, control (C-bSP) or miR-498 (M-bSP) sponges were stably transfected into BG-1 cells. The stable clones were treated with EtOH (Control), 10⁻⁷ mol/L VD, leptin (100 ng/mL), or 10⁻⁷ mol/L VD plus leptin (100 ng/mL) for 3 days. Total RNAs were extracted and the expression of hTERT was determined by qRT-PCR and normalized to GAPDH. The values are expressed as fold of the control. B, cells were treated as in A but for 8 days. Cell growth was measured in the MTT assays. C, cells were treated as in B and stained with PI. Cell-cycle distribution was assessed by flow cytometry. D, cells were treated as in B. Apoptosis was measured by FITC Annexin-V analyses. Data represent three independent experiments and are presented as the mean ± SD. Statistical analyses were performed with the Student t test (n = 3 in A, C, and D; n = 10 in B).

1,25(OH)2D3 analogue EB1089 suppresses HFD-induced ovarian tumor growth in mice through miR-498

HFD is known to increase serum leptin levels, cause leptin resistance, and promote the development of obesity and cancer (37). The dominance of 1,25(OH)2D3 over leptin through miR-498 makes it likely for 1,25(OH)2D3 to suppress HFD-induced ovarian tumor growth. To test this idea, BG-1 cells stably transfected with firefly luciferase together with either control or miR-498 sponges were injected into peritoneal cavity of nude mice. The mice were then treated for up to 60 days with a vehicle or synthetic 1,25(OH)2D3 analogue EB1089 that is known to be less calcemic and more potent in tumor suppression. Tumor growth was monitored by live luciferase imaging (Fig. 5A and B), counting numbers and size of tumor nodules (Supplementary Fig. S1A) and measuring tumor weight (Supplementary Fig. S1B). As shown in Fig. 5A and B, the growth of tumors expressing control sponges was significantly suppressed by EB1089 in mice fed with VDD diet. The suppression was largely diminished in tumors expressing miR-498 sponges, showing the importance of miR-498. More importantly, HFD stimulated BG-1 tumor growth by up to 6-fold, which was remarkably suppressed by EB1089 in groups expressing control sponges but not in those expressing miR-498 sponges. In parallel studies, tumors expressing miR-498 sponges grew faster than those expressing control sponges in vehicle-treated groups, revealing a role of basal miR-498 expression in suppressing basal and HFD-induced ovarian tumor growth. Ki67 staining and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assays of tumor tissue sections revealed that the tumor suppression by EB1089 is associated with decreased proliferation (Supplementary Fig. S2) and increased apoptosis (Supplementary Fig. S3). Overall, the data show that EB1089 suppresses the HFD-induced ovarian tumor growth and that miR-498 is an important mediator of this antitumor effect.

As expected, mice fed with HFD were heavier than mice on VDD diet and the difference became statistically significant (P < 0.0001) since 4 weeks of the experiment (Fig. 6A). Serum leptin levels at the end of the experiment were also significantly increased in mice on HFD as compared with those on VDD diet (Fig. 6B). Neither EB1089 nor miR-498 sponges altered the effect of HFD on body weight and serum leptin levels. EB1089 increase serum calcium levels but did not induce hypercalcemia (Supplementary Fig. S4), qRT-PCR (Fig. 6C) and Western blot (Fig. 6D) analyses showed that levels of hTERT mRNA and protein expression in tumors were significantly suppressed by EB1089 in both VDD and HFD groups, and that miR-498 sponges diminished the suppressive effect of EB1089. The data suggest that HFD increased leptin to stimulate ovarian tumor growth in vivo and the stimulation of tumor growth was suppressed by EB1089 through miR-498-mediated hTERT downregulation.
In vivo suppression of leptin actions in ovarian cancer cells. The studies described above have established the concept that 1,25(OH)₂D₃ may prevent obesity-associated apoptosis, to become cancerous (38). Because of the critically short telomere length, telomerase activity remains rate-limiting in cancer cells, explaining why tumor-promoting factors need to increase telomerase activity to increase tumor growth. In the present studies, ICI 182,780, a pure ERα antagonist, diminished the ability of leptin to induce hTERT expression, cell growth, and ERα transcriptional activity, defining ERα-mediated hTERT induction as the main mechanism underlying leptin stimulation of estrogen-sensitive ovarian cancer growth. 1,25(OH)₂D₃ suppressed such a leptin action through miR-498-mediated hTERT destruction, which was translated into the suppression of HFD-induced hTERT expression and ovarian tumor growth in vivo in mice (Fig. 7E). In addition to BG-1 cells, miR-498 induction and hTERT downregulation by 1,25(OH)₂D₃ were also detected in ERα-positive PE-04, MCF-7, and Ishikawa cells, showing that the concept that 1,25(OH)₂D₃ may prevent obesity-associated cancers can be generalized to estrogen-sensitive ovarian as well as breast and uterine cancers.

Previous studies in estrogen-insensitive ovarian cancer cells have defined miR-498 as a primary 1,25(OH)₂D₃ target gene that directly binds to the 3′-UTR of hTERT mRNA to induce cell death (26). The present studies are the first to document a cross-talk between leptin and 1,25(OH)₂D₃ and place telomerase and the miRNA pathway at the center stage. It is important to point out that our studies do not project miR-498-mediated hTERT degradation as the sole mechanism underlying the 1,25(OH)₂D₃ suppression of estrogen and leptin actions in cancer cells. 1,25(OH)₂D₃ has been reported to decrease ERα expression and activity in MCF-7 cells (39, 40). In BG-1 cells, 1,25(OH)₂D₃ caused a modest reduction in ERα protein expression (Fig 1C) and decreased its transcriptional activity in reporter assays (data not shown), suggesting that the effect of 1,25(OH)₂D₃ on ERα expression and/or activity may also contribute to its suppression of leptin actions in ovarian cancer cells.

Discussion

Telomerase is a hallmark of cancer that cells must possess to overcome two proliferative barriers, senescence and crisis-associated apoptosis, to become cancerous (38). Because of the critically short telomere length, telomerase activity remains rate-limiting in cancer cells, explaining why tumor-promoting factors need to increase telomerase activity to increase tumor growth. In the present studies, ICI 182,780, a pure ERα antagonist, diminished the ability of leptin to induce hTERT expression, cell growth, and ERα transcriptional activity, defining ERα-mediated hTERT induction as the main mechanism underlying leptin stimulation of estrogen-sensitive ovarian cancer growth. 1,25(OH)₂D₃ suppressed such a leptin action through miR-498-mediated hTERT destruction, which was translated into the suppression of HFD-induced hTERT expression and ovarian tumor growth in vivo in mice (Fig. 7E). In addition to BG-1 cells, miR-498 induction and hTERT downregulation by 1,25(OH)₂D₃ were also detected in ERα-positive PE-04, MCF-7, and Ishikawa cells, showing that the concept that 1,25(OH)₂D₃ may prevent obesity-associated cancers can be generalized to estrogen-sensitive ovarian as well as breast and uterine cancers.

Previous studies in estrogen-insensitive ovarian cancer cells have defined miR-498 as a primary 1,25(OH)₂D₃ target gene that directly binds to the 3′-UTR of hTERT mRNA to induce cell death (26). The present studies are the first to document an important role for miR-498 in mediating the suppressive effect of 1,25(OH)₂D₃ on estrogen stimulation of cancer growth. The studies are also the first to document a cross-talk between leptin and 1,25(OH)₂D₃ and place telomerase and the miRNA pathway at the center stage. It is important to point out that our studies do not project miR-498-mediated hTERT degradation as the sole mechanism underlying the 1,25(OH)₂D₃ suppression of estrogen and leptin actions in cancer cells. 1,25(OH)₂D₃ has been reported to decrease ERα expression and activity in MCF-7 cells (39, 40). In BG-1 cells, 1,25(OH)₂D₃ caused a modest reduction in ERα protein expression (Fig 1C) and decreased its transcriptional activity in reporter assays (data not shown), suggesting that the effect of 1,25(OH)₂D₃ on ERα expression and/or activity may also contribute to its suppression of leptin actions in ovarian cancer cells.
Nevertheless, the fact that miR-498 sponges relieved majority of the suppressive effect of 1,25(OH)\textsubscript{2}D\textsubscript{3} on leptin and HFD-induced ovarian cancer growth shows that the miR-498 pathway is the main mediator of the 1,25(OH)\textsubscript{2}D\textsubscript{3} effect on leptin.

Although present studies have focused on how 1,25(OH)\textsubscript{2}D\textsubscript{3} suppresses ER\textalpha-mediated leptin effect on telomerase, there are no reasons to believe that the impact of 1,25(OH)\textsubscript{2}D\textsubscript{3} action through miR-498 on telomerase will be limited to leptin action or ER\textalpha-positive tumors. Recently, hypercholesterolemia, another obesity comorbidity and an independent cancer risk factor in postmenopausal women, has been shown to activate ER\textalpha and liver X receptor to control breast cancer growth and invasion after converting into 27-hydroxycholesterol (41). Although remaining to be determined, 27-hydroxycholesterol may increase telomerase activity through ER\textalpha and 1,25(OH)\textsubscript{2}D\textsubscript{3} may prevent hypercholesterolemia-associated cancer risk through miR-498. Likewise, leptin has been shown to increase telomerase activity in breast and hepatocellular carcinoma cells through STAT3 (42, 43) and c-Myc (43), which may not depend on ER\textalpha. 1,25(OH)\textsubscript{2}D\textsubscript{3} should also be able to suppress the ER\textalpha-independent leptin effect on telomerase through the same miR-498 pathway.

Although controversial, literature information, in general, supports an association between obesity and the risk of ovarian cancer (44–46). Similarly, the estrogen sensitivity of human ovarian cancer has been implicated by the facts that hormone replacement therapy increased ovarian cancer incidence (47) and that its decreased usage after the Women’s Health Initiative studies was associated with a reduction (48). Despite the estrogen sensitivity of their cancers, patients with ovarian cancer do not benefit significantly from treatments with anti-estrogens or aromatase inhibitors. 1,25(OH)\textsubscript{2}D\textsubscript{3} may offer an
effective approach to block ERα actions, which may be used to selectively reduce ovarian cancer risk in obese women. Consistent with this idea, the well-publicized Cohort Consortium Vitamin D Pooling Project of Rare Cancers revealed an inverse correlation between serum 25-hydroxyvitamin D3 and ovarian cancer risk that reached statistical significance only in obese women (49).

The concept that vitamin D may prevent cancers has been supported by epidemiologic and molecular studies from many laboratories. However, population-based studies aiming to advance vitamin D into clinics for cancer intervention are complicated by many issues such as timing, dosages, and duration, etc. With personalized medicine concept in mind, one may argue that the key to success will be the identification of a subpopulation of human subjects who are highly sensitive to vitamin D. The present studies argue that two groups of women may benefit greatly from vitamin D intervention. One group would be women who are overweight or obese. The idea is consistent with literature information that obesity is usually correlated with low serum levels of 25-hydroxyvitamin D3, which was projected to be responsible for 20% of the cancer risk linked to increased body mass index (50). Another group would be postmenopausal women who receive hormone replacement therapy, which are known to increase their cancer risks. Well-controlled clinical trials should be carried out to determine whether vitamin D will offer a nontoxic and economic way of cancer intervention in overweight and obese women and whether its addition to the current hormone replacement therapy formula will cut down the increase in cancer risks.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: R. Kasiappan, W. Bai
Development of methodology: R. Kasiappan, Y. Sun, W. Bai
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References

50. Lagunova Z, Porojnicu AC, Grant WB, Brundal O, Moan JE. Obesity and increased risk of cancer: does decrease of serum 25-hydroxyvitamin D level with increasing body mass index explain some of the association? Mol Nutr Food Res 2010;54:1127–33.
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