Identification of microRNA-98 as a Therapeutic Target Inhibiting Prostate Cancer Growth and a Biomarker Induced by Vitamin D

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Background: MicroRNAs play an important role in the anti-tumor effect of vitamin D in prostate cancer.

Results: Vitamin D regulates miR-98 which suppresses CCNJ and prostate cancer cell growth.

Conclusion: miR-98 mediates vitamin D anti-proliferative effect and correlates with vitamin D treatment in blood.

Significance: miR-98 can inhibit the growth of prostate cancer and measure vitamin D treatment.

The anti-tumor effect of vitamin D has been well recognized but its translational application is hindered by side effects induced by supra-physiological concentration of vitamin D required for cancer treatment. Thus, exploring the vitamin D tumor suppressive functional mechanism can facilitate improvement of its clinical application. We screened miRNA profiles in response to vitamin D and found that a tumor suppressive miRNA, miR-98, is transcriptionally induced by 1α,25-dihydroxyvitamin D3 (1,25-VD) in LNCaP. Mechanistic dissection revealed that 1,25-VD-induced miR-98 is mediated through both a direct mechanism, enhancing the VDR binding response element in the promoter region of miR-98, and an indirect mechanism, down-regulating LIN-28 expression. Knockdown of miR-98 led to a reduction of 1,25-VD anti-growth effect and overexpression of miR-98 suppressed the LNCaP cells growth via inducing G2/M arrest. And CCNJ, a protein controlling cell mitosis, is down-regulated by miR-98 via targeting 3′-untranslated region of CCNJ. Interestingly, miR-98 levels in blood are increased upon 1,25-VD treatment in mice suggesting the biomarker potential of miR-98 in predicting 1,25-VD response. Together, the finding that growth inhibitive miR-98 is induced by 1,25-VD provides a potential therapeutic target for prostate cancer and a potential biomarker for 1,25-VD anti-tumor action.

The anti-tumor effects of vitamin D against advanced prostate cancer (PCa) are well recognized in preclinical and clinical studies. However, the therapeutic window of vitamin D treatment is extremely narrow and effective doses cannot be administered without inducing hypercalcemia. Therefore, much effort has been directed toward the identification of new analogs with potent cell-regulatory action as well as decreased risk of inducing adverse calcemic side effects (1, 2). One of the most promising synthetic analogs is Seocalcitol (EB1089, Leo Pharmaceutical Products) (3). In addition, combination treatments consisting of vitamin D analogs and other agents such as dexamethasone or Taxotere, a chemotherapy drug, have also been tested on PCa cells and in clinical trials (4, 5). All those compounds or vitamin D combined therapies have decreased side effects, yet their efficacy in human clinical trials has not been validated. The anti-tumor effect of vitamin D includes anti-proliferation through accumulation of cells in the G0/G1 or G2/M phase (6–9), induction of apoptosis (10, 11), inhibition of angiogenesis (12), and anti-metastasis (13, 14). In addition, it appears that PCa cells have diverse sensitivity to vitamin D, and can become resistant to the tumor suppressive effects of vitamin D. In our experimental model system, long-term treatment of PCa cells with the vitamin D active metabolite, 1α,25-dihydroxyvitamin D3 (1,25-VD), can induce vitamin D resistance in PCa cell culture. Our efforts in improving the anti-tumor effects of 1,25-VD led us to explore the potential role of microRNAs (miRNAs) in mediating this effect.

miRNAs are small non-coding single-stranded RNAs of ~22 nucleotides that destabilize and inhibit the translation of target messenger RNAs (mRNAs). The fact that one single miRNA can control hundreds of target mRNAs is striking and makes miRNAs as powerful as transcription factors in regulating whole cell proteomes. The multifaceted roles of miRNAs in cancer, including PCa, have gained increased appreciation after recently accumulated evidence. More importantly, miRNA roles in influencing cancer biology, including proliferation, apoptosis, and invasiveness, have proven indispensable. Recently, reports provide evidence supporting the role of certain miRNAs in PCa progression, such as loss of miR-15/16 and miR-101 are often associated with advanced PCa (15, 16). Indeed, introduction of miR-15/16 into PCa inhibits cell growth, while knockdown induces tumorigenicity and invasiveness of normal prostate cells (15). Introduction of miR-101 into PCa cells could suppress growth, invasiveness, and xenograft tumor growth, while knockdown increases invasiveness of benign cells (16). In addition, miR-221 and miR-222 are found to promote PCa cell growth while knockdown of both decrease PCa clonogenicity (17), suggesting oncogenic properties of...
miR-221 and miR-222. The idea of targeting miRNAs in PCa therapy has been pre-clinically tested and showed promising results. Exploiting miRNAs in improving the efficacy of current therapy could broaden the strategies for battling advanced PCa.

The roles of miRNAs in mediating the vitamin D anti-tumor action remain unclear and demand investigation. It was reported that VDR level is repressed by miR-125b, thus overexpression of miR-125b attenuates the anti-tumor effect of 1,25-VD (18). Vitamin D and testosterone can regulate miRNAs in an additive or synergistic manner in LNCaP (19). Among these miRNAs, the role of miR-22 in mediating the anti-proliferative and anti-migratory effect of vitamin D has been proven important in colon cancer (20). To identify which miRNAs participate in the anti-tumor effect of vitamin D, we profiled miRNAs expression by microarray in the 1,25-VD-treated PCa cell line, LNCaP, and validated that miR-98, a tumor suppressive miRNA, is regulated by 1,25-VD. We further discovered the dysregulation of miRNAs expression in vitamin D resistant LNCaP sublines. Modulation of miR-98 affects the anti-tumor effect of vitamin D suggesting that miR-98 can be a potential target for developing therapies for PCa.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Reagents—**Ambion® Pre-miRTM control and miR-98 precursors, and Anti-miRTM control and miR-98 inhibitors were purchased from Invitrogen (Carlsbad, CA). Transfection of anti-miRNA and miRNA precursors was performed using siPORT® NeoFX™ (Invitrogen). The plasmid pcDNA3.1(-)miR-98 was constructed by PCR amplification of miR-98 from genomic DNA of LNCaP cells using primer pairs Xhol-miR98-s: 5'-CCGCTCTGAGTTTGCCTGCG-TGCCCTTAATTAG-3' and BglII-miR98-as: 5'-CGAGATCT-CAACACTGCTAAGACTAAGTG-3' then inserted into the Xhol and BglII sites of pcDNA3.1(-) vector. The plasmid pGL3-p-LUC-CCNJ 3'-UTR is constructed by inserting CCNJ 3'-UTR, amplified by PCR using primers CCNJ-3'-UTR-f: 5'-GGGGAGCAGCATGTATCTGACGTT-3' (forward) and 5'-GCTCTAGAAAGTTACCACAGGCAC-GCC-3' (reverse); VDRE-C: 5'-TGAGGTAGTAAAGTTGATTGGG-3' and(forward) and 5'-GGAGGTAGTAAAGTTGATTGGG-3' (reverse); and 5'-CCCTCTGAGTTTGATGGTT-3' (reverse); LIN28A and LIN28B antibodies were purchased from Abcam (Cambridge, MA).

**Establishing Stable Cell Lines—**LNCaP stable cell lines expressing shRNA-targeting VDR (shVDR) and control shRNA (SC) were generated as described in a previous publication (21). LNCaP stable cell line expressing miR-98 (LNCaPmiR98) was established by transfecting LNCaP with pcDNA3.1(-)miR-98 plasmid. Two days after transfection, cells were selected by 350 μg/ml G418 (Invitrogen) for 10 days. Single clones were selected by series dilution. The overexpression of miR-98 was confirmed by Q-PCR.

** Colony Formation Assay—** Cells were seeded on 6-well dishes at a density of 10^4 cells/well. Cells were treated with EtOH or 1,25-VD of different concentration every other day. Two weeks after treatment, colonies were fixed and stained by crystal violet. After taking images by Chemidoc (Bio-Rad), colonies were counted by QuantityOne software (Bio-Rad).

**ChIP Assay—** ChIP assays were performed according to a previous publication (22). DNA fragments containing VDREs in miR-98 promoter were amplified by the following specific primer pairs. VDRE-A: 5’-GGAGCACGATGTATCTGACGTTGGGC-3’ (forward) and 5’-GGGGAGGTAGTAAAGTTGATTGGG-3’ (reverse); VDRE-B: 5’-GCTCTAGAAGTTACCACAGGCAC-GCC-3’ and(forward) and 5’-TGAGGTAGTAAAGTTGATTGGG-3’ (reverse); VDRE-C: 5’-TGAGGTAGTAAAGTTGATTGGG-3’ (forward) and 5’-GGAGGTAGTAAAGTTGATTGGG-3’ (reverse); and 5’-CCCTCTGAGTTTGATGGTT-3’ (reverse).

**Quantitative PCR (Q-PCR) Assays and Primers—** Total RNA was harvested from cells by Trizol (Invitrogen) and converted to cDNA using nCode™ VILO™ miRNA cDNA synthesis kit (Invitrogen). Q-PCR was performed as in the previous report (22). Primers used are U6: 5’-CTTGTGGGGCTCACAGACGA-A-3’; miR-98: 5’-AGGGAGGTAGTAAAGTTGATTGGG-3’; and 5’-CCCTCTGAGTTTGCCTGGA-3’ (forward) and 5’-CTGAGATGCTAAGACTAAGTG-3’ (reverse).

**Cell Cycle Analysis—** After indicated treatments, cells were trypsinized, washed by PBS, and fixed in 70% EtOH overnight. Cells were then pelleted and incubated in PBS containing 0.05 mg/ml RNase A for 30 min at room temperature. After washing, the cells were stained with 10 μg/ml propidium iodide for 10 min then washed three times with PBS. Cells were resuspended in PBS at 10^6 cells/ml. Cell cycle profiles were determined by flow cytometry using FACSCanto-II (BD, Franklin Lakes, NJ) and analyzed by FlowJo software.

**Mouse Models—** The study was approved by the University of Rochester Committee on Animal Resources, and the mice were kept in a specific pathogen-free environment at the animal facility of the University of Rochester Medical Center. PTEN(-/-) and TRAMP mice were obtained from The Jackson Lab (Bar Harbor, Maine). Adult male mice at age 24–26 weeks of age were intraperitoneally injected with 1,25-VD (25 ng/g of mice weight) in corn oil. After 24 h, mice were anesthetized for drawing cardiac blood which was then mixed with EDTA (0.2%) to prevent coagulation. Total blood cells were spun down and RNA extracted by Trizol.

**Cell viability assay, Western blotting assay, transient transfection, and luciferase assays were performed according to previous publications (22, 23).**

**RESULTS**

**Vitamin D Induces miR-98 Expression in LNCaP Cells—** In search of miRNAs mediating the anti-proliferative effect of 1,25-VD, we performed systemic miRNA array profiling comparing LNCaP cells treated with ethanol (EtOH) vehicle control and 1,25-VD (LC Sciences). We performed two independent experiments in microarray study. There are 8 miRNAs consistently up- or down-regulated by 1,25-VD with statistical significance in individual experiments (Fig. 1A). Further in-depth analysis provided by LC Science shows that miR-98 is the miRNA showing strong signals (>500) in array; therefore it was chosen for further validation and exploration for its roles in mediating 1,25-VD anti-tumor effects. First, we confirmed 1,25-VD effects on the miR-98 expression by Q-PCR in LNCaP sublines...
with different vitamin D responsiveness: vitamin D-resistant line (LNCaP-r) versus parental cells (LNCaP-p) (24). As shown in Fig. 1B, miR-98 expression was induced by 1,25-VD in LNCaP-p cell, but this miR-98 induction by 1,25-VD was diminished in the LNCaP-r cells. This suggests the regulation of miR-98 is through 1,25-VD/VDR transcriptional activity. To further verify VDR’s involvement in this regulation, we knocked down VDR by shRNA against VDR and examined the miR-98 expression upon 1,25-VD treatment. We found that VDR expression was reduced in VDR knockdown clones as compared with scramble shRNA controls (Fig. 1C). VDR expression in LNCaP cell lines stably expressed with scramble shRNA (subline sc8 and sc10) or shRNA against VDR (subline shVDR3 and shVDR7). Total RNA harvested from cells was reverse-transcribed to cDNA. The mRNAs of VDR and actin were measured by Q-PCR. After being normalized with actin, the relative mRNA expression of VDR compared with LNCaP-sc8 cells was calculated and mean ± S.D. plotted. *, p < 0.05 compared with sc sublines.

FIGURE 1. The vitamin D induction of miR-98 is dependent on VDR transactivity in LNCaP cells. A, array profiling of miRNAs expression in 1,25-VD treated LNCaP cells showed 8 miRNAs significantly regulated by 1,25-VD treatment. LNCaP cells were treated with EtOH or 1,25-VD (100 nM) for 24 h then harvested for RNA extraction. Total RNAs were submitted to LC Science for array profiling of miRNAs expression. The relative expression of miRNAs in 1,25-VD-treated group compared with EtOH-treated groups with statistical significance (p < 0.01) in two independent experiments was summarized. 8, vitamin D-induced miR-98 in LNCaP parental cells but not in vitamin D-resistant cells. Cells were treated with EtOH or 1,25-VD 100 nM for 24 h, then total RNAs harvested from cells were reverse-transcribed to cDNA using VILO NCode kit. The expression of miR-98 and U6 was measured by Q-PCR. The relative miR-98 expression, after being normalized with U6 in 1,25-VD-treated group compared with EtOH-treated group, was calculated and mean ± S.D. plotted. *, p < 0.05 compared with EtOH-treated group in each subline. C, VDR expression in LNCaP cell lines stably expressed with scramble shRNA (subline sc8 and sc10) or shRNA against VDR (subline shVDR3 and shVDR7). Total RNA harvested from cells was reverse-transcribed to cDNA. The mRNAs of VDR and actin were measured by Q-PCR. After being normalized with actin, the relative mRNA expression of VDR compared with LNCaP-sc8 cells was calculated and mean ± S.D. plotted. *, p < 0.05 compared with sc sublines. D, vitamin D induction of miR-98 is diminished in the VDR knockdown LNCaP cells. Cells were treated and harvested for detecting miR-98 expression as in B. The relative expression of miR-98 normalized by U6 was calculated and mean ± S.D. plotted. *, p < 0.05 1,25-VD treated group compared with EtOH-treated group in each subline.

The Induction of miR-98 Contributes to the Anti-proliferative Effect of 1,25-VD—MiR-98 belongs to the Let-7 family which is well categorized as a tumor suppressive miRNA. It has been shown to regulate HMGA2, a gene controlling growth and tumorigenesis (25). Therefore, we suspect that miR-98 is involved in the anti-proliferative mechanism of 1,25-VD. Cells were transfected with antagonirs of miR-98 (anti-miR-98) to knock down miR-98 expression; the 1,25-VD responsiveness was examined. As expected, the 1,25-VD induction of miR-98 was diminished in the anti-miR-98 transfected cells (Fig. 2A); consequently, 1,25-VD-mediated growth inhibition effect was significantly reduced in the anti-miR-98 cells as compared with cells that were transfected with control antagonirs (Fig. 2B). This indicates the importance of miR-98 in mediating the anti-proliferative effect of 1,25-VD. On the other hand, overexpression of miR-98 alone can suppress LNCaP cells growth up to 30% and treatment with 1,25-VD in the miR-98 overexpressed cells does not further suppress cell growth (Fig. 2D). We further examined the growth inhibitive effect of miR-98 by colony formation assay detecting cell survival and cell viability and by direct cell counting. In order to perform this study, we established miR-98 stably expressed LNCaP cells (LNCaPmiR98) (Fig. 2F). The
result showed dramatic inhibition of cell survival and cell viability and by miR-98 when it was stably expressed in LNCaP cells (Fig. 2, F and G). Treatment of 1,25-VD further suppressed the colony numbers and cell viability. This suggests miR-98 is a key mediator of the anti-proliferative effect of 1,25-VD.

1,25-VD Regulates miR-98 Expression via Direct and Indirect Mechanisms—The transcriptional regulation of miR-98 has not yet been studied. Its transcription start (TSS) site has been predicted to be located at 3070 bp in 5’ upstream, within the host gene HUWE1 (26). We searched for putative VDR

### FIGURE 2. Modulation of miR-98 affects the anti-growth effect of 1,25-VD in LNCaP.

- **A.** expression of miR-98 was knocked down by antagomir against miR-98. One day after being transfected with antagomirs, LNCaP cells were treated with EtOH or 1,25-VD for 24 h. Total RNA was harvested then reverse-transcribed to cDNA. The relative expression of miR-98 was measured and plotted as described in Fig. 1. *p < 0.05. B. anti-growth effect of 1,25-VD in LNCaP was decreased by antagomir of miR-98. LNCaP cells were transfected with control antagomirs and miR-98 antagomirs. After 24 h, cells were treated with EtOH or 100 nM 1,25-VD every 2 days. Viable cells were detected and quantified by MTT assay at 2, 4, 6 days after treatment. The viable cells of the EtOH-treated group at the day of assay was set as 100% then relative viable cells were calculated and mean ± S.D. plotted (*p < 0.05 and **p < 0.01 when compared with the EtOH-treated group). C. RNA was harvested as described in A. Amount of miR-98 was quantified by Q-PCR. The relative expression level compared with control pre-miRNA transfected, EtOH-treated group was calculated and mean ± S.D. plotted. **p < 0.01 compared with control group of same treatment. D. LNCaP cells were seeded and transfected with control or pre-miR98 oligonucleotides. After 24 h, cells were treated with EtOH or 1,25-VD (1–100 nM) every 2 days. At day 6 after treatment, viable cells were measured by MTT assay. The relative percentages of survival cells compared with the group transfected with control pre-miRNA and treated with EtOH were calculated and plotted. **p < 0.01 compared with control group of same treatment. E. LNCaP stable line overexpressing miR-98 was established by transfecting pcDNA3.1(-)miR-98 followed by neomycin selection. The expression of miR-98 in parental and the stable line LNCaPmiR98 was examined by Q-PCR. F. colony formation assay was performed by seeding 10^4 LNCaP and LNCaPmiR98 cells in 6-well culture dishes. One day after seeding, cells were treated with EtOH or indicated concentration of 1,25-VD every other day. Two weeks after treatment, cells were stained and counted. The colony forming percentage was calculated by setting colony number of EtOH-treated LNCaP cells as 100% then mean ± S.D. plotted. G, cell viability assay were performed by seeding 25,000 cells/well of 12 wells, and treated with EtOH, or indicated concentration of 1,25-VD every other day. Cells were trypsinized at day 6 for counting by Vi-CELL XR 2.03 (Beckman Coulter, Inc.)
response elements (VDREs) within 2 kb flanking TSS sites by ChIP mapper online program, and four putative VDREs were identified (Fig. 3A). We performed a ChIP assay to test the binding between VDR and putative VDREs and found that VDR bound all VDREs in a ligand-independent manner, except VDRE-B (Fig. 3B, and quantified result in C), suggesting that VDR can bind to VDREs in 5′ H11032 promoter of miR-98 to induce its expression. In addition to direct regulation of miR-98 by VDR, we also tested the possibility that 1,25-VD induces miR-98 via an indirect mechanism, such as through regulation of LIN28 proteins that have been shown to bind to the let-7 family pre-microRNA and block production of the mature microRNAs (27). We found that 1,25-VD suppressed LIN28A and LIN28B expression (Fig. 3D, and E), and therefore could consequently result in miR-98 induction. Overall, these results suggest 1,25-VD regulates miR-98 via both direct and indirect mechanisms.

**MiR-98 Induces G2/M Arrest and Down-regulates CCNJ**—The significant growth suppressive effect of miR-98 in LNCaP was further investigated by cell cycle analysis. LNCaP cells were stably transfected with miR-98 or control vector, then cells were treated with 1,25-VD and analyzed for cell cycle distribution. The result showed that 1,25-VD treatment alone increased G1 distribution while miR-98 overexpression induced G2/M arrest (Fig. 4, A and B). Interestingly, miR-98-overexpressed cells were further arrested in the G1 cell cycle by 1,25-VD. To investigate the miR-98’s molecular target that controls G2/M cell cycle, we searched targets of miR-98 by TargetScan and found cyclin J (CCNJ), which has been linked with G2/M cell cycle in early Drosophila embryogenesis (28). We therefore examined the expression of CCNJ in 1,25-VD-treated and miR-98-overexpressed cells and found both 1,25-VD and miR-98 suppressed CCNJ expression but the combination did not have an additive effect (Fig. 4C). The molecular mechanism by which miR-98 regulates CCNJ was further investigated where we inserted 3′-UTR of CCNJ that contains the putative miR-98 target site into 3′-UTR region of LUC cDNA in pGL3-p-LUC plasmid (Fig. 4D). Overexpression of miR-98 reduced LUC expression while knockdown miR-98 induced LUC expression (Fig. 4E). These results suggested that miR-98 down-regulates CCNJ, hence impairing mitosis progression.

**The Level of miR-98 in Blood Is Correlated with 1,25-VD Treatment in Mice**—The potential of miRNA as a biomarker in body fluid has been reported. We expect miR-98 will be a useful functional biomarker predicting 1,25-VD response. The indi-
individual variation in response to 1,25-VD is contributed by a variety of factors, including metabolizing enzyme level, VDR polymorphism, cellular context of coregulators, etc. Functional biomarker measuring response to 1,25-VD will be useful in predicting individual response to vitamin D-based therapy. To prove the principle, mice including wild type and prostate cancer mice models, PTEN heterozygous deletion and TRAMP, were treated with 1,25-VD, and their blood sample was harvested for measuring miR-98 level. The result showed an induction of miR-98 in blood cells from mice treated with 1,25-VD regardless of genetic background (Fig. 5). These data indicated that miR-98 can be measured from the blood and 1,25-VD can
miR-98 expression was detected by Q-PCR then the relative expression compared with EtOH-injected mice was calculated and mean ± S.D. plotted. *, p < 0.05; **, p < 0.01 compared with EtOH-treated group.

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induce its expression, thus, miR-98 level can potentially be developed as a biomarker to predict 1,25-VD anti-tumor activity.

**DISCUSSION**

Exploring miRNAs regulation as a novel mechanism through which vitamin D inhibits cell growth identifies the proliferation inhibitory effect of miR-98. Previous studies identify miR-22 and miR-32 induced by vitamin D in colon cancer cells and human myeloid leukemia cells, respectively (20, 29). The function of miR-22 contributes to the anti-growth and anti-migration effect of 1,25-VD in colon cancer cells (20). On the other hand, vitamin D induced miR-32 down-regulates the pro-apoptotic protein Bim and leads to anti-apoptotic effect of vitamin D. It is therefore suggested that down-regulation of miR-32 can enhance the therapeutic efficacy in leukemia patients (29).

Considerable preclinical and epidemiologic data suggest that vitamin D plays a critical role in pathogenesis, progression and therapy for PCa. However, the outcomes from the clinical trials that used 1,25-VD as a second line of therapy for advanced PCa patients are inconclusive. This is mainly due to lack of suitable pharmaceutical preparation that optimizes biologic dose and/or maximum-tolerated dose of 1,25-VD for clinical study. Thus, developing the vitamin D based therapy aiming to reduce hypercalcemic side effects has been one of the major efforts in the field. The discovery of oncomirs, tumor-suppressive, and oncogenic miRNAs, provides novel therapeutic targets for cancer therapy. There are promising results showing that locked-nucleic acid modified oligonucleotides knockdown miR-122, hence inhibiting HCV replication in liver; and intranasal administration of let-7 effectively reduces lung cancer in K-ras mutant mouse model (30). In addition to directly targeting oncomirs, miRNAs modulators can also sensitize cancer to radiation, chemotherapy, and hormone depletion therapy (31–33). Therefore, miRNAs modulators are suitable for developing combination therapy to sensitize cancer to vitamin D treatment. The findings of others and our study indicate that miR-22 and miR-98 mediate the growth inhibitory effect of 1,25-VD. These provide a therapy strategy where combining 1,25-VD and miRNAs modulators, such as ectopically introducing tumor suppressive miRNAs (miR-22 and miR-98) or knockdown VDR signal down-regulating miRNAs (miR-125b and miR-27), could reduce the dosage of 1,25-VD, hence avoiding the hypercalcemic side effect. Nonetheless, developing safe and tissue specific delivery methods to knockdown or replaced miRNAs for PCa therapy is necessary to prevent toxicity and off-target effects.

The vitamin D regulation of miR-98 is correlated with VDR transactivity. We therefore seek VDREs in the promoter region of miR-98, which is located at its host gene HUWE1. We confirmed VDR binding of putative VDREs in the promoter region. In addition to direct regulation, we also found 1,25-VD indirectly regulates miR-98 via down-regulation of LIN28, the miRNAs processing proteins. The mechanism of 1,25-VD regulation of LIN28 is possible through regulation of NFκB activity that needs to be further investigated. The balance between LIN28 and Let-7 family is found to control the stemness of stem cells (34, 35). A recent study identifies that miR-125b, the microRNA down-regulating VDR, can suppress the differentiation of hair follicles and maintains stemness of stem cell progenies. Treatment with 1,25-VD can rescue the hair growth defect in mice with doxycycline-induced expression of miR-125b in skin (36). We therefore suspect 1,25-VD treatment could control the balance between quiescence/self-renewal and differentiation status of stem cells through regulation of LIN28.

The known targets of miR-98 include an oncogene (HMGAA2), a tumor suppressor (FUS1), and a immune-modulator, cytokine-inducible src homology 2-containing protein (CIS) (25, 37, 38). We observed the G2/M arrest in cells over-expressing miR-98 and identified a mitosis controlling cyclin, CCNJ, which is down-regulated by miR-98 via targeting its 3’-UTR. Interestingly, treatment of 1,25-VD in miR-98 overexpressed cells results in increasing G1 arrest as the effect of 1,25-VD alone. We therefore suspect other mechanisms through which miR-98 triggers can sensitize the 1,25-VD-in...
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produced G1 arrest in LNCaP. One possible mechanism is through reciprocal suppression of LIN28B, which is one of the predicted miR-98 targets in TargetScan. The down-regulation of LIN28B promotes differentiation of LNCaP cells, thereby enabling cells responding to the G1 arrest effect of 1,25-VD. Another interesting candidate target of miR-98 is enhancer of zeste homolog 2 (EZH2), which functions in protein complex to trimethylate histone H3 lysine 27 leading to epigenetic silencing (16). It is suspected that by down-regulation of EZH2, anti-proliferative genes, especially those involved in G1 arresting, is de-silenced, therefore enabling 1,25-VD to induce VDR up-regulating their expression. Whether CCN3 or other not-yet identified miR-98 targets contribute to the role of miR-98 in mediating the growth inhibitory effect of 1,25-VD requires further study.

Biomarker potential of miRNAs relies on their high stability and existence in formalin-fixed tissue, cell free serum/plasma, and urine (39). The biological response to vitamin D varied among individuals. Factors contributing to a broad range of sensitivity to vitamin D include genomic polymorphisms of VDR and metabolism enzymes and pathological conditions altering VDR activity. Biomarkers representing biological response of vitamin D treatment will be more meaningful than measurement of serum vitamin D level. A recent study investigates the serum level of miRNAs in subjects given high dose vitamin D3 (20,000 and 40,000 IU) weekly for a year (40). Although there is not yet a conclusive result, measuring miRNAs in body fluid to identify the response to vitamin D is worth pursuing. We therefore explore the biomarker potential of miR-98 representing vitamin D treatment in mice bearing prostate cancer. The cellular fraction of blood contains miR-98 whose expression correlates well with vitamin D treatment in both wild type and cancerous mice. Further investigation correlating expression levels of miR-98 and other vitamin D-regulated anti-tumor miRNAs (such as miR-22) in blood or other body fluid with anti-tumor effect of vitamin D is demanded.

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