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The sum of many small changes: microRNAs are specifically and potentially globally altered by vitamin D_3 metabolites

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Abstract

Vitamin D_3 deficiency is rampant which may contribute to increased risk of many diseases including cancer, cardiovascular disease and autoimmune disorders. Genomic activity of the active metabolite 1,25-dihydroxyvitamin D (1,25D) mediates most vitamin D_3's actions and many gene targets of 1,25D have been characterized. As the importance of non-coding RNAs has emerged, the ability of vitamin D_3 *via* 1,25D to regulate microRNAs (miRNAs) has been demonstrated in several cancer cell lines, patient tissue and sera. In vitamin D_3 intervention patient trials, significant differences in miRNAs are observed between treatment groups and/or between baseline and followup. In patient sera from population studies, specific miRNA differences associate with serum levels of 25D. The findings thus far indicate that dietary vitamin D_3 in patients and 1,25D *in vitro* not only regulate specific miRNA(s), but may also globally upregulate miRNA levels.

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Keywords

Vitamin D_3; microRNAs

1. Introduction

The dietary and UV-induced prohormone vitamin D_3 has pleiotropic effects that include regulation of calcium homeostasis, anti-inflammatory and potentially cancer prevention and/or treatment properties [1]. Population studies have shown that a low vitamin D_3 status is associated with increased risk of colon [2], breast [3,4], prostate [5,6] and other cancers [7]. Moreover, a recent cohort analysis showed that low vitamin D_3 status increased risk of death from all cancers [8].

In addition to natural dietary and supplemental vitamin D_3, sun exposure triggers formation of vitamin D_3 (cholecalciferol) in the skin. Cholecalciferol is metabolized into 25-hydroxyvitamin D_3 (25D), primarily by the liver. 25D, or "circulating vitamin D", is typically measured as an indicator of "vitamin D status". Circulating 25D is further metabolized by the kidney to the active metabolite, 1,25-dihydroxyvitamin D_3 (1,25D) [1], which regulates gene transcription *via* binding of 1,25D to the vitamin D receptor (VDR). VDR interacts with vitamin D response elements (VDREs) in the DNA, positively or

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negatively regulating gene transcription [1]. ChIP-seq data for VDR in immune cells has identified thousands of VDREs in the genome [9,10], but ChIP-seq identification of VDR binding sites is yet to be determined in other tissue types. Interestingly, 1,25D-regulated genes highly differ between tissues and cell types, a phenomenon that Zhang et al. recently suggested may be due to ligand-induced and DNA binding-induced alterations in VDR structure and activity [11]. Non-genomic "rapid actions" of vitamin D_3 have been reported and are dependent on membrane VDR [12].

Given the strong genomic actions of 1,25D, and the reality that only 1.5% of our genome contains protein-coding genes [13, 14], it is likely that 1,25D also regulates the expression of some of the remaining genome with includes non-coding RNAs (ncRNAs). Non-coding areas are among the ultra-conserved elements (>95% identity with chicken, dog, mouse, rat) in the genome [15]. microRNAs (miRNAs) are a class of small ncRNAs that function by binding to imperfect complementary sites in the 3′-untranslated region (UTR) of target mRNAs, decreasing mRNA stability and/or decreasing protein translation [16]. miRNA are predicted to regulate 30% of all coding genes [17]. Mature miRNAs (18–25 nucleotides) are the result of post-transcription processing of longer nuclear encoded-hairpin pri-miRNAs. Drosha is a ribonuclease that resides in the nucleus and processes the full length pri-miRNA transcript into a hairpin pre-miRNAa which is exported into the cytosol for further processing by Dicer into the mature single stranded miRNA [18]. Pri-miRNAs can be transcribed independently when they are situated in intergenic regions or located on the antisense strands of annotated genes [19]. Other pri-miRNAs are encoded in intronic regions of host genes [19]. The transcriptional control of pri-miRNA expression remains incomplete. In the cytosol, mature miRNAs incorporate into the RNA-induced silencing complex (RISC) to silence mRNAs [18]. The pleotropic effect of miRNAs lies in their promiscuity and ability for one miRNA to regulate the expression of multiple target mRNAs.

Aberrant miRNA levels are found in cancers [20] and some miRNAs have been shown to influence the initiation and progression of human cancer. The importance of miRNAs in cancer was first demonstrated in the deletion of miRs-15 and 16-1 and the subsequent observation of increased expression of Bcl-2 in B-cell chronic lymphocytic leukemia (CLL) [21–23]. Since that seminal discovery, miRNA profiling in cancers has exploded. Tumor-associated changes in miRNAs have been shown to occur by gene deletion/amplification, epigenetic mechanisms and by alterations in the miRNA processing machinery [20]. Expression profiling of cancers has identified miRNA signatures in cancers that associate with diagnosis, staging, progression, prognosis and response to treatment [20]. A defined role for individual miRNA changes has not been characterized for many of the cancer-related miRNAs because their function(s) is still unknown.

Interestingly, human tumors show a marked widespread reduction in miRNA levels [24]. This reduction in miRNA levels may be an attribute of stem-like properties and contribute to epigenetic abnormalities in cancer as reduction of global miRNAs expression disrupts maintenance of DNA hypermethylation [25]. Specific roles for miRNAs have emerged in the stem cells of developing animals [26]. It follows that in cancer, where cells regain "stemness", miRNAs would be also involved.

Irrespective of the role of miRNAs during carcinogenesis, miRNAs are potential powerful biomarkers as they are aberrantly expressed in cancer and resistant to degradation in serum and tissues [27,28]. Multiple freeze–thaw cycles and storage at ambient temperature have no effect on miRNA detection in plasma and serum [28]. As well, miRNAs remain stable in archival formalin-fixed paraffin-embedded (FFPE) tissues [29,30]. Because of their remarkable stability, miRNAs are ideal biomarkers that can be explored in both archival and prospective specimens.

Despite the well characterized genomic actions of 1,25D and the vital role of miRNAs in fundamental cell biology, there are very few studies that examine regulation of miRNAs by 1,25D directly or as a result of dietary vitamin D_3. Here we review the current findings on miRNAs that are altered by dietary vitamin D_3 or directly *via* its metabolites.

2. Regulation of miRNAs by 1,25D and vitamin D_3

2.1. Prostate cancer

Vitamin D_3 has anti-cancer and chemopreventive activities in the prostate. Laboratory and *in vivo* rodent studies strongly support an anti-cancer activity for vitamin D_3 in the prostate, whereas epidemiologic evidence obtained from serum levels of vitamin D_3 metabolites show mixed results (reviewed in [1,31]). Interestingly, low serum levels of 25D are consistently associated with increased risk of P PCa mortality [5,32], but not consistently associated with overall PCa risk [31], suggesting vitamin D_3 may be important in protecting against aggressive forms of PCa. Another layer to local vitamin D_3 action is that PCa cells have reduced vitamin D_3 1α-hydroxylase activity, which may lead to a disconnect between serum 25D levels and prostatic 1,25D bioavailability [33]. 1,25D regulates the expression of hundreds of genes in normal prostate cells and PCa cells, as shown by cDNA microarray analysis [34,35].

MiRNA expression signatures specific to PCa have been reported [36–42]. These PCa-related miRNA signatures provide a foundation for future research, but they need to be tested in a larger number of specimens and the biological role of the PCa-specific miR alterations have yet to be studied. In PCa, several studies have shown global repression of miRs [24] or an imbalance of more down-regulated miR than upregulated miRs [24,38,40,43]. Dicer levels are increased in PCa and associate with an aggressive cancer phenotype, which does not implicate dicer as the mechanism for widespread miRNA reduction in PCa [44].

Three studies have examined the effect of 1,25D on miRNA expression in prostate cells and one study examined patient prostate tissue. Wang et al. [45] found that fifteen miRNAs were differentially regulated by 2.0-fold by combination treatment with 1,25D (100 nM) and testosterone (5 nM) (T + D) in LNCaP cells. Overall, around 80% of the regulated miRNAs were upregulated by T + D particularly; miR-134, miR-22, and miR-29a/b while only miR-17 and miR-20a/b were downregulated (Table 1). Wang et al. suggest that the synergistic effect of 1,25D and testosterone have more significant effects on miRNA expression than either on their own. In another study, miR-106b alone was shown to be upregulated by 1,25D in prostate cells and contribute to p21 mediated cell-cycle arrest [46].

Our group has analyzed the regulation of miRNAs by 1,25D (50 nM) and vitamin D_3 (cholecalciferol) in prostate cells and in patient tissue respectively [93]. *In vitro*, miR-100 and miR-125b, were upregulated while their targets PLK1 and E2F3 were down-regulated by 1,25D in a VDR-dependant manner [93] (Table 1). Validation of the cell culture findings in PCa patients given oral vitamin D_3 for 3–8 weeks prior to radical prostatectomy demonstrated local prostatic 1,25D concentrations positively correlated with the miRs (miR-100, miR-125b, miR-106b, miR-141, miR-331-3p, miR-103, let-7a, and let-7b) in normal and/or PCa epithelium (Table 2). As well, there was an overall downregulation of miRNAs in PCa regions compared to benign [93]. These results show that vitamin D_3 may globally augment miRNAs in both benign and PCa tissue in patients.

2.2. Breast cancer

Low serum 25D levels are associated with decreased breast cancer risk [47]. Goodwin et al. [48] found that in a study of 535 women 75 years in age or younger, vitamin D_3 deficiency

was associated with distant recurrence and mortality. They also found similar results to Neuhouser and colleagues who found that 75% of breast cancer survivors were deficient in vitamin D_3 by serum 25D levels [49]. In contrast, a nested-case control study of 512 breast cancer patients and matched controls, found no significant association between recurrence, survival, and serum 25D after breast cancer treatment [50]. While epidemiological studies show varied results, *in vivo* 1,25D analog EB1089 reduced growth of breast cancer tumors in mice [51] by inducing apoptosis [51]. In mice, supplementation with vitamin D_3 and calcium prevented western style diet (high fat diet)-induced mammary hyperproliferation [52].

MiRNA expression can differentiate breast cancer from benign tissue with high accuracy, as well, particular features associated with breast cancer such as estrogen or progesterone receptor expression, lymph node metastasis, vascular invasion, proliferation, and p53 can be identified from miRNA profiles [21]. Iorio et al. identified miR-10b, miR-125b, and miR-145 as miRNAs that were consistently expressed at a lower level in tumor areas while miR-21 and miR-155 were upregulated. The expression of miR-206, miR-335, and miR-126, tumor suppressive miRNAs, is absent in metastatic tumor cells and these miRNAs suppress breast cancer metastasis to lung and bone [53]. Dicer expression may also be a predictor of breast tumor and metastasis as dicer levels were decreased in patients with recurrence and in breast cancer cell lines and low levels were associated with metastasis [54].

In MCF12F breast cancer cells, 25D regulated stress-induced miRNAs [55]. Microarray profiling in stressed cells (induced by serum starvation) upregulated miR-26b, miR-182, and let-7a and downregulated miR-18a, miR-106, and miR-30c by 2.0-fold. Pretreatment with 25D (250 nmol/L) inhibited/reversed the stress-induced miRNA changes in expression (Table 1). MiRNA expression in 25D-treated cells without stress was not included in this study. Stressors in the form of hypoxia, oxidative stress, serum starvation, inflammatory stress, and heat-shock-induced stress are potential induces of cancer and can alter cancer progression. The ability of 25D to target stress-induced miRNAs suggests a mechanism involved in its chemopreventive role in breast cancer [55].

2.3. Colon cancer

In 1989 a US study first reported that serum 25D was inversely correlated with colorectal cancer [2]. A meta-analysis of that pivotal 1989 study and eight additional clinical studies further validated the inverse association between serum 25D levels and colorectal cancer risk [47]. In clinical patient specimens VDR levels are increased early in colon cancer but are greatly reduced in the later stages [56]. *In vivo*, Balb/c mice given a vitamin D_3-deficient diet had larger tumors, and decreased VDR and CYP27B1 expression than vitamin D-sufficient mice [57]. Mice given a western style diet (with low calcium and vitamin D_3 and high fat) develop colonic tumors while supplementation with calcium and vitamin D_3 reduced tumor progression, suggesting that vitamin D_3 deficiency is involved in tumor progression [58].

Similarly to other cancers, miRNAs are dysregulated in colon cancer. In colon cancer miRNAs have been shown to be diagnostic (miR-17-3p and miR-92a) and prognostic markers (miR-21) and suitable predictors of treatment outcome (let-7 and miR-181b) (as reviewed by [59]).

Alvarez-Diaz et al. demonstrated that tumor suppressive, miR-22 is induced by 1,25D in a VDR-dependent manner and exerts anti-proliferative and anti-migratory effects when overexpressed in human colon cancer cells [60] (Table 1). A 48 h treatment with 1,25D (100nM) augmented miR-22 expression and regulated vitamin D target genes *OGN,*

NELL2, HNRH1, RERE and NFAT5. In matched normal and tumor samples from colon cancer patients, miR-22 levels were lower in 78% of tumor and miR-22 expression positively correlated with the VDR expression, which was also lower (72%) in tumor samples. Independent of vitamin D_3, others found that miR-22 regulates angiogenesis, *via* suppression of hypoxia inducible factor 1 and VEGF, in colon cancer cells and confirmed that miR-122 levels are lower in colon cancer *versus* benign colonic epithelium [61]. Therefore, miR-22 may be a significant part of the chemopreventive activity of vitamin D_3 in colon cancer.

2.4. Melanoma

Vitamin D_3 is synthesized in the skin from sun exposure and that UV exposure is also the major risk factor for skin cancers [62], making the role of vitamin D_3 in melanoma somewhat complicated. *In vitro*, Colston et al. first demonstrated that melanoma cells express the VDR and that 1,25D is anti-proliferative [63]. A cohort study also demonstrated that high 25D levels at diagnosis were protective of melanoma relapse and mortality suggesting that vitamin D_3 supplementation may protect melanoma patients from relapse [64]. Another study found that a VDR promoter polymorphism, A-1012G, increased occurrence and metastasis development of malignant melanoma [65].Various studies have profiled miRNAs in melanoma tissue and cells [66–68]. One study identified miRNAs (4 upregulated/11 downregulated in 8 melanoma cells lines) that could distinguish melanoma from other solid tumors [66]. Due to inconsistent published findings on melanoma and miRNAs, Philippidou and colleagues profiled 9 melanoma cell lines and 20 patient samples (3 benign/20 melanoma metastasis) where they identified miR-200c, as did Gaur et al. [66], as downregulated both *in vitro* and in patients [68]. Dicer expression is also altered in melanoma where protein levels where increased in melanoma tissue [69].

Essa et al. examined the effect of 1,25D (10nM) on melanoma cells and identified miR-125b as downregulated when VDR was increased [70]. However, knockdown of miR-125b did not alter VDR mRNA and protein levels [70]. Basal levels of miR-125b were also lower in 1,25D-responsive melanoma cell lines (MeWo and SK-Mel28) compared to primary melanocytes [70]. miR-125b may be involved in the early progression of melanoma as it was downregulated in early metastasis of cutaneous melanoma [71] and knockdown of miR-125b enhanced the metastatic capability though the inhibition of senescence and apoptosis [72].

2.5. Leukemia

Differentiation therapy is a useful treatment in hematological malignancies. The earliest report of 1,25D's involvement in differentiation of leukemia cells was in the human promyelocytic leukemia cell line, HL60 [73] Then Munker et al. demonstrated that 1,25D was also anti-proliferative in leukemia cells [74]. Numerous other studies have further shown the anti-proliferative and pro-differentiating property of vitamin D_3 *in vivo* and *in vitro*. Analysis of UVB exposure and leukemia incidence around the world, demonstrated that serum vitamin D_3 status may be predictive of leukemia risk [75,76]. Human studies with vitamin D_3 (or analogs) alone or in combination therapy have seen minimal effects on survival and/or rates/duration or remission as reviewed by [77]. Further studies to understand the mechanism of vitamin D_3 on the immune system as well as clinical trials with more effective vitamin D_3 analogs may be beneficial in the treatment of leukemia's with vitamin D_3.

The seminal study that demonstrated miRNA involvement cancer was in chronic lymphocytic leukemia (CLL) patients where miR-15a and miR-16-1 were downregulated due to a genomic deletion [78]. Further studies identified differential miRNA patterns

between normal and leukemia patient tissue, aberrant miRNA expression in malignancy, and miRNAs involved in hematoposisis (as reviewed by [79]).

In acute myeloid leukemia cells, both miR-181a/b and miR-32 are regulated by 1,25D [80,81]. HL60 and U937 cells treated with 1,25D (0.1–100nM) for 48h downregulated both miR-181a and more so miR-181b. Wang et al. demonstrated that 1,25D augmented cell cycle regulator $p27^{Kip1}$ and transfection with a pre-miR-181a abrogated that effect suggesting that 1,25D and miR-181 are involved in the control of cell cycle transition in myeloid leukemia [81]. 1,25D also can alter pro-survival mechanisms in HL60 and U937 cells through its regulation of miR-32 [80]. Gocek et al. found that 1,25D-induced miR-32, regulated pro-apoptotic Bim. Further, decreased miR-32 expression made AML cells more susceptible to agents like AraC that are used to treat this disease [80].

2.6. Serum

miRNAs are differentially expressed in both serum and plasma of cancer patients, and may serve as potential biomarkers for cancer diagnosis and prognosis [82,83]. In pregnant women, Enquobahrie et al. demonstrated that vitamin D_3-deficiency was associated with differential miRNAs levels (10 downregulated and 1 upregulated) (Table 2) compared to women with normal serum 25D. Pregnant women often have vitamin D_3 insufficiency or deficiency [84] which may lead to increased risk of pregnancy-related complications such as gestational diabetes, preeclampsia, and bacterial vaginosis [85,86].

Jorde and colleagues examined a 730 miRNAs in two pilot studies of patients given 4000IU/day of vitamin D_3 for 12 months and identified 18 miRNAs that were unregulated by vitamin D_3 and 8 that were downregulated [77] (Table 2). 12 miRNAs were examined in their main study and only miR-532-3p had weak correlation to serum 25D [77].

3. Mechanism

While studies show that vitamin D_3 or its metabolites alter the levels of some miRNAs, the mechanism of regulation is not necessarily straight forward. Canonical VDR-mediated regulation of miRNAs *via* VDREs (vitamin D response element) has been demonstrated for several miRNAs and may mediate regulation of other miRNAs. Briefly, Peng et al. analyzed the 1 kb 5′ flanking sequence of the miR-132 and let-7a pri-miRNAs and found multiple VDR/RXR binding sites in some of the miRs regulated by stress/25D [55]. Adding complexity to the mechanism, there also appears to be a negative feedback loop between miRNAs and VDR. Mohri et al. demonstrated that overexpression of miR-125b reduced VDR/RXRα protein levels post-transcriptionally in MCF-7 cells [92], although direct regulation of miRNAs by 1,25D was not assessed in this study.

Taking a birds' eye look at the studies so far, it appears that in addition to direct VDRE-mediated regulation of specific miRNAs, vitamin D_3 may globally augment miRNA expression. All of the *in vitro* and patient studies that did full miRNA profiling identified more miRNAs upregulated by vitamin D_3 (or 1,25D) than downregulated miRNAs [59,60,77,85], with the exception of one study which did not analyze miRNAs regulated by 1,25D in the absence of stress [55]. Several mechanisms may cause such a global effect. One explanation is that vitamin D_3 alters the miRNA processing machinery, but this has not been reported. Another mechanism for global regulation of miRNAs by vitamin D_3 is a VDR-dependent chromatin opening which increases pri-miRNA transcription globally. In support of this mechanism, Disanto et al. found that VDR binding alters chromatin states that determine areas of the genome that are accessible to transcription factor binding and to the activation or inhibition of transcription [87]. Given the heterogeneity in VDRE consensus sequences and the discovery of very distal VDREs up to 100kb away from the

transcription start site [9,10], it maybe technically challenging to assess non-VDRE-dependent mechanisms of 1,25D-regulated miRNAs.

Non-genomic VDR-dependent activity of 1,25D may also alter miRNA levels by effecting miRNA stability or processing pathway, although in the literature there is not yet evidence that this has been examined. Consistent with this hypothesis, KHSRP and TARDBP, proteins involved in miRNA processing and regulation of miRNA biogenesis and maturation [88,89], were upregulated by 1,25D in colon cancer cells [69].

4. Significance and conclusions

Many of the vitamin D-regulated miRNAs (let-7a, let-7b, miR-100, miR-125b, miR-100, miR-106b, miR-141, miR-103, miR-331-3p) alter cell phenotypes in a manner consistent with tumor suppressor activity and other activities of vitamin D_3. However, miRNAs identified thus far have been cell type specific with little overlap, consistent with tissue-specific VDR activity. Notably, the potential for vitamin D_3 to induce widespread miRNA upregulation is of high significance to cancer in which there is a global suppression of miRNAs [24,37,42,90,91]. Further examination of vitamin D_3 effects on global miRNA levels by RNAseq in animal models and in clinical trial specimens are needed to validate and describe this potentially powerful action of vitamin D_3. Vitamin D_3 may also be important in maintaining normal miRNA expression as Peng and colleagues, demonstrated that 1,25D reversed the stress-induced changes in miRNA expression in breast cancer cells back to baseline [55].

Although regulation of any one miRNA by vitamin D_3 may not seem significant in cell function, these small changes may add up to the preservation of overall health in persons with vitamin D_3 sufficiency. It makes sense that vitamin D_3, an essential hormone, modulates many aspects of normal cell function. It is the recent prevalence of vitamin D_3 deficiency, and diseases linked to that deficiency, that has brought the identification of mechanisms of vitamin D_3 action to forefront of research. In general health practice vitamin D_3 is not an intervention or drug, but rather part of overall health and that maintenance of vitamin D_3 sufficiency is as important as other markers of health (*i.e.* low serum cholesterol). In regards to non-coding RNAs, basic research in this area is still in its infancy and what we do not know far outweighs what we do know. The reality that many of the ultraconserved elements of the genome are non-coding regions, signifies that these regions are vital to mediating our genetic code. It is then not surprising that maintaining vitamin D_3 sufficiency is also important in safeguarding the expression of our non-coding genome. While miRNAs are the focus of this review and the majority of ncRNA research, regulation of long-ncRNAs, snoRNAs and pseudogenes (among other ncRNAs) by 1,25D may also contribute to the role of vitamin D_3 in maintaining cell health.

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Table 1

MiRNAs regulated by 1,25D or 25D *in vitro.*

	Cells	Organ	Dose vitamin D	miRNAs					References
				Total profiled/method		Up-regulated		Down-regulated	
in vitro	LNCaP cells	Prostate	100 nM $1,25(OH)_2D_3$(48 H)	866	miRNA microarray	miR-542-5p	miR-371-5p	miR-17	Wang et al. [45]
						miR-29b	miR-663	miR-20a	
						miR-1207-5p	miR-134	miR-20b	
						miR-22	miR-135a*		
						miR-1915	miR-1181		
						miR-29a	miR-629*		
	Melanoma cells	Skin	10^{-8} M $1,25(OH)_2D_3$(24 H)	2	qRT-PCR	n/a		miR-125b	Essa et al. [70]
								miR-27b	
	MCF12F cells	Breast	[250nmol/L $25(OH)_2D_3$ + stress]	1350	miRNA microarray	miR-98	miR-26b	let-7e	Peng et al. [55]
						miR-21	miR-182	let-7b	
						miR-422b	miR-203	miR-205	
						miR-30c	let-7a	miR-200b	
						miR-93	miR-191	miR-92	
						miR-20b	let-7f	let-7d	
						miR-106a	miR-200c		
						miR-18a	miR-16		
	Colon cancer cells (SW480-ADH)	Colon	10^{-7} M $1,25(OH)_2D_3$ (2 48, 96 H)	1350	miRNA Microarray	miR-22		miR-93	Alvarez-Diaz
						miR-21		miR-20b	et al. [60]
						miR-224		miR-106a	
						miR-222		miR-18a	
						miR-146a/b			
	Human myeloid leukemia cells (HL60-G and U937)	Blood	10nmol/L $1,25(OH)_2D_3$ (U937) 1 nmol/L $1,25(OH)_2D_3$ (HL60)	245	miRNA microarray (Garzon 2007)	miR-32		n/a	Gocek et al. [80]
	Human myeloid leukemia cells (HL60-G and U937) Blood	Blood	10nmol/L $1,25(OH)_2D_3$(U9371 nmol/ L 1,25(OH) D (HL60) 245	245	miRNA microarray (Garzon 2007)	n/a		miR-181a	Wang et al. [81]
								miR-181b	
	RWPE-1 cells	Prostate	$1,25(OH)_2D_3$ (100nM)	1	qRT-PCR	miR-106b		n/a	Thorne et al. [46]

Cells	Organ	Dose vitamin D	miRNAs					References
			Total profiled/method		Up-regulated		Down-regulated	
Primary	Prostate	50nM $1,25(OH)_2D_3$	667	qRT-PCR array	**1 H:**	miR-320	**1 H:**	Giangreco et al. [93]
prostate cells		(1 H or 24 H)			miR-92	miR-132	miR-103	
					miR-27b	miR-135b	miR-339-3p	
					24 H:	miR-103	miR-130a	
					miR-24	miR-365	**24 H:**	
					miR-140-5p	miR-99b	miR-196b	
					miR-339-3p	miR-125a-5p		
					miR-301a	miR-141		
					miR-342-3p	miR-138		
					miR-345	miR-26a		
					miR-374b	miR-29a		
					miR-30c	miR-28-3p		
					miR-106b	miR-429		
					miR-708	miR-31		
					miR-100**	miR-29c		
					miR-331-3p	miR-452		
					miR-125b**	miR-744		
						miR-126		

Table 2

MiRNAs associated with serum or tissue levels of vitamin D3 metabolites in patients.

	Cells/tissue	Organ	Dose vitamin D	miRNAs					References
				Total profiled/method		Up-regulated		Down-regulated	
Patient specimens	Plasma (males)	Plasma	Vitamin D3 supplementation in males: *Pilot study* 1: 40,000 IU/ week for 1 year (N=5) *Pilot study 2:* for 1 year (N=5) *Main study:* for 12 months [20,000 (N=19) or 40,000 (N= 21) IU/ week or placebo (N=37)]-males	730 Pilot 1	qRT-PCR	let-7f	miR-543	miR-106b	Jorde et al. [77]
				742 Pilot 2		miR-133b	miR-766	miR-19a	
				12 Main		miR-26a	miR-15b	miR-22	
						miR-28-5p	miR-191	miR-424	
						miR-338-3p	miR-221	miR-548b-3p	
						let-7a	miR-331-3p	miR-660	
						let-7d	miR-339-5p	miR-324-5p	
						miR-146a	miR-374b	mR-532-3p	
						miR-151-3p/5p	miR-99b		
	Plasma (pregnant women)	Plasma	Participants (N= 13) with low (<25.5 ng./ml) and high (>31.7 ng/ml) $25(OH)_2D_3$	1884	OneArray™ miRNA microarray	miR-589	miR-320d	miR-574-5p	Enquobahrie et al. [85]
						miR-601	miR-423-3p		
						miR-573	miR-484		
						miR-196a*	miR-93		
						miR-92b			
						miR-138			
						(Comparing high to low 25D concentrations)			
	LCM-collected human prostate epithelium	Prostate	Vitamin D3 [400, 10,000, 40,000 IU/day for 3-8 weeks]	12	qRT-PCR	Benign:	PCa:	n/a	Giangreco et al. [93]
						miR-141	miR-100		
						miR-103	miR-125b		
						miR-100	miR-106b		
						miR-125b	miR-141		
						let-7a	miR-103		
						let-7b	miR-331-3p		
							let-7a		
							let-7b		
						(miRNA with positive correlation to 1,25 and/or25D)			