Vitamin D Impacts the Expression of Runx2 Target Genes and Modulates Inflammation, Oxidative Stress and Membrane Vesicle Biogenesis Gene Networks in 143B Osteosarcoma Cells

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Abstract: Osteosarcoma (OS) is an aggressive malignancy of bone affecting children, adolescents and young adults. Understanding vitamin D metabolism and vitamin D regulated genes in OS is an important aspect of vitamin D/cancer paradigm, and in evaluating vitamin D as adjuvant therapy for human OS. Vitamin D treatment of 143B OS cells induced significant and novel changes in the expression of genes that regulate: (a) inflammation and immunity; (b) formation of reactive oxygen species, metabolism of cyclic nucleotides, sterols, vitamins and mineral (calcium), quantity of gap junctions and skeletogenesis; (c) bone mineral density; and (d) cell viability of skeletal cells, aggregation of bone cancer cells and exocytosis of secretory vesicles. Ingenuity pathway analysis revealed significant reduction in Runx2 target genes such as fibroblast growth factor -1, -12 (FGF1 and FGF12), bone morphogenetic factor-1 (BMP1), SWI/SNF related, matrix associated actin dependent regulator of chromatin subfamily a, member 4 (SMARCA4), Matrix extracellular phosphoglycoprotein (MEPE), Integrin, β4 (ITGBP4), Matrix Metalloproteinase -1, -28 (MMP1 and MMP28), and signal transducer and activator of transcription-4 (STAT4) in vitamin D treated 143B OS cells. These genes interact with the inflammation, oxidative stress and membrane vesicle biogenesis gene networks. Vitamin D not only inhibited the expression of Runx2 target genes MMP1, MMP28 and kallikrein related peptidase-7 (KLK7), but also migration and invasion of 143B OS cells. Vitamin D regulated Runx2 target genes or their products represent potential therapeutic targets and laboratory biomarkers for applications in translational oncology.
1. Introduction

Osteosarcoma (OS) is the primary malignancy of bone affecting children, adolescents and young adults and accounts for 20%–45% of all bone tumors. In the United States, about 500–1000 new cases are diagnosed annually. Development of lung metastasis is the main cause of death in OS patients [1–3]. With the advent of adjuvant chemotherapy, the five-year survival rate is approximately 70%. Despite aggressive chemotherapy and surgical treatments, one third of patients usually relapse with pulmonary metastases [4–6]. Several reports indicate that OS patients have decreased bone density, aberrations in vitamin D regulatory system, sub optimal vitamin D levels, oncogenic osteomalacia and increased incidence of pathological fractures which tend to increase lung metastases [7–12]. There is an unmet need to identify novel disease and/or therapeutic biomarkers for applications in laboratory medicine and translational oncology to stratify OS patients for response to therapy and enhance their survival.

Data from cellular, preclinical and epidemiological studies support the role of vitamin D in cancer chemoprevention/therapy, and clearly explain why and how vitamin D can affect tumor growth and proliferation, and that higher serum levels of 25(OH)D₃ correlate to better survival and response to therapy [13]. 1α,25(OH)₂D₃ is the biologically active form of vitamin D and functions as a ligand for vitamin D receptors. Expression and activities of vitamin D metabolizing enzymes namely 1-α(OH)ase (encoded by gene CYP27B1) and 24 hydroxylase (encoded by gene CYP24A1) help in the regulation of steady state levels of 1α,25(OH)₂D₃. Different types of cancers have altered expression and activities of genes encoding vitamin D metabolizing enzymes or vitamin D modulators such as Fibroblast growth factor-23 (FGF23) [14–26]. FGF23 exerts autocrine effects on the proliferation of tumor cells as several solid tumor cells express FGF receptors [26]. FGF23 exerts a highly regulated feedback control on 1α,25(OH)₂D₃ mediated functions [27]. Presence of excess of FGF23 and matrix extracellular phosphoglycoprotein (MEPE) in oncogenic osteomalacia indicates similarities with genetically inherited rickets such as X-linked and autosomal dominant hypophosphatemic rickets [28]. In the bone microenvironment, it is the osteocytes which express FGF23, Dentin matrix protein-1 (DMP1) or phosphate regulating endopeptidase homolog, X-linked (Phex) [29]. Interestingly, a recent study suggests osteocyte as the cell of origin for osteosarcomagenesis [30]. In that study, the authors report abundant expression of DMP1 in murine, canine and human OS and evidence of osteoblastic/osteolytic lesions in mice injected with MLO-Y4 mouse osteocyte-like cell line [30]. Another study reported that FGF23 up regulates DMP1 mRNA in MLOY4 cells [31]. Neither the source and status of FGF23 nor its impact on oncogenic osteomalacia in OS is clear.

Runx2 is a transcription factor important for osteogenic differentiation and normal skeletal development. Recent studies highlight the role of Runx2 as a reliable OS biomarker for evaluating disease status and/or therapeutic response as there is high incidence of Runx2 genomic amplification and increased expression of Runx2 mRNA and protein in OS biopsy samples, tumor tissues from OS-mouse models, and its positive correlation with chemoresistance [32–34]. The role of vitamin D in regulating Runx2 expression and activity is not clear. Some studies indicate that Runx2 expression and activity is dependent on the expression levels of vitamin D receptor (VDR) and the differentiation status of the cell [32,33]. Another study reports that cholecalciferol (dietary vitamin D) modulates Runx2–DNA interactions and preferentially inhibits proliferation of breast cancer, and endothelial and bone cells [34]. All the above studies led us to investigate the role of 1,25(OH)₂D₃ in inhibiting expression of Runx2 and Runx2 target genes in 143B OS cells.

The molecular mechanisms underlying the antineoplastic properties of 1α,25(OH)₂D₃ are mainly mediated by modulation of expression of genes that regulate cellular proliferation, differentiation, apoptosis, angiogenesis, and oxidative stress [35,36]. We and others have previously reported that

**Keywords:** biomarkers; calcitriol; fibroblast growth factor 23; membrane vesicle biogenesis; microarray; inflammation; osteosarcoma; oxidative stress; Runx2
1α,25(OH)₂D₃ exerts its antineoplastic effect by inducing differentiation and apoptosis of cancer cells [35,37]. The role of vitamin D and vitamin D regulatory system in OS is not clear and needs in depth genomic and proteomic investigational studies.

The main goal of this study is to evaluate 1α,25(OH)₂D₃ regulated gene expression in a metastatic human osteosarcoma cell line, 143B, at different stages of their growth by microarray gene expression profiling. The reason for choosing 143B cell line for our study is that these cells are extremely aggressive and show evidence of pulmonary metastasis when injected in vivo. Using this cell line, we have generated a pre-clinical bioluminescent osteosarcoma orthotopic mouse (BOOM) model [38]. It is our hypothesis that a number of 1α,25(OH)₂D₃ regulated genes are differentially expressed during proliferation, post-proliferation, and differentiation of 143B human OS cell line, and regulate cell cycle, cellular growth, proliferation and development, cell death, cell–cell and cell–matrix interactions, and cellular function and oxidative stress. To test the proposed hypothesis, we have compared vitamin D mediated changes in the expression of Runx2, Runx2 target genes and vitamin D regulatory system (VDR, CYP27B1 and CYP24A1) at specific time points i.e., day 3, 9, and 15 as these match with the designated growth stages proliferation, post-proliferation, and differentiation, respectively, based on the results obtained from previous studies [32–34,37,39]. Knowledge gained from this study is innovative and significant, as it will identify key vitamin D target genes impacting potential cancer pathway signatures, and novel diagnostic biomarkers and will provide foundation for validating mechanism(s) underlying antineoplastic effects of vitamin D in the preclinical BOOM model [38].

2. Results

2.1. 1α,25(OH)₂D₃ Induces Stage-Specific Expression of Target Genes in 143B Human OS Cells

Microarray analysis of 143B OS cells treated with control vehicle (0.01% ethanol) or 100 nM 1α,25(OH)₂D₃ for 3, 9 and 15 days revealed a total of 500 differentially expressed genes (Figures 1 and 2). Notably, 94 statistically significant ($p < 0.05$) (fold changes > 1.2) target genes including 31 up regulated and 63 down regulated genes in the proliferation group; a total of 240 statistically significant target genes including 173 up regulated and 67 down regulated genes in the post-proliferation group; and a total of 178 statistically significant target genes including 64 up regulated and 114 down regulated genes in the differentiation group were modified in the vitamin D treated relative to vehicle treated groups (Figure 2). The genes whose expression levels were most significantly changed by 1α,25(OH)₂D₃ in 143B and relevant to bone biology and bone tumor microenvironment specifically regulate: (a) inflammation and immunity; (b) formation of reactive oxygen species, metabolism of cyclic nucleotides, sterols, vitamins and calcium, quantity of gap junctions and skeletogenesis; and (c) bone mineral density, cell viability of skeletal cells, aggregation of bone cancer cells and exocytosis of secretory vesicles (Table 1).
Figure 1. Heat map of Vitamin D target genes in 143B Osteosarcoma (OS) cells. Heat map of 1α,25(OH)2D3 induced gene expression fold changes (A) along with the names of the vitamin D-target genes (B) in 143B human OS cells during proliferation, post-proliferation, and differentiation relative to control (vehicle). Color bar represents log fold change values, red indicates up-regulated while green represents down regulated genes. Numbers 1–3 represents different growth stages of 143B cells post vitamin D treatment vs. control (ethanol or vehicle treated), namely proliferation, three days; post proliferation, nine days; and differentiation, 14 days. The colors red and green indicate genes that are up and down regulated, respectively.

Figure 2. Comparison of number of statistically significant 1α,25(OH)2D3 induced target genes in 143B human OS cells during proliferation, post-proliferation, and differentiation relative to control (vehicle).
Table 1. Ingenuity pathway analysis (IPA) ranked vitamin D modulated biofunctions relevant to bone biology and bone tumor microenvironment.

| Stage               | Disease or Function                                      | p Value       | Molecules                                                                 |
|---------------------|----------------------------------------------------------|---------------|---------------------------------------------------------------------------|
| Proliferation       | formation of bone cells                                   | $9.69 \times 10^{-3}$ | TSHR                                                                      |
|                     | metabolic bone disease                                    | $2.63 \times 10^{-2}$ | BMP1 and RGN                                                             |
|                     | eggression of natural killer cells; non-canonical wnt signaling | $4.85 \times 10^{-3}$ | RORC                                                                      |
|                     | inflammation                                              | $4.85 \times 10^{-3}$ | ITGAM                                                                     |
|                     | cellular assembly and vesicle trafficking                 | $4.85 \times 10^{-3}$ | RAB7A                                                                     |
|                     | Formation of reactive oxygen species                      | $2.48 \times 10^{-3}$ | APOE, CD28, GRIN1, P2RX7, PIK3CG, SOD2 (activation z score 1.66)           |
|                     | metabolism of cyclic nucleotides                         | $1.15 \times 10^{-4}$ | APOE, CASP2, CHRM2, CRH, CRHR1, GALR2, GRM1, NPY4R, OPRD1, PDE4C, PDA2, PIK3CG, PYY, RAMP2 (activation z score 1.66) |
|                     | catabolism of sterol                                      | $2.52 \times 10^{-4}$ | APOE, CEL, CYP24A1                                                        |
|                     | quantity of gap junctions                                 | $5.88 \times 10^{-3}$ | APOE, GJB1, GRIN1, PCDHGA3 (activation z score: 1.73)                    |
|                     | vitamin and mineral metabolism (quantity of calcium ions) | $9.39 \times 10^{-3}$ | APOE, CACNA1H, CD28, CD38, CHRM2, CRH, GRIN1, GRM1, IBSP, MLN, P2RX7, PIK3CG, PSEN2, PYY, THY1 (activation z score 2.6) |
|                     | Deformation of bone                                       | $1.42 \times 10^{-2}$ | HBB, PAX8                                                                 |
| Differentiation     | Bone mineral density                                     | $1.80 \times 10^{-3}$ | DCN, ESR1, IGF1, PRLR, PTH, RGN                                         |
|                     | cell viability of bone cell lines                         | $5.83 \times 10^{-3}$ | PTH                                                                      |
|                     | aggregation of bone cancer cells                          | $1.16 \times 10^{-2}$ | CDH1                                                                     |
|                     | exocytosis of secretory vesicles                          | $1.16 \times 10^{-2}$ | IGF1                                                                     |

Table 2 shows a list of top five biological functions (ranked by their statistical significance) of 1α,25(OH)₂D₃ regulated genes during proliferation, post-proliferation and differentiation growth stages of 143B cells. From the list of top ten genes differentially regulated in vitamin D treated 143B cells vs. vehicle treated 143B cells during proliferation, post-proliferation and differentiation, it is obvious that 1α,25(OH)₂D₃ modulated genes have functions that have either biological or clinical relevance as biomarkers for evaluating disease progression, diagnosis, prognosis and/or efficacy (Supplementary Tables; ST1A-F). These genes include kallikrein related peptidases-3 and -7 (KLK3 and KLK7), a disintegrin and metallopeptidase domain 21 (ADAM21), hypermethylated in cancer (HIC1), retinoic acid receptor beta (RARB), secreted frizzled receptor 5 (sFRF5), corticotropin releasing hormone (CRH), PRKC apoptosis WT1 regulator (PAWR), and adenosine A2a receptor (ADORA2A). Ingenuity system pathway analyses revealed a number of vitamin D down regulated expression of Runx2 modulators or Runx2 target oncogenes such as FGF1, FGF12, bone morphogenetic protein 1 (BMP1), MEPE, SWI/SNF related, matrix associated actin dependent regulator of chromatin subfamily a, member 4 (SMARCA4), parathyroid hormone (PTH), estrogen receptor 1 (ESR1), and chemokine (C–C) motif receptor 1 (CCR1) which either directly enhance neoplastic properties and/or interact with the inflammation, oxidative stress and membrane vesicle biogenesis genetic networks and modulate tumor microenvironments [40,41] (Figures 3 and 4; Figure S1A–C (inflammation); Figure S2A–C (oxidative stress); and Figure S3A–C (vesiculation)).
Table 2. Ingenuity pathway analysis (IPA) ranked vitamin D mediated changes in top five biofunctions (based on their \( p \) values) in 143B osteosarcoma cells.

| Category | Diseases or Functions | \( p \) Value | Molecules |
|----------|-----------------------|---------------|-----------|
| **Proliferation** | | | |
| Cancer | thyroid cancer | \( 6.10 \times 10^{-5} \) | FLT1, GDF15, KLK10, KLK7, RARB, TSHR |
| Endocrine System Disorders | thyroid cancer | \( 6.10 \times 10^{-5} \) | FLT1, GDF15, KLK10, KLK7, RARB, TSHR |
| Cell-To-Cell Signaling and Interaction | communication of cells | \( 2.05 \times 10^{-4} \) | ACVR1B, CAPN3, CASP1, FGF12, FLT1, GDF15, ITGA3, PAK2, RAP3, RARB, RASGRF1, SMAD5-AS1, TACSTD2, TLR6, TSHR |
| Cellular Movement | cell movement of prostate cancer cell lines | \( 4.68 \times 10^{-4} \) | CTSZ, GDF15, HIC1, PAK2 (activation \( z \) score: 1.97) |
| Cell-To-Cell Signaling and Interaction | signal transduction | \( 5.35 \times 10^{-4} \) | ACVR1B, CAPN3, CASP1, FGF12, FLT1, GDF15, PAK2, RAP3, RARB, RASGRF1, SMAD5-AS1, TACSTD2, TLR6, TSHR |
| **Post-Proliferation** | | | |
| Behavior | behavior | \( 2.31 \times 10^{-5} \) | ABCA2, APOE, BCR, CACNB1, CARTPT, CD36, CDKL5, CDH1, CHRM2, CRH, CRHR1, CTNNB2, CTNNBD2, DBH, EGF, GALR1, GATA2, GRIN1, GRM1, HBB, HOX8B, KCNJ5, LAMA4, LSAMP, MBD2, NFR3, NPR3, NPY4R, NTRK2, P2RX7, PAWR, PSEN2, PTPRN, PYH, SOD2 |
| Small Molecule Biochemistry | sulfation of raloxifene | \( 9.22 \times 10^{-5} \) | SULT1C2, SULT2A1, SULT2B1 |
| Neurological Disease | seizures | \( 9.25 \times 10^{-5} \) | ADAM22, ANKRD6, ATP6V0A4, CACNA1H, CRH, DBH, GJB1, GRP162, GRIK3, GRIN1, GRM1, HBB, HBD, NTRK2, PSEN2, PTPRN, SLC4A10, SOD2, SSTR3 |
| Cell Morphology | abnormal morphology of myelin sheath | \( 1.13 \times 10^{-4} \) | ABCA2, APOE, ERCC6, GJB1, LAMA4 |
| Nervous System Development and Function | abnormal morphology of myelin sheath | \( 1.13 \times 10^{-4} \) | ABCA2, APOE, ERCC6, GJB1, LAMA4 |
| **Differentiation** | | | |
| Tissue Development | development of mammary alveolus | \( 7.10 \times 10^{-6} \) | CDH1, IGF1, PRLR, TGFA |
| Digestive System Development and Function | abnormal morphology of digestive system | \( 8.20 \times 10^{-6} \) | ABCB1, CCR1, DCN, ESRA, GJB1, IKZF1, KRT6A, PRLR, RAD23B, RGN, SOSTDC1, STAT4, TGFA |
| Organ Development | response of liver | \( 1.21 \times 10^{-5} \) | ABCB11, ADOR1, CAPN1, CXCL5, ESRA1, IGF1, STAT4, STAT6, TGFA (activation \( z \) score: 0.179) |
| Carbohydrate Metabolism | deposition of polysaccharide | \( 1.55 \times 10^{-5} \) | ESRA1, IGF1, PTH |
| Skeletal and Muscular System Development and Function | bone mineral density | \( 1.80 \times 10^{-5} \) | DCN, ESRA1, IGF1, PRLR, PTH, RGN |
Vitamin D Target Genes in 143B Cells and Human OS Tissue Microarrays were not however significant at the experimentally tested time points (Figure S4). This was mainly due to the time points selected in the study (Day 3, 9 and 15) as previous studies indicate maximal expression of CYP24.

2.2. Real Time Quantitative Polymerase Chain Reaction, Western Blotting, and Immunohistochemistry Detects Vitamin D Target Genes in 143B Cells and Human OS Tissue Microarrays

Microarray data showed an increased expression of CYP24 mRNA in 1α,25(OH)2D3 treated 143B cells during post-proliferation, which was confirmed by RT-qPCR. The changes in the relative expression of CYP24, CYP27B1, and VDR in 1α,25(OH)2D3 treated vs. untreated 143B OS cell line were not however significant at the experimentally tested time points (Figure S4). This was mainly due to the time points selected in the study (Day 3, 9 and 15) as previous studies indicate maximal...
changes in the gene expression (especially for CYP24) within the 24 h [42]. The expression of vitamin D target genes (CYP24, CYP27B1, and VDR) at the protein level was detected by Western blotting, which confirmed qPCR results (Figure S4). RT-qPCR studies demonstrated inhibition of expression of Runx2 (proliferation) and Runx2 target genes matrix metalloproteinases, MMP1 (post-proliferation) and MMP28 (post-proliferation) in the same samples which were used for microarray profiling studies (Figure 5). Vitamin D mediated down regulation of MMP 28 (Figure 5) and KLK7 (proliferation) (a MMP processing protease (Figure 6)) expression by RT-qPCR confirms microarray results (Table 2A and Table S2A,C). Interestingly, osteoblastic OS core group of bone cancer tissue microarray (TMA) displayed intense expression of VRS compared to fibroblastic and talangiectactic OS (Figure 7) but the expression varied with tumor site (Figure S5). Increased expression of VDR and FGF23 relative to other VRS components is interesting, especially in the context of increased Runx2 expression as previously observed in the same OS-core type, and also in the tumor tissue isolated from the BOOM model [38]. The immunostaining of VRS in the disease free healthy bone was very weak or absent compared to the tumor tissue.

**Figure 5.** Effect of vitamin D in inhibiting the gene expression of: Runx2 (A); and Runx2 target genes MMP1 and MMP28 (B), in 143B osteosarcoma cells. Star indicates statistical significance of \( p \leq 0.05 \).

**Figure 6.** Detection of reduced KLK7 mRNA expression in 1α,25(OH)2D3 treated 143B cells by RT-qPCR. Asterisk represents statistical significance of \( p \leq 0.05 \).
2.3. 1α,25(OH)₂D₃ Inhibits Migration and Invasion of 143 Cells

Calcitriol or 1α,25(OH)₂D₃ significantly inhibited not only the migration of 143B cells, but also their invasion through Matrigel (Figure 8). This observation is consistent with the real time qPCR data showing decreased Runx2, MMP1 and MMP28 gene expression in 1α,25(OH)₂D₃ treated 143B cells, and microarray data which revealed that 1α,25(OH)₂D₃ regulated the expression of a number of genes involved in cellular movement, for example, ADAM21, MMP28, and adherens junction associated protein 1 (AJAP1) (Table S1B,C,F).

3. Discussion

In recent years, there has been a burgeoning interest in identifying biomarkers/molecular signatures, which have great potential to revolutionize genomic and/or personalized medicine. Advances in molecular technologies allow the application of biomarkers as an invaluable cost-effective detection tool either used alone or in combination with existing imaging methods for early screening or risk assessment, detection and diagnosis, and developing effective cancer therapies. This study provides a
novel and valuable insight into global gene expression profiling of 1α,25(OH)2D3-mediated-growth stage specific changes in 143B, a metastatic OS cell line.

Identification of 1α,25(OH)2D3 regulated genes such as RARB, CASP1, ARL3, PAWR, SMARCA4, ADORA2A and STAT4 and their interaction with the inflammation, oxidative stress and membrane vesicle biogenesis gene networks is very interesting. Most of the genes constituting this panel serve as biomarkers for efficacy, prognosis, diagnosis, disease progression (targeting inflammation, oxidative stress and vesiculation gene networks) and response to therapy [43–57]. Vitamin D mediated genetic networks such as those identified in our study, for example inflammation, oxidative stress and membrane vesicle biogenesis pathways in 143B cells, shed insight into the mechanism(s) underlying antineoplastic effects of vitamin D in human OS.

Immunodetection of VDR especially VDR and FGF23 in osteoblastic core of OS-TMAs is interesting as previous studies have reported tumor promoting effects of FGF23 either directly or indirectly by affecting the bioavailability and catabolism of 1α,25(OH)2D3 which in turn is important for mediating antineoplastic functions [26,58]. FGF/FGFR signaling increases the transcripational activity of Runx2, an osteogenic transcription factor [59], which is overexpressed in several cancers including osteosarcoma [60]. Mice overexpressing FGF23 display increased expression of Runx2 and alkaline phosphatase, receptor activator of NFκB ligand or RANKL and osteoprotegerin transcripts, MMP9 and cathepsin K immunoexpression along with increased serum concentrations of C terminal telopeptide of collagen (CTX) with increased bone resorptive activity [61]. Vitamin D mediated down regulation of FGF1 in OS cells is significant as previous studies have detected increased levels of serum FGF1 and FGFR1 amplification in OS patients [62,63]. FGF1 mediated activation of PKA and PKC signaling pathways induces nucleoside triphosphate pyrophosphohydrolase (NTPPPH) expression in OS cells [64]. Both FGF1 and FGFR2 stimulate FGF23 transcriptional activity, in OS cells, which in turn was blocked in the presence of FGF1 inhibitor, PD173704 [65]. Future studies will investigate the role of FGF23 in stimulating expression of Runx2 and Runx2 target genes and enhancing transcriptional activity of Runx2 leading to increased biogenesis of tumor supportive EMVs that drive the vicious cycle and contribute to vitamin D deficiency. We and others have shown that NITTPPPH overexpression results in osteomalacia in long bones, and is localized in EMVs [66,67]. We have previously reported the role of calcium influx and cAMP signaling in EMV biogenesis in OS cells [68]. Whether FGF1 stimulates EMV biogenesis in OS via calcium or cAMP/PKA signaling in OS cells is unknown. Vitamin D mediated down regulation of FGF12 mRNA expression in microarray gene expression studies supports the neoplastic role of FGFs in osteosarcoma pathobiology as it stimulates proliferation, extra cellular matrix remodeling, inflammation, and angiogenesis. CCR1 is important for migration and invasion of osteosarcoma cells. Overexpression of FGFR3 in MM cells stimulates secretion of CCL3, a ligand for CCR1 and activates RAS-MAPK pathway. Inactivation of CCR1 suppresses not only cancer cells but also cells in the bone tumor microenvironment reducing the overall osteolytic tumor burden [69]. Whether FGFs stimulate FGFR mediated CCL3/CCR1 signaling and its downstream effects on Runx2 target genes such as RANKL, MMPs, parathyroid hormone related peptide (PTHrP), survivin, and vascular endothelial growth factor (VEGF) are unknown in OS. The role of FGF ligands (FGF1, FGF12 or FGF23) or receptors (FGFR1) as biomarkers of OS disease progression or therapeutic response seems promising for stratification of patients to improve survival outcomes.

Vitamin D regulation of Runx2 gene interactions in our study highlights the role of Runx2 and Runx2 target genes in OS pathobiology. Previous studies report that Runx2 increases the expression of prometastatic Runx2 target genes like integrins, focal adhesion kinase FAK/PTK2 or Talin (TLN), MMPs, PTHrP, VEGF, bone sialoprotein (BSP), osteopontin, survivin, etc. [70–77]. Runx2 expression has a pro-survival role in rapidly proliferating tumor cells in the bone microenvironment by promoting PTH or PTHrP mediated antiapoptotic effect and inducing the expression of survivin [78]. In OS, a number of growth factors such as BMPs, PTH/PTHrP, TGF-β, and FGF23 activate Runx2 either directly or indirectly and promoting Runx2 phosphorylation. Runx2 gene product PTHrP can activate PTHrP/PTHrPRI signaling and raise intracellular cyclic-adenosine monophosphate cAMP and calcium
levels which in turn results in cytoskeleton changes and potentially lead to exosomal biogenesis. Vitamin D mediated down regulation of Runx2 target gene integrin, β4 (ITGB4) is interesting as this integrin regulates the expression and function of ezrin, an important biomarker and mediator of OS pulmonary metastasis [79]. Detection of ITGB4 in the cancer exosomes and its role in metastatic organotropism, especially lung tropism, opens up an important question whether vitamin D mediated inhibition of ITGB4 impacts pulmonary metastasis via reduced amounts in exosomes/EMVs derived from OS cells and inhibition of exosomal uptake by lung fibroblasts [80]. Recently, a novel role of Runx2 in tumor cell survival became evident as studies reveal that Runx2 inhibited the apoptotic pathway by activating the expression of survivin and Bcl2 [81]. Survivin is an important cancer biomarker of OS and its expression correlates well with relapse and chemoresistance [82,83]. Runx2 overexpression leads to osteopenia and multiple fractures, through increased receptor activator of NF-κB ligand (RANKL) expression which in turn could stimulate OCL activity [84,85]. Vitamin D mediated down regulation of expression of Runx2 and Runx2 target genes such as MMP1, MMP28 is important as Runx2 expression is associated with poor chemotherapy response in OS [86]. Elegant genomic occupancy and chromatin immunoprecipitation (ChIP) studies reveal that in OS cell lines, Runx2 regulates the functions of genes of focal adhesion pathway, which regulate cell motility and adhesion (TLN1 and FAK) [77].

To further validate the biological and therapeutic relevance of vitamin D in inhibiting expression and/or activity of Runx2 target genes in OS, future studies will include: (a) determining the effect of vitamin D in Runx2 overexpressing or Runx2 siRNA treated OS cell lines differing in their p53 and/or ki-ras status (U2OS, SaOS2, HOS and 143B) and that display differential neoplastic activity such as aggressiveness (migration and invasion), angiogenesis, EMV biogenesis, metastasis and chemoresistance; (b) evaluating the role of vitamin D in modulating differential expression of miRNAs in OS cell lines; and (c) elucidating the role of vitamin D in inhibiting Runx2 mediated osteosarcoma bone disease and metastases in vivo, using the BOOM model.

4. Materials and Methods

4.1. Cell Culture

Osteosarcoma cell line 143B was obtained from American Type Culture Collection (Manassas, VA, USA). We have validated and established the oncogenic activity of 143B OS cells in the BOOM model [38]. The cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 100 U/mL Penicillin, and 100 µg/mL Streptomycin, 10% Fetal Bovine Serum, and 1% non-essential amino acids under an atmosphere of 5% CO₂ at 37 °C in a humidified incubator. Cells were seeded at a density of 0.5 × 10⁴ cells per well in a 6-well tissue-culture plate. 143B cells were treated with control vehicle (0.01% ethanol) or vitamin D or 1α,25(OH)₂D₃ (100 nM) and medium was changed every other day. To stimulate differentiation, L-ascorbic acid 2-phosphate (50 µg/mL) and β-glycerophosphate (5 mM) were added to the cultures. Treatment sets were repeated for three different experiments.

4.2. RNA Isolation and Assessment of RNA Quality and Purity

Total RNA was isolated from 143B OS cells that were at different stages of growth, i.e., proliferative (72 h), post-proliferative (9 days) and differentiation (15 days), using RNeasy Mini Kit (Qiagen, Santa Clara, CA, USA). The quality and purity of isolated RNA was evaluated by Agilent bioanalyzer and only those samples with high values for RNA integrity number (RIN) were selected for hybridization studies (Table S2 and Figure S6).

4.3. Microarray Data Analysis

Preparation of cRNA targets was done using standard Affymetrix protocols. The cRNA fragments were allowed to hybridize to the sequences on the chip of Human Genome U133A 2.0 arrays. This array consists of ~18,400 transcripts representing over 14,500 genes. The probe intensity values
were corrected for background noise, and subsequently normalized and summarized using Robust Multi-array Average procedure [87]. The resulting log (base 2) intensity values were used for differential expression calculations. Fold change and p-values were calculated for genes that were differentially expressed in vitamin D treated proliferation, post-proliferation and differentiation vs. control (vehicle treated) groups. Fold change statistics for individual genes were derived based on previously published statistical methods [88,89]. Each treatment and control group consisted of biological triplicates for analysis. Genes with an absolute fold-change greater than or equal to 1.2 and having a p-value less than or equal to 0.05 were considered significantly regulated. All computations were performed in Matlab (R2012b, The MathWorks Inc., and Natick, MA, USA) and the Partek Genomic suite (v 6.5, Partek Inc., St. Louis, MO, USA). Biological functions, pathways and upstream regulators associated with significantly perturbed genes were identified using the Ingenuity Pathways Analysis software (IPA, Ingenuity Systems, available online: www.Ingenuity.com). IPA identifies significant molecular networks, biological functions and upstream regulators associated with a set of genes based on information gathered in the Ingenuity Pathway Knowledge Base (IPKB).

4.4. Validation of Selected Vitamin D Regulated Target Genes by Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) and Western Blotting in 143B Human Osteosarcoma Cell Line

For detection and validation of selected vitamin D regulated target genes, total RNA from 143B (±1α,25(OH)2D3) was isolated and probed with primers for VRS comprising of VDR, CYP27B1, CYP24, Runx2 and Runx2 target genes (MMP-1 and MMP28). (Primer sequences and PCR cycling conditions are provided as supplementary information). Real time qPCR was performed according to the standard protocol recommended by Applied Biosystems 7500 Sequence Detection system and software (Applied Biosystems, Foster City, CA, USA), and iCycler (Bio-Rad, Hercules, CA, USA). Relative quantitation of target mRNA expression, normalized to an endogenous control and relative to a calibrator (osteoblast RNA) was calculated using the mathematical expression for fold change, i.e., $2^{-\Delta\Delta C_T}$ (fold), as described by Livak et al, where $\Delta C_T = C_T$ of the target gene $- C_T$ of the endogenous control gene (GAPDH), and $\Delta\Delta C_T = \Delta C_T$ of the samples for target gene $- \Delta C_T$ of the calibrator for the target gene [90].

For the detection of expression of VDR, 1-α OHase, 24-hydroxylase and Runx2 proteins, Western blot analyses was performed. Twenty-five to fifty micrograms of crude cell-lysate (protein) was solubilized in SDS-sample buffer, electrophoresed on 12% denaturing polyacrylamide gels and visualized by Comassie blue stain. For immunoblotting, the proteins from the gel was transferred on to a PVDF membrane and incubated with following VRS primary antibodies: anti-VDR, anti-1-α hydroxylase, anti-24-hydroxylase, and anti-Runx2. The primary antibodies for VRS regulatory system were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and used at a concentration of 1:200. The immunostained bands were visualized using an ECL chemiluminescent detection system (Amersham Biosciences, Piscataway, NJ, USA). Extracts of breast cancer cells MCF7 and kidney tissue were used as positive control [39].

4.5. Detection and Immunolocalization of VRS in Human OS Tissue Microarrays

A bone cancer tissue arrays containing at least 6 cases of OS (osteoblastic, parosteal, fibroblastic, talarjactatic, conventional OS of left lower limb, and proximal humerus) in duplicates, and 2 cores of disease-free healthy bone tissue per array were purchased from US Biomax (Rockville, MD, USA). All the OS cores were classified as malignant, stage II b, and T2N0M0 grade. Immunohistochemistry for markers of vitamin D regulatory system, i.e., VDR, 1-α hydroxylase and 24-hydroxylase was performed as described below. Briefly, the arrays were fixed in 4% paraformaldehyde and standard immunostaining procedures was performed using the ABC staining kit from Santa Cruz Biotechnology Inc. Primary antibodies for VDR, 1-α hydroxylase, 24-hydroxylase, FGF23 were purchased from Santa Cruz Biotechnology Inc. All the primary antibodies were used at 1:200 dilutions. Immunostaining
intensity in the OS cores was compared to the disease free bone tissue cores or the control group. As negative control, primary antibody was excluded in the immunostaining.

4.6. Statistical Analysis

For quantitation of real time qPCR data, excel software was used to calculate mean and standard errors of means, and t test was used to analyze differences in 1α,25(OH)2D3 vs. untreated samples. A p value of <0.05 was considered as statistically significant. For analysis of microarray data, statistical methods are described in the microarray data analysis sub-section.

5. Conclusions

In conclusion, our data highlights the role of vitamin D in targeting Runx2 pathway in 143B OS cell line, specifically in the inhibition of genes critical for cell cycle, cellular proliferation, survival, migration and invasion, cell–cell and cell–matrix interactions, microtubule dynamics and cytoskeletal rearrangements, EMV biogenesis and chemo-resistance. This study suggests a novel role of vitamin D regulated Runx2 target genes/or their products as clinically relevant biomarkers in vitamin D mediated chemoprevention strategies or in adjuvant therapy for OS disease management.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/1422-0067/18/3/642/s1.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

BMP Bone morphogenetic protein
BOOM Bioluminescent osteosarcoma orthotopic mouse
EMV Extracellular membrane vesicles
FGF Fibroblast growth factor
FGFR Fibroblast growth factor receptor
ITGBP4 Integrin, β4
MEPE Matrix extracellular phosphoglycoprotein
MMP Matrix Metalloproteinase
OS Osteosarcoma
PTHrP Parathyroid hormone related peptide
Runx2 Runt-related transcription factor 2
SMARCA4 SWI/SNF related, matrix associated actin dependent regulator of chromatin subfamily a, member 4
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