Mutt, S. J., Karhu, T., Lehtonen, S., Lehenkari, P., Carlberg, C., Saarnio, J., et al. (2012). Inhibition of cytokine secretion from adipocytes by 1,25-dihydroxyvitamin D_3 via the NF-κB pathway. *FASEB J.* 26, 4400–4407. doi: 10.1096/fj.12-210880

Ng, K. Y., Ma, M. T., Leung, K. K., and Leung, P. S. (2010). Vitamin D and vitamin A receptor expression and the proliferative effects of ligand activation of these receptors on the development of pancreatic progenitor cells derived from human fetal pancreas. *Stem Cell Rev.* 7, 53–63. doi: 10.1007/s12015-010-9146-1

Oh, Y. S., Kim, E. J., Schaffer, B. S., Kang, Y. H., Binderup, L., MacDonald, R. G., et al. (2001). Synthetic low-calcaemic vitamin D_3 analogues inhibit secretion of insulin-like growth factor II and stimulate production of insulin-like growth factor-binding protein-6 in conjunction with growth suppression of HT-29 colon cancer cells. *Mol. Cell. Endocrinol.* 183, 141–149. doi: 10.1016/S0303-7207(01)00598-6

Okuda, N., Takeda, S., Shinomiya, K., Muneta, T., Itoh, S., Noda, M., et al. (2007). ED-71, a novel vitamin D analog, promotes bone formation and angiogenesis and inhibits bone resorption after bone marrow ablation. *Bone* 40, 281–292. doi: 10.1016/j.bone.2006.08.017

Pálmer, H. G., González-Sancho, J. M., Espada, J., Berciano, M. T., Puig, I., Baulida, J., et al. (2001). Vitamin D_3 promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of β-catenin signaling. *J. Cell Biol.* 154, 369–387. doi: 10.1083/jcb.200102028

Pálmer, H. G., Sánchez-Carbayo, M., Ordóñez-Morán, P., Larriba, M. J., Cordón-Cardó, C., and Muñoz, A. (2003). Genetic signatures of differentiation induced by 1α,25-dihydroxyvitamin D_3 in human colon cancer cells. *Cancer Res.* 63, 7799–7806.

Pan, Q., and Simpson, R. U. (1999). c-Myc intron element-binding proteins are required for 1,25-dihydroxyvitamin D_3 regulation of c-myc during HL-60 cell differentiation and the involvement of HOXB4. *J. Biol. Chem.* 274, 8437–8444. doi: 10.1074/jbc.274.13.8437

Parikh, G., Varadinova, M., Suwandhi, P., Araki, T., Rosenwaks, Z., Poretsky, L., et al. (2010). Vitamin D regulates steroidogenesis and insulin-like growth factor binding protein-1 (IGFBP-1) production in human ovarian cells. *Horm. Metab. Res.* 42, 754–757. doi: 10.1055/s-0030-1262837

Pickup, M., Novitskiy, S., and Moses, H. L. (2013). The roles of TGFβ in the tumour microenvironment. *Nat. Rev. Cancer* 13, 788–799. doi: 10.1038/nrc3500

Qadan, L. R., Perez-Stable, C. M., Schwall, R. H., Burnstein, K. L., Ostenson, R. C., Howard, G. A., et al. (2000). Hepatocyte growth factor and vitamin D cooperatively inhibit androgen-unresponsive prostate cancer cell lines. *Endocrinology* 141, 2567–2573. doi: 10.1210/endo.141.7.7546

Reichrath, S., and Reichrath, J. (2012). No evidence for induction of key components of the Notch signaling pathway (Notch-1, Jagged-1) by treatment with UV-B, $1,25(OH)_2D_3$, and/or epigenetic drugs (TSA, 5-Aza) in human keratinocytes *in vitro*. *Dermatoendocrinology* 4, 44–52. doi: 10.4161/derm.19027

Ren, Z., Li, W., Zhao, Q., Ma, L., and Zhu, J. (2012). The impact of 1,25-dihydroxyvitamin D_3 on the expressions of vascular endothelial growth factor and transforming growth factor-β_1 in the retinas of rats with diabetes. *Diabetes Res. Clin. Pract.* 98, 474–480. doi: 10.1016/j.diabres.2012.09.028

Rodilla, V., Villanueva, A., Obrador-Hevia, A., Robert-Moreno, A., Fernandez-Majada, V., Grilli, A., et al. (2009). Jagged1 is the pathological link between Wnt and Notch pathways in colorectal cancer. *Proc. Natl. Acad. Sci. U.S.A.* 106, 6315–6320. doi: 10.1073/pnas.0813221106

Ryynänen, J., and Carlberg, C. (2013). Primary 1,25-dihydroxyvitamin D_3 response of the interleukin 8 gene cluster in human monocyte- and macrophage-like cells. *PLoS ONE* 8:e78170. doi: 10.1371/journal.pone.0078170

Salehi-Tabar, R., Nguyen-Yamamoto, L., Tavera-Mendoza, L. E., Quail, T., Dimitrov, V., An, B. S., et al. (2012). Vitamin D receptor as a master regulator of the c-MYC/MXD1 network. *Proc. Natl. Acad. Sci. U.S.A.* 109, 18827–18832. doi: 10.1073/pnas.1210037109

Schauber, J., Dorschner, R. A., Coda, A. B., Buchau, A. S., Liu, P. T., Kiken, D., et al. (2007). Injury enhances TLR2 function and antimicrobial peptide expression through a vitamin D-dependent mechanism. *J. Clin. Invest.* 117, 803–811. doi: 10.1172/JCI30142

Schlaeppi, J. M., Gutzwiller, S., Finkenzeller, G., and Fournier, B. (1997). 1,25-Dihydroxyvitamin D_3 induces the expression of vascular endothelial growth factor in osteoblastic cells. *Endocr. Res.* 23, 213–229.

Schnabel, M., Fichtel, I., Gotzen, L., and Schlegel, J. (2002). Differential expression of Notch genes in human osteoblastic cells. *Int. J. Mol. Med.* 9, 229–232.

Seoane, S., and Pérez-Fernández, R. (2006). The vitamin D receptor represses transcription of the pituitary transcription factor Pit-1 gene without involvement of the retinoid X receptor. *Mol. Endocrinol.* 20, 735–748. doi: 10.1210/me.2005-0253

Sertznig, P., Dunlop, T., Seifert, M., Tilgen, W., and Reichrath, J. (2009a). Cross-talk between vitamin D receptor (VDR)- and peroxisome proliferator-activated receptor (PPAR)-signaling in melanoma cells. *Anticancer Res.* 29, 3647–3658.

Sertznig, P., Seifert, M., Tilgen, W., and Reichrath, J. (2009b). Activation of vitamin D receptor (VDR)- and peroxisome proliferator-activated receptor (PPAR)-signaling pathways through $1,25(OH)_2D_3$ in melanoma cell lines and other skin-derived cell lines. *Dermatoendocrinology* 1, 232–238. doi: 10.4161/derm.1.4.9629

Shah, S., Islam, M. N., Dakshanamurthy, S., Rizvi, I., Rao, M., Herrell, R., et al. (2006). The molecular basis of vitamin D receptor and β-catenin crossregulation. *Mol. Cell* 21, 799–809. doi: 10.1016/j.molcel.2006.01.037

Shalhoub, V., Shatzen, E. M., Ward, S. C., Young, J. I., Boedigheimer, M., Twehues, L., et al. (2010). Chondro/osteoblastic and cardiovascular gene modulation in human artery smooth muscle cells that calcify in the presence of phosphate and calcitriol or paricalcitol. *J. Cell. Biochem.* 111, 911–921. doi: 10.1002/jcb.22779

Shen, Q., and Christakos, S. (2005). The vitamin D receptor, Runx2, and the Notch signaling pathway cooperate in the transcriptional regulation of osteopontin. *J. Biol. Chem.* 280, 40589–40598. doi: 10.1074/jbc.M504166200

Shen, X., Mula, R. V. R., Li, J., Weigel, N. L., and Falzon, M. (2007). PTHrP contributes to the anti-proliferative and integrin α6β4-regulating effects of 1,25-dihydroxyvitamin D_3. *Steroids* 72, 930–938. doi: 10.1016/j.steroids.2007.08.003

Staal, A., Birkenhäger, J. C., Pols, H. A. P., Buurman, C. J., Vink-van Wijngaarden, T., Kleinekoort, W. M., et al. (1994). Transforming growth factor β-induced dissociation between vitamin D receptor level and 1,25-dihydroxyvitamin D_3 action in osteoblast-like cells. *Bone Miner.* 26, 27–42. doi: 10.1016/S0169-6009(08)80160-2

Staal, A., Geertsma-Kleinekoort, W. M. C., Van Den Bemd, G. J. C. M., Buurman, C. J., Birkenhäger, J. C., Pols, H. A. P., et al. (1998). Regulation of osteocalcin production and bone resorption by 1,25-dihydroxyvitamin D_3 in mouse long bones: interaction with the bone-derived growth factors TGF-β and IGF-I. *J. Bone Miner. Res.* 13, 36–43. doi: 10.1359/jbmr.1998.13.1.36

Staal, A., Van Wijnen, A. J., Desai, R. K., Pols, H. A. P., Birkenhäger, J. C., Deluca, H. F., et al. (1996). Antagonistic effects of transforming growth factor-β on vitamin D_3 enhancement of osteocalcin and osteopontin transcription: reduced interactions of vitamin D receptor/retinoid X receptor complexes with vitamin E response elements. *Endocrinology* 137, 2001–2011. doi: 10.1210/endo.137.5.8612541

Stambolsky, P., Tabach, Y., Fontemaggi, G., Weisz, L., Maor-Aloni, R., Siegfried, Z., et al. (2010). Modulation of the vitamin D_3 response by cancer-associated mutant p53. *Cancer Cell* 17, 273–285. doi: 10.1016/j.ccr.2009.11.025

Sun, J., Kong, J., Duan, Y., Szeto, F. L., Liao, A., Madara, J. L., et al. (2006). Increased NF-κB activity in fibroblasts lacking the vitamin D receptor. *Am. J. Physiol. Endocrinol. Metab.* 291, E315–E322. doi: 10.1152/ajpendo.00590.2005

Swami, S., Krishnan, A. V., Peng, L., Lundqvist, J., and Feldman, D. (2013). Transrepression of the estrogen receptor promoter by calcitriol in human breast cancer cells via two negative vitamin D response elements. *Endocr. Relat. Cancer* 20, 565–577. doi: 10.1530/ERC-12-0281

Tang, J. Y., Xiao, T. Z., Oda, Y., Chang, K. S., Shpall, E., Wu, A., et al. (2011). Vitamin D_3 inhibits hedgehog signaling and proliferation in murine basal cell carcinomas. *Cancer Prev. Res.* 4, 744–751. doi: 10.1158/1940-6207.CAPR-10-0285

Tang, W. B., Ling, G. H., Sun, L., and Liu, F. Y. (2010). Smad anchor for receptor activation (SARA) in TGF-β signaling. *Front. Biosci.* 2, 857–860. doi: 10.2741/E147

Tavera-Mendoza, L., Wang, T. T., Lallemant, B., Zhang, R., Nagai, Y., Bourdeau, V., et al. (2006). Convergence of vitamin D and retinoic acid signalling at a common hormone response element. *EMBO Rep.* 7, 180–185. doi: 10.1038/sj.embor.7400594

Thorne, J. L., Maguire, O., Doig, C. L., Battaglia, S., Fehr, L., Sucheston, L. E., et al. (2011). Epigenetic control of a VDR-governed feed-forward loop that regulates $p21^{waf1/cip1}$ expression and function in non-malignant prostate cells. *Nucleic Acids Res.* 39, 2045–2056. doi: 10.1093/nar/gkq875

Ting, H. J., Bao, B. Y., Hsu, C. L., and Lee, Y. F. (2005). Androgen-receptor coregulators mediate the suppressive effect of androgen signals on vitamin D receptor activity. *Endocrine* 26, 1–9. doi: 10.1385/ENDO:26:1:001

Tong, W.-M., Hofer, H., Ellinger, A., Peterlik, M., and Cross, H. S. (1999). Mechanism of antimitogenic action of vitamin D in human colon carcinoma cells: relevance for suppression of epidermal growth factor-stimulated cell growth. *Oncol. Res.* 11, 77–84.

Tong, W.-M., Kállay, E., Hofer, H., Hulla, W., Manhardt, T., Peterlik, M., et al. (1998). Growth regulation of human colon cancer cells by epidermal growth factor and 1,25-dihydroxyvitamin D_3 is mediated by mutual modulation of receptor expression. *Eur. J. Cancer* 34, 2119–2125. doi: 10.1016/S0959-8049(98)00267-6

Toropainen, S., Väisänen, S., Heikkinen, S., and Carlberg, C. (2010). The down-regulation of the human MYC gene by the nuclear hormone 1α,25-dihydroxyvitamin D_3 is associated with cycling of corepressors and histone deacetylases. *J. Mol. Biol.* 400, 284–294. doi: 10.1016/j.jmb.2010.05.031

Towers, T. L., Staeva, T. P., and Freedman, L. P. (1999). A two-hit mechanism for vitamin D3-mediated transcriptional repression of the granulocyte-macrophage colony-stimulating factor gene: vitamin D receptor competes for DNA binding with NFAT1 and stabilizes c-Jun. *Mol. Cell. Biol.* 19, 4191–4199.

Uhmann, A., Niemann, H., Lammering, B., Henkel, C., Hess, I., Nitzki, F., et al. (2011). Antitumoral effects of calcitriol in basal cell carcinomas involve inhibition of hedgehog signaling and induction of vitamin D receptor signaling and differentiation. *Mol. Cancer Ther.* 10, 2179–2188. doi: 10.1158/1535-7163.MCT-11-0422

Uhmann, A., Niemann, H., Lammering, B., Henkel, C., Hess, I., Rosenberger, A., et al. (2012). Calcitriol inhibits Hedgehog signaling and induces vitamin D receptor signaling and differentiation in the patched mouse model of embryonal rhabdomyosarcoma. *Sarcoma* 2012, 357040. doi: 10.1155/2012/357040

Vink-van Wijngaarden, T., Pols, H. A. P., Buurman, C. J., Birkenhäger, J. C., and van Leeuwen, J. P. T. M. (1996). Inhibition of insulin- and insulin-like growth factor-I-stimulated growth of human breast cancer cells by 1,25-dihydroxyvitamin D_3 and the vitamin D_3 analogue EB1089. *Eur. J. Cancer* 32A, 842–848. doi: 10.1016/0959-8049(95)00647-8

Wang, W. L., Chatterjee, N., Chittur, S. V., Welsh, J., and Tenniswood, M. P. (2011). Effects of 1α,25 dihydroxyvitamin D_3 and testosterone on miRNA and mRNA expression in LNCaP cells. *Mol. Cancer* 10, 58. doi: 10.1186/1476-4598-10-58

Woeckel, V. J., Bruedigam, C., Koedam, M., Chiba, H., van der Eerden, B. C. J., and van Leeuwen, J. P. T. M. (2013a). 1α,25-dihydroxyvitamin D_3 and rosiglitazone synergistically enhance osteoblast-mediated mineralization. *Gene* 512, 438–443. doi: 10.1016/j.gene.2012.07.051

Woeckel, V. J., Koedam, M., van de Peppel, J., Chiba, H., van der Eerden, B. C. J., and van Leeuwen, J. P. T. M. (2012). Evidence of vitamin D and interferon-β cross-talk in human osteoblasts with 1α,25-dihydroxyvitamin D_3 being dominant over interferon-β in stimulating mineralization. *J. Cell. Physiol.* 227, 3258–3266. doi: 10.1002/jcp.24020

Woeckel, V. J., van der Eerden, B. C. J., Schreuders-Koedam, M., Eijken, M., and Van Leeuwen, J. P. T. M. (2013b). 1α,25-dihydroxyvitamin D_3 stimulates activin A production to fine-tune osteoblast-induced mineralization. *J. Cell. Physiol.* 228, 2167–2174. doi: 10.1002/jcp.24388

Wu, F. S., Zheng, S. S., Wu, L. J., Teng, L. S., Ma, Z. M., Zhao, W. H., et al. (2007). Calcitriol inhibits the growth of MHCC97 heptocellular cell lines by down-modulating c-met and ERK expressions. *Liver Int.* 27, 700–707. doi: 10.1111/j.1478-3231.2007.01487.x

Yanagi, Y., Suzawa, M., Kawabata, M., Miyazono, K., Yanagisawa, J., and Kato, S. (1999). Positive and negative modulation of vitamin D receptor function by transforming growth factor-β signaling through Smad proteins. *J. Biol. Chem.* 274, 12971–12974.

Yanagisawa, J., Yanagi, Y., Masuhiro, Y., Suzawa, M., Watanabe, M., Kashiwagi, K., et al. (1999). Convergence of transforming growth factor-β and vitamin D signaling pathways on SMAD transcriptional coactivators. *Science* 283, 1317–1321.

Yu, X. P., Bellido, T., and Manolagas, S. C. (1995). Down-regulation of NF-κB protein levels in activated human lymphocytes by 1,25-dihydroxyvitamin D_3. *Proc. Natl. Acad. Sci. U.S.A.* 92, 10990–10994.

Yuan, W., Pan, W., Kong, J., Zheng, W., Szeto, F. L., Wong, K. E., et al. (2007). 1,25-dihydroxyvitamin D_3 suppresses renin gene transcription by blocking the activity of the cyclic AMP response element in the renin gene promoter. *J. Biol. Chem.* 282, 29821–29830. doi: 10.1074/jbc.M705495200

Zhang, G. Y., Cheng, T., Luan, Q., Liao, T., Nie, C. L., Zheng, X., et al. (2011). Vitamin D: a novel therapeutic approach for keloid, an *in vitro* analysis. *Br. J. Dermatol.* 164, 729–737. doi: 10.1111/j.1365-2133.2010.10130.x

Zhao, X. Y., Ly, L. H., Peehl, D. M., and Feldman, D. (1999). Induction of androgen receptor by 1α,25-dihydroxyvitamin D_3 and 9-cis retinoic acid in LNCaP human prostate cancer cells. *Endocrinology* 140, 1205–1212. doi: 10.1210/endo.140.3.6561

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 10 January 2014; paper pending published: 21 January 2014; accepted: 30 January 2014; published online: 18 February 2014.

Citation: Larriba MJ, González-Sancho JM, Bonilla F and Muñoz A (2014) Interaction of vitamin D with membrane-based signaling pathways. Front. Physiol. ***5****:60. doi: 10.3389/fphys.2014.00060*

This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology.

frontiers in PHYSIOLOGY

REVIEW ARTICLE
published: 29 April 2014
doi: 10.3389/fphys.2014.00164

Vitamin D and the epigenome

Irfete S. Fetahu, Julia Höbaus and Enikő Kállay*

Department of Pathophysiology and Allergy Research, Center of Pathophysiology, Infectiology and Immunology, Comprehensive Cancer Center, Medical University of Vienna, Vienna, Austria

Edited by:
Carsten Carlberg, University of Eastern Finland, Finland

Reviewed by:
Patsie Polly, The University of New South Wales, Australia
Moray J. Campbell, Roswell Park Cancer Institute, USA

***Correspondence:**
Enikő Kállay, Department of Pathophysiology and Allergy Research, Medical University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna, Austria
e-mail: enikoe.kallay@meduniwien.ac.at

Epigenetic mechanisms play a crucial role in regulating gene expression. The main mechanisms involve methylation of DNA and covalent modifications of histones by methylation, acetylation, phosphorylation, or ubiquitination. The complex interplay of different epigenetic mechanisms is mediated by enzymes acting in the nucleus. Modifications in DNA methylation are performed mainly by DNA methyltransferases (DNMTs) and ten-eleven translocation (TET) proteins, while a plethora of enzymes, such as histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and histone demethylases (HDMs) regulate covalent histone modifications. In many diseases, such as cancer, the epigenetic regulatory system is often disturbed. Vitamin D interacts with the epigenome on multiple levels. Firstly, critical genes in the vitamin D signaling system, such as those coding for vitamin D receptor (*VDR*) and the enzymes 25-hydroxylase (*CYP2R1*), 1α-hydroxylase (*CYP27B1*), and 24-hydroxylase (*CYP24A1*) have large CpG islands in their promoter regions and therefore can be silenced by DNA methylation. Secondly, VDR protein physically interacts with coactivator and corepressor proteins, which in turn are in contact with chromatin modifiers, such as HATs, HDACs, HMTs, and with chromatin remodelers. Thirdly, a number of genes encoding for chromatin modifiers and remodelers, such as HDMs of the Jumonji C (JmjC)-domain containing proteins and lysine-specific demethylase (LSD) families are primary targets of VDR and its ligands. Finally, there is evidence that certain VDR ligands have DNA demethylating effects. In this review we will discuss regulation of the vitamin D system by epigenetic modifications and how vitamin D contributes to the maintenance of the epigenome, and evaluate its impact in health and disease.

Keywords: VDR, VDRE, 1,25-dihydroxyvitamin D3, CYP27B1, CYP24A1, DNA methylation, histone modifications, CpG island

INTRODUCTION

The role of vitamin D in regulating gene expression has become increasingly evident since the discovery of the transcription factor vitamin D receptor (VDR), a member of the steroid nuclear receptor superfamily. The effect of liganded VDR depends on the epigenetic landscape of the target gene. Genome wide analysis in the human leukemia cell line THP-1 showed that VDR binds mainly at loci of open chromatin. Upon treatment with the VDR ligand 1,25-dihydroxyvitamin D_3 (1,25-D_3), chromatin accessibility further increases in more than 30% of these loci (Seuter et al., 2013). The mechanism of action of the liganded VDR is dependent on binding and action of histone acetyltransferases (HATs) and histone methyltransferases (HMTs). It has been shown that co-treatment of cells with 1,25-D_3, and histone deacetylase or DNA methyltransferase inhibitors often have synergistic effects (Pan et al., 2010).

Many common diseases have both genetic and epigenetic components, which communicate in an intricate and multilayered manner. Currently, it is not clear to what extent epigenetic alterations contribute to onset and progress of common diseases, such as cancer. Epigenetics refers to processes that alter gene activity without changing the DNA sequence. They play an important role in regulating key processes during development, including embryonic developmental events, gene imprinting, and inactivation of chromosome X in females (Bird, 2002; Meissner et al., 2008; Tsai and Baylin, 2011). Maintenance of normal functioning of these biological processes is dependent on the intricate interaction between several epigenetic mechanisms, such as DNA methylation, histone modifications, and non-coding RNAs (Jones and Baylin, 2007). Therefore, at a given promoter the marks arising from DNA methylation and histone modifications determine whether the chromatin is in an open (active) or a closed (repressed) state. Deregulation of the epigenetic mechanisms can lead to aberrant DNA methylation patterns and chromatin architecture, which is a common feature in cancer (Baylin and Jones, 2011; Tsai and Baylin, 2011; Helin and Dhanak, 2013).

EPIGENETIC CHANGES MEDIATED BY THE VITAMIN D RECEPTOR AND ITS LIGANDS

The effect of nutrition on the methylation equilibrium of the genome is already accepted as one of the mechanisms preventing either promoter hyper- or global hypomethylation. Several nutrients are renowned for their impact on DNA methylation, such as folic acid, vitamin B, green tea, and alcohol (Arasaradnam et al., 2008). The effect of vitamin D is currently under debate.

Primary epigenetic effects of vitamin D are linked to histone modifications, mainly acetylation. The VDR/RXR dimer interacts with HATs to induce transcriptional activation (Karlic and Varga,

2011). Several studies have suggested that vitamin D may affect also DNA methylation. A recent study associated severe vitamin D deficiency with methylation changes in leukocyte DNA, although the observed differences were relatively small (Zhu et al., 2013). This study suggested that subjects with vitamin D deficiency were more likely to show reduced synthesis and increased catabolism of active vitamin D. Whether this was the cause of the vitamin D deficiency or the consequence thereof is not clear and needs further studies.

EFFECT OF VITAMIN D ON DNA METHYLATION

DNA methylation is the most extensively studied epigenetic mark (Esteller, 2008). In humans, DNA methylation occurs on cytosines followed by guanine (CpG) (Bird, 1980; Gruenbaum et al., 1981). Regions of DNA enriched in CpG clusters form CpG islands (CGI) (Wang and Leung, 2004). DNA methylation is necessary for regulating and orchestrating key biological processes, including cell cycle, differentiation, as well as genomic imprinting (Feinberg et al., 2002; Reik and Lewis, 2005; Jones and Baylin, 2007). DNA hypermethylation is mainly found in intergenic regions and repetitive genomic sequences to maintain these in a transcriptionally inactive chromatin state (Herman and Baylin, 2003).

DNA methyltransferases (DNMTs) mediate DNA methylation (Robertson, 2005). DNMT1 encodes for a maintenance methyltransferase, whereas DNTM3A/3B encode for de novo methyltransferases, which are pivotal to maintain and establish genomic methylation (Okano et al., 1998, 1999; Jin and Robertson, 2013). However, *in vivo* studies suggest that all three DNMTs might exert both de novo and maintenance functions (Rhee et al., 2000, 2002; Kim et al., 2002; Esteller, 2007a). Recently, a new group of enzymes that induce active demethylation of the DNA was discovered, the ten-eleven translocation (TET) enzyme family, which plays an important role both in development and tumorigenesis (Kriaucionis and Heintz, 2009; Ficz et al., 2011; Williams et al., 2011; Yamaguchi et al., 2012; Hackett et al., 2013).

Alterations in the cancer epigenome are generally associated with loss of global DNA methylation and gain of methylation in specific gene promoters (Ting et al., 2006). Loss of global methylation may lead to chromosomal instability (Eden et al., 2003), loss of imprinting (Cui et al., 2003; Bjornsson et al., 2007), and activation of transposable elements, thereby leading to disturbances in the genome (Bestor, 2005; Esteller, 2008). Conversely, hypermethylation of promoter regions of tumor suppressor genes (Greger et al., 1989; Sakai et al., 1991; Esteller, 2008) leads to loss of expression of key genes affecting pathways involved in maintenance of cellular functions, including cell cycle, apoptosis, and DNA repair (Esteller, 2007b). Several bona fide tumor suppressor genes are silenced by promoter hypermethylation in tumors. For instance, hypermethylation of the promoter of the DNA repair gene *hMLH1* is associated with early stages of endometrial and colon cancer, and microsatellite instability phenotype (Esteller et al., 1999). Epigenetically mediated silencing of cyclin-dependent kinase inhibitor 2A, which is crucial for control of cell cycle has been reported in several cancers (Brock et al., 2008; Liau et al., 2014). Additionally, pathways regulated by microRNAs have been associated with DNA hypermethylation-dependent silencing (Saito et al., 2006).

Besides methylating cytosines, DNMTs may coordinate other chromatin-mediated aspects of gene expression at sites of gene promoters (Herman and Baylin, 2003). For example, hypermethylation of promoters of tumor suppressor genes is associated with recruitment of proteins belonging to the methyl CpG-binding domain (MBD) family, MeCP2, MBD1, MBD2, MBD3, and MBD4 (Ballestar and Esteller, 2005). It has been shown that MeCP2 represses transcription of methylated DNA by recruiting histone deacetylases (HDACs), providing the first evidence for interactions between DNA methylation and histone modifications (Jones et al., 1998; Nan et al., 1998).

There is evidence that 1,25-D_3 is able to induce DNA demethylation, however, the mechanisms behind the effect of 1,25-D_3 on DNA methylation are not clear. In most cases it is probably passive demethylation that happens over several cycles of DNA replication. However, in some cases demethylation occurs within 1–4 h, indicative of an active process (Doig et al., 2013). The fact that vitamin D can alter methylation of DNA in the promoter of certain genes is novel. Tapp and colleagues suggested that in healthy subjects global, age-related CGI methylation of human rectal mucosa was influenced not only by gender, folate availability, and selenium, but also by vitamin D status (Tapp et al., 2013). The authors show negative association between serum 25-D_3 level and CGI methylation of the adenomatous polyposis coli (*APC*) promoter region, a tumor suppressor often inactive in colorectal cancer. Interestingly, they observed a weak positive correlation of vitamin D level with methylation of *LINE-1* (genomic long interspersed nuclear element-1), a mammalian autonomous retrotransposon, increasing stability of this region (Tapp et al., 2013). A recent study in colorectal cancer patients investigating two Canadian populations (from Newfoundland and Ontario) found that high dietary vitamin D intake was associated with lower methylation of the two WNT antagonists *dickkopf 1* (*DKK1*) and *WNT5A* (Rawson et al., 2012). This relationship became even more significant in females in the Newfoundland population, while in the Ontario population the association between vitamin D intake and lower methylation was observed only in early stage tumors, but not in late stage tumors (Rawson et al., 2012). These data confer further insights in the mechanisms regulating the transcriptional activating effect of vitamin D on *DKK1* expression described *in vitro* (Aguilera et al., 2007; Pendas-Franco et al., 2008).

Moreover, treatment of the triple negative breast cancer cell line MDA-MB-231 with 1,25-D_3 reduced DNA methylation of the *e-cadherin* promoter (Lopes et al., 2012), while another study showed that 1,25-D_3 induced demethylation of the *PDZ-LIM* domain-containing protein 2 promoter, leading to increased expression (Vanoirbeek et al., 2014). In non-malignant and malignant prostate epithelial cells, treatment with 1,25-D_3 caused clear changes in site-specific methylation of the *p21* promoter, in a cell line-specific manner (Doig et al., 2013).

An interesting interaction between vitamin D and DNA methylation is induction of the expression of GADD45 (growth arrest and DNA damage) protein by 1,25-D_3 in several tumor

cells (Jiang et al., 2003; Zhang et al., 2006; Bremmer et al., 2012). GADD45A is one of the enzymes that promote epigenetic gene activation by repair mediated DNA demethylation in Xenopus laevis (Barreto et al., 2007).

In summary, alterations in DNA methylation lead to aberrant gene expression and disruptions of genomic integrity, which contribute to development and progression of diseases. Vitamin D can regulate these processes; the mechanisms behind need further investigations.

INTERACTIONS OF VITAMIN D WITH CHROMATIN MODULATORS AND REMODELERS

Nuclear receptors, such as the VDR contain DNA-binding domains that mediate binding to the DNA, presuming the DNA is available and is not wound tightly around nucleosomes. The chromatin context determines nuclear receptor binding and determines which epigenetic modifications will occur thereafter. Upon binding to their genomic response elements, nuclear hormone receptors will then recruit different regulatory cofactor complexes (Lee et al., 2001). The unliganded VDR is able to bind also genomic DNA, where it usually forms complexes with corepressor proteins that either exert HDAC activity, e.g., ALIEN (Polly et al., 2000), or are associated with HDACs, such as NCOR1 and SMRT. The corepressors dissociate upon binding of 1,25-D_3, and are replaced by coactivator complexes.

The chromatin environment dictates gene activity throughout the genome. Post-translational modifications of the N-terminal tails of histone proteins allow nucleosomes to shift, the chromatin to relax, and genes to become activated. Histone modifications change in response to environmental stimuli (Meyer et al., 2013). Histones are major protein components of chromatin that undergo post-translational modifications, including acetylation of lysines, methylation of lysines and arginines, and phosphorylation of serine and threonine residues (Esteller, 2008). In epigenetically silenced genes, hypermethylation of CGIs is often associated with loss of acetylation on histone 3 and 4 (H3 and H4), loss of methylation of lysine (K) 4 on H3 (H3K4), and gain of methylation of K9 and K27 on H3 (H3K9 and H3K27) (Esteller, 2008).

Histone acetylation generally correlates with transcriptional activation (Hebbes et al., 1988; Kouzarides, 2007) and is dependent on a dynamic interaction between histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Marks et al., 2001). The balance between the actions of these enzymes is crucial in controlling gene expression, and governs several developmental processes and disease states (Haberland et al., 2009). Generally, HATs are defined as activators of transcription, whereas HDACs as transcription repressors (Parbin et al., 2014). In various cancer types, including prostate, gastric, and breast cancers, overexpression of HDAC1 is often associated with poor clinical outcome (Choi et al., 2001; Halkidou et al., 2004; Zhang et al., 2005). In colorectal cancer patients HDAC1, 2, and 3 are overexpressed, and high HDAC1 and 2 expression is linked with reduced patient survival (Zhu et al., 2004; Wilson et al., 2006). Overexpression of HDAC1 plays a crucial role in regulating proliferation by repressing the expression of the cyclin-dependent kinase inhibitor p21 (Lagger et al., 2003). Additionally, silencing of HDAC4 leads to re-expression of p21, which in turn induces cell growth arrest and tumor growth inhibition, both *in vitro* and *in vivo* in a human glioblastoma model (Mottet et al., 2009). In addition to classical HDACs, another group of enzymes, the sirtuins (silent information regulator 2 proteins) are involved in histone deacetylation (Schwer and Verdin, 2008). Sirtuins have been linked to metabolic disorders, cancer, aging, and also regulation of the circadian rhythm (Guarente, 2006; Longo and Kennedy, 2006; Jung-Hynes et al., 2010).

Many of the coactivators recruited by the VDR, including p160 steroid receptor coactivator proteins (SRC1, 2, and 3), p300, or CBP have lysine acetyltransferase activity (**Figure 1**). Indeed, treatment of THP-1 cells with 1,25-D_3 increased H3K27ac at the promoter of several early VDR target genes (Seuter et al., 2013). In genetic hypercalciuric stone forming rats inhibition of bone morphogenetic protein 2 (BMP2) by 1,25-D_3, seems to involve H3 deacetylation and H3K9 di-methylation (Fu et al., 2013).

In MDA-MB453 breast cancer cells 1,25-D_3 treatment regulates expression of p21 through a mechanism involving both histone acetylation and methylation, probably by dynamic

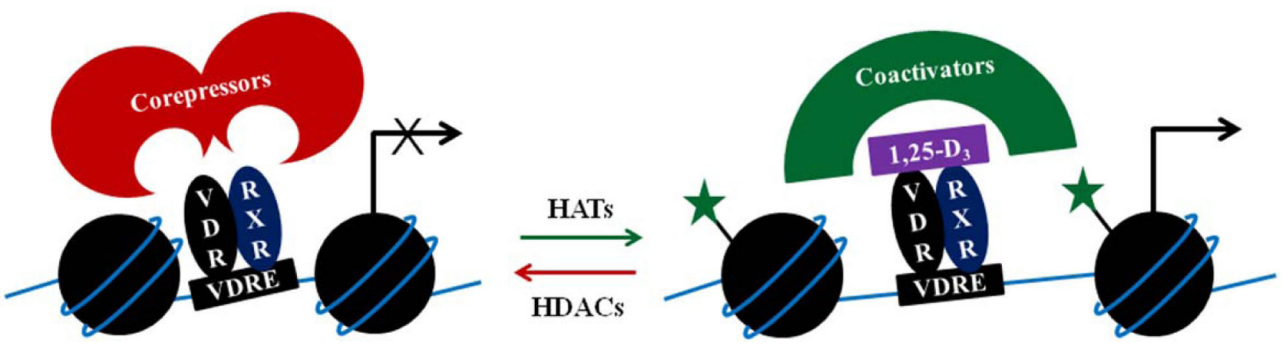

FIGURE 1 | Simplified illustration of a two-step coregulator model. The VDR/RXR complex in absence of the 1,25-D_3 may bind corepressors that would lead/maintain gene repression, partly by attracting histone deacetylases (HDACs). Upon ligand binding to the VDR/RXR complex, corepressors are replaced by coactivators, which include histone acetyltransferases (HATs). Acetylation of histones (indicated by the green stars) enables chromatin relaxation and gene transcription.

chromatin looping from distal 1,25-D_3 responsive elements to the TSS of *p21* (Saramäki et al., 2009).

Histone methylation can lead either to gene activation or repression, depending on the histone site that is methylated, the degree of methylation (e.g., mono-methylation, di-methylation, or tri-methylation), amino acid residues affected, and their position in the histone tail (Esteller, 2008). Methylation of histones depends on a dynamic process arising from the actions of methyltransferases (HMTs) and demethylases (HDMs) (Shi and Whetstine, 2007; Mosammaparast and Shi, 2010; Greer and Shi, 2012). So far, two protein families capable of demethylating lysines are known, the amine oxidases (Shi et al., 2004) and jumonji C (JmjC)-domain-containing proteins (Cloos et al., 2006; Tsukada et al., 2006). The first histone demethylase discovered was the lysine-specific demethylase 1 (LSD1/KDM1A), an amine oxidase, which demethylates H3K4me2/me1 (**Table 1**) (Shi et al., 2004). High expression of KDM1A in various cancers, including colorectal cancer, prostate cancer, and neuroblastomas is associated with increased cancer recurrence and poor clinical outcome (Kahl et al., 2006; Schulte et al., 2009; Ding et al., 2013). Additionally, it has been shown that LSD1 is essential for androgen and estrogen receptor-dependent gene activation via H3K9me2/me1 demethylation (Metzger et al., 2005; Garcia-Bassets et al., 2007; Perillo et al., 2008). There is a reciprocal regulatory effect between the activity of VDR and histone demethylases. In the colon cancer cell line SW480-ADH 1,25-D_3 increased the expression of the lysine-specific demethylase 1 and 2 (Pereira et al., 2012).

1,25-D_3 treatment affected also the expression of a series of different JmjC histone demethylases. The first identified member of the JmjC family was KDM2A/JHDM1A (Tsukada et al., 2006). Expression profiling data showed altered expression of KDM2A and KDM2B in several tumors, however, it seems that their pro- or antioncogenic functions are tissue-dependent (Frescas et al., 2007, 2008; Pfau et al., 2008). 1,25-D_3 inhibited the expression of several histone demethylases (e.g., KDM4A/4C/4D/5A/2B, JMJD5/6, PLA2G4B), and induced the expression of others, JARID2 and KDM5B (Pereira et al., 2012). Members of the KDM4 family catalyze tri-demethylation of H3K9 and/or H3K36 (Cloos et al., 2006; Fodor et al., 2006; Klose et al., 2006; Whetstine et al., 2006; Lin et al., 2008). H3K9me3 is a mark for heterochromatin and demethylation of H3K9 is suggested to be linked with chromosomal instability (Cloos et al., 2006). Inhibition of expression of KDM4 family members by 1,25-D_3 could thus contribute to genome stability. Members of KDM5 cluster catalyze demethylation of H3K4me3/me2, which is a mark for open chromatin (Christensen et al., 2007; Iwase et al., 2007; Klose et al., 2007; Tahiliani et al., 2007) and their upregulaton upon 1,25-D_3 treatment might lead to gene repression (Pereira et al., 2011). Overexpression of KDM5B has been reported in breast and prostate cancers (Lu et al., 1999; Xiang et al., 2007). Deletion of *kdm5b* inhibits tumor growth in a syngeneic mouse mammary

Table 1 | A simplified list of the members of the two classes of histone demethylases (mentioned in the manuscript).

Class of histone demethylases	Histone demethylase family	Histone demethylase	Histone substrate	Gene expression
Amine oxidases	KDM1	KDM1A	H3K4me2/me1 H3K9me2/me1	Repression Activation
		KDM1B	H3K4me2/me1	Repression
Jumonji C-domain-containing proteins	KDM2	KDM2A	H3K36me2/me1	Repression
		KDM2B	H3K4me3 H3K36me2/me1	
	KDM3	KDM3A	H3K9me2/me1	Activation
		KDM3B	H3K9me3/me2/me1	
	KDM4	KDM4A	H3K9me3/me2	Activation
		KDM4B	H3K36me3/me2	Repression
		KDM4C		
		KDM4D	H3K9me3/me2	Activation
	KDM5	KDM5A	H3K4me3/me2	Repression
		KDM5B		
		KDM5C		
		KDM5D		
	KDM6	KDM6A	H3K27me3/me2	Activation
		KDM6B		
	PHF	JHDM1D	H3K9me2/me1 H3K27me2/me1	Activation
		PHF8	H3K9me2/me1	

Reviewed in Pedersen and Helin (2010), Greer and Shi (2012). The main histone demethylase families and submembers are indicated. Degree of methylation and site of lysine residue are given. References for individual enzymes can be found throughout the text. H, Histone; K, lysine; me1, mono-methylation; me2, di-methylation; me3, tri-methylation; KDM1A, lysine-specific demethylase 1A; JHDM1D, JmjC-domain-containing histone demethylation protein 1D; PHF, plant homeodomain finger protein.

tumor (Yamane et al., 2007), suggestive of its potential role in tumor development. 1,25-D_3 induced the expression of the histone demethylase KDM6B as well, which is the only other known enzyme, besides KDM6A that is able to demethylate H3K27me3, a histone mark that correlates with gene repression. Furthermore, the authors showed positive correlation between KDM6B and VDR in 96 colon tumor patients, and inverse correlation of KDM6B with SNAIL1, which is involved in epithelial to mesenchymal transition, indicating that probably the antiproliferative role of 1,25-D_3 via KDM6B upregulation might take place *in vivo* (Pereira et al., 2011). Interestingly, treatment of SW480-ADH cells with 1,25-D_3 had no effect on global H3K27me3 levels, in spite of KDM6B upregulation (Pereira et al., 2011, 2012). The effect of 1,25-D_3 on the expression of histone demethylases may well be indirect and could be mediated by microRNAs (Padi et al., 2013). KDM2A is one of the direct targets of microRNA-627. 1,25-D_3-dependent upregulation of the microRNA-627 expression both *in vitro*, in the HT-29 colorectal cancer cells and *in vivo*, in tumor xenografts, led to lower KDM2A levels (Padi et al., 2013).

In different pathologies, the expression pattern of the nuclear receptor cofactors is altered, compromising the effect of 1,25-D_3 (Doig et al., 2013; Singh et al., 2013). The initial interactions between VDR and coactivators are the seed for the assembly of intricate multiprotein complexes that remodel the chromatin structure, recruit the core transcriptional machinery, and induce expression of 1,25-D_3 target genes (**Figure 1**). Often, differences in responsiveness to 1,25-D_3 depend on the expression pattern of the coregulators of VDR. In prostate cancer cells, the temporal distribution of the nuclear corepressor NCOR1 at VDR target genes is different in 1,25-D_3 responsive cells compared with unresponsive cells (Doig et al., 2013; Singh et al., 2013).

The liganded VDR is able both to transactivate and transrepress target genes. The mechanisms of action are probably different between transactivation and transrepression, and also highly dependent on the motifs of the vitamin D response elements. A highly complex mechanism regulates the ligand-dependent repression of CYP27B1 (Kim et al., 2007a). CYP27B1 repression requires two epigenetic modifications: deacetylation of histones and methylation of the *CYP27B1* gene promoter and exon regions. This is dependent on the presence of the VDR interacting repressor (VDIR) and the chromatin remodeler Williams Syndrome transcription factor. In the absence of 1,25-D_3, VDIR is bound directly to the E-box motifs in the negative VDRE and recruits histone acetyltransferases to induce *CYP27B1* gene transcription. In the presence of 1,25-D_3, VDIR acts as a scaffold for the 1,25-D_3-VDR complex to repress transcription of *CYP27B1* through recruitment of HDAC2, DNMT1, and DNMT3B (Kim et al., 2007a). It seems that VDIR and HDAC2 are involved also in the 1,25-D_3-dependent transrepression of the human parathyroid hormone gene (Kim et al., 2007b). It is not clear, whether this mechanism of transrepression by liganded VDR also applies to other genes. In mesenchymal stem cells 1,25-D_3 represses gene expression by binding to promoters with enhanced H3K9Ac and H3K9me2 (Tan et al., 2009). Whether H3K9 acetylation/methylation enabled VDR binding or VDR binding caused H3K9 acetylation, is not clear.

Proper orchestration of histone modifications in crosstalk with other chromatin regulators is crucial in maintaining the epigenetic landscape and governing gene expression. Any disturbances in these constellations may lead to aberrant gene expression. Whether 1,25-D_3 affects regulation of other chromatin modulators as well, is not yet known.

REGULATION OF THE VITAMIN D SYSTEM

The vitamin D system has pleiotropic functions and regulates approximately 3% of the human genome (Bouillon et al., 2008). To maintain balance, a strict regulation of the vitamin D system genes is of utmost importance. The main role of liganded VDR in tissues not involved in calcium homeostasis is to control expression of genes that regulate cell proliferation, differentiation, and apoptosis. One major limitation in the therapeutic exploitation of these effects is the resistance of cancer cells to 1,25-D_3. Epigenetic corruption of VDR signaling is suggested to be one of the mechanisms that leads to reduced responsiveness to 1,25-D_3 actions. This can be caused by promoter methylation of key vitamin D system genes or by skewed accumulation of VDR-associated corepressors, preferentially at promoters of anti-proliferative target genes (Abedin et al., 2006).

Expression of the vitamin D degrading and metabolizing enzymes is regulated through binding of 1,25-D_3-liganded VDR to vitamin D responsive elements (VDREs). However, the major regulators of 1,25-D_3 levels and signaling CYP2R1, CYP24A1, CYP27B1, and VDR, "the vitamin D tool" genes, are prone to epigenetic regulation. CpG islands span the promoters of *CYP2R1*, *CYP24A1*, and *VDR*, while a CpG island is located within the *CYP27B1* gene locus (**Figure 2**). Therefore, DNA methylation and histone modifications in these regions can change the chromatin state from an open to closed conformation and lead to transcriptional repression of these genes. Expression of vitamin D tool genes becomes deregulated in various types of cancer, and these changes may be partially attributed to epigenetic alterations (reviewed in Hobaus et al., 2013). As early as 1984, Yoneda et al. (1984) have shown that the histone acetyltransferase inhibitor butyrate augments 1,25-D_3 actions. Several studies confirmed these findings (e.g., Rashid et al., 2001) and have suggested that the action of butyrate could be through upregulation of VDR or CYP27B1 expression (Gaschott and Stein, 2003). Whether this effect is mediated by direct acetylation of the *VDR* or *CYP27B1* promoters, has not been determined.

In this chapter we discuss evidence for epigenetic regulation through DNA methylation of these genes in health and disease.

EPIGENETIC REGULATION OF THE VITAMIN D RECEPTOR

The VDR is a nuclear receptor mediating 1,25-D_3 signaling. It is expressed by at least 38 cell types in the human body (Norman and Bouillon, 2010). In the absence of its ligand 1,25-D_3, VDR is mainly found in the cytoplasm (Nagpal et al., 2005). Upon ligand binding, VDR heterodimerizes with the retinoid X receptor (RXR) and translocates to the nucleus, where it binds to vitamin D responsive elements (VDREs) to regulate transcription of 1,25-D_3 target genes. This is achieved through recruitment of coactivators or corepressors to the VDR-RXR complex bound to DNA (Nagpal et al., 2005; Pike et al., 2012; Haussler et al., 2013;

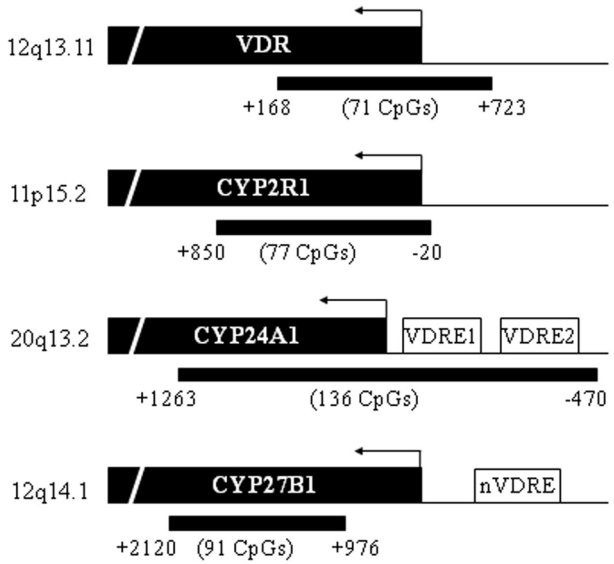

FIGURE 2 | Location of CpG islands in the promoter region of vitamin D tools genes. VDR is located on 12q13.11 (chr12:48235320-48298814), CYP2R1 on chromosome 11p15.2 (chr11:14899556-14913751), CYP24A1 on chromosome 20q13.2 (chr20:52769988-52790516), and CYP27B1 on 12q14.1 (chr12:58156117-58160976). The locations of the CpG islands are indicated (black bars) relative to start of the gene locus (not to TSS) according to UCSC Genome Browser Homepage (GRCh37/hg19) (Karolchik et al., 2014). Number of CpGs located within each island is stated. Two vitamin D responsive elements (VDRE) are located in the proximal CYP24A1 promoter region and one nVDRE is located in the CYP27B1 promoter region.

Pike and Meyer, 2013). As VDR is rarely mutated during carcinogenesis (Miller et al., 1997), the disturbance of the vitamin D signaling and apparent 1,25-D_3 insensitivity in cancer (Marik et al., 2010) must be attributed to other alterations, which may include epigenetic changes, such as promoter methylation.

The *VDR* gene is located on the long arm of chromosome 12 (12q13.11) and contains 4 potential promoter regions. Exons 1a, 1c, and 1d of the *VDR* are well conserved, while 1b, 1e, and 1f show low homology (Halsall et al., 2007). Exon 1a appears to contain a strong promoter, including several transcription factor binding sites (AP-2 and SP1). Transcription was reported to originate also in exons 1d, 1e, and 1f, while translation starts in exon 2 (Halsall et al., 2007). Marik and colleagues performed an *in silico* analysis of the *VDR* gene sequence and reported three CpG islands located in exon 1a spanning from −790 bp to 380 bp relative to the TSS in exon 1a (Marik et al., 2010). According to the UCSC Genome browser, however, only one large CpG island spanning 892 bp in length is found in the *VDR* promoter region (Gardiner-Garden and Frommer, 1987; Karolchik et al., 2014). This discrepancy is likely due to the different search parameters used for CpG island identification.

Epigenetic silencing of VDR was suggested to cause the slow normalization of VDR levels in the parathyroid glands of uremic rats after kidney transplantation (Lewin et al., 2002; Hofman-Bang et al., 2012). However, sequencing of the *VDR* promoter [−250 to 300 bp relative to exon 1 (43 CpGs)] in normal and uremic rats showed no difference between methylation patterns (Hofman-Bang et al., 2012). Further, the authors reported that methylation levels coincided with the negative control, thus showing that promoter methylation does not play a role in regulating VDR expression in the parathyroid glands.

In contrast, promoter methylation was reported to cause repression of *VDR* gene expression in HIV infected T cells. In normal T cells, activation or priming causes an upregulation of VDR expression (Von Essen et al., 2010). In comparison, infection of previously activated T cells with human immunodeficiency virus (HIV) led to upregulation of DNMT3B, increased promoter methylation of *VDR* (45–70%), and decreased *VDR* gene expression (Chandel et al., 2013). This downregulation of VDR could be reversed upon treatment with 5-azacytidine (AZA) suggesting that the decreased expression of VDR by HIV is, at least partially, caused by DNA methylation (Chandel et al., 2013). There is evidence for an inverse correlation between the vitamin D status and infections, however, many trials failed to show a protective effect of vitamin D (reviewed in Yamshchikov et al., 2009). Thus, reduced sensitivity to vitamin D metabolites due to, e.g., downregulation of VDR may account for inconclusive trials. This is supported by a study investigating methylation of the 3′end of *VDR* in two South African groups revealing differences with respect to ethnicity and tuberculosis status of the patients (Andraos et al., 2011).

In breast tumors, methylation of exon 1a of the *VDR* gene was significantly higher (65% of CpGs methylated) compared with normal breast tissue (15% of CpGs methylated) (Marik et al., 2010). *In vitro*, in breast cancer cell lines, three hypermethylated regions in exon 1a became demethylated after treatment with the DNMT1 inhibitor 5-aza-2′-deoxycytidine (DAC) and VDR mRNA expression increased. These regions were in proximity to the SP1 binding sites (approximately 790 bp from TSS), NFκB binding sites (approximately −480 from TSS), and the exon 1a TSS. Treatment with 1,25-D_3 had no effect on methylation of these regions (Marik et al., 2010). In other types of cancer, e.g., the choriocarcinoma-derived trophoblast cell lines JEG-3 and JAR, the *VDR* promoter was densely methylated (Novakovic et al., 2009). In contrast, no methylation of the VDR promoter region was observed in colon cancer cell lines, and treatment with DAC did not increase gene expression (Habano et al., 2011; Höbaus et al., 2013a). In parathyroid tumors the expression of VDR is decreased (Gogusev et al., 1997; Carling et al., 2000), however, no differences in DNA methylation of *VDR* were observed between parathyroid tumors and healthy controls (Sulaiman et al., 2013). Similar results were seen in parathyroid adenoma samples, which showed decreased expression of VDR, but showed no promoter methylation (Varshney et al., 2013).

Additionally, it has been suggested that expression of 5′ truncated variants of VDR is linked to methylation of the *VDR* promoter. These variants are predominantly found in breast cancer compared with the full length variants expressed in normal breast tissue. Treatment with DAC restored expression of the active transcript variant of VDR in breast cancer cell lines, indicating promoter methylation as cause of truncated protein expression (Marik et al., 2010). The significance of these potentially untranslated truncated variants remains to be investigated, however, as

they are not found in normal breast tissue aberrant expression of truncated isoforms may further disrupt vitamin D signaling in tumor tissue.

A recent study suggested that in colorectal cancer metastases, VDR becomes the target of the polycomb group protein enhancer of zeste homolog 2 (EZH2) that mediates VDR downregulation by H3K27 trimethylation in the VDR promoter (Lin et al., 2013). The histone deacetylase HDAC3, one of the most frequently upregulated genes in cancer, seems to inhibit VDR expression. In two colorectal cancer cell lines, HCT116 and SW480 knock down of HDAC3 increased VDR expression and restored sensitivity of these cells to 1,25-D_3 (Godman et al., 2008).

Taken together, there is evidence that in various diseases the decreased tissue sensitivity to 1,25-D_3 could have been caused by the epigenetic silencing of the VDR.

EPIGENETIC REGULATION OF THE CYP2R1

CYP2R1 is a microsomal P450 enzyme, which hydroxylates both vitamin D_2 and D_3 at position C-25 to form the circulating storage form of vitamin D 25-D_3. The promoter region of *CYP2R1* is located within a CpG island, which can be subjected to epigenetic regulation. So far, only two studies investigated the promoter methylation status of this gene. Genome wide association studies found increased *CYP2R1* promoter methylation in leukocyte DNA from individuals with severe vitamin D deficiency compared with control group (Zhu et al., 2013). Further, methylation levels of *CYP2R1* promoter decreased within 12 months of vitamin D supplementation in DNA extracted from serum of non-Hispanic white American post-menopausal women aged $\geq$55 years (Zhu et al., 2013), indicating an effect of vitamin D supplementation on *CYP2R1* promoter methylation. These data indicate that under low vitamin D serum levels, the promoter of the major 25-hydroxylase *CYP2R1* may become methylated, and that event appears to be reversible upon exposure to increased vitamin D.

EPIGENETIC REGULATION OF THE CYP27B1

CYP27B1 is an inner mitochondrial membrane P450 enzyme that converts 25-D3 to its active form 1,25-D3. It is mainly expressed in the proximal tubule of the kidneys, but it is also expressed in many vitamin D target tissues, albeit at lower levels (Hendrix et al., 2004). The *CYP27B1* gene harbors a CpG island. However, recent sequence updates (Ensembl 74, November 2013) shift the CpG island from the *CYP27B1* promoter region into the gene coding sequence (Flicek et al., 2014). This explains the differences between the location of the CpG island depicted in **Figure 2** and the location of the CpG island described in literature. For simplicity, statements on nVDRES and CpG island location below refer to reports in the published articles and not to **Figure 2**.

The promoter region of *CYP27B1* contains a negative VDRE (nVDRE) located at around 500 bp, consisting of two E-box like motifs (Murayama et al., 2004). This region is responsible for 1,25-D_3-dependent transrepression, which seems to be achieved through recruitment of both HDACs and DNMTs by VDR/RXR to the promoter region of *CYP27B1* (Takeyama and Kato, 2011). For further details see subsection Interactions of Vitamin D with Chromatin Modulators and Remodelers.

In cancer, expression of CYP27B1 is often downregulated. This may be explained by increased methylation of the CpG island located within *CYP27B1*. In the breast cancer cells MDA-MB-231, *CYP27B1* hypermethylation led to gene silencing, which could be reversed by treatment with deoxyC (Shi et al., 2002). In prostate cancer cell lines, combination of the DNMT1 inhibitor DAC and the HDAC inhibitor TSA resulted in increased activity of CYP27B1 (Khorchide et al., 2005). In the choriocarcinoma cell lines BeWo and JAR the promoter of *CYP27B1* was densely methylated (Novakovic et al., 2009). The *CYP27B1* promoter was hypermethylated (61%) in Non-Hodgkin's lymphoma, but not in benign follicular hyperplasia. Two out of four non-Hodgkin's lymphoma cell lines showed strong methylation of the *CYP27B1* promoter. Interestingly, all four responded to DAC-TSA treatments with upregulation of gene expression independent of the methylation status of their *CYP27B1* promoter, which may be explained by other regions prone to methylation not investigated in this study or by differences in silencing mechanisms (Shi et al., 2007). Further, the methylation level of *CYP27B1* was increased in primary lymphoma and leukemia cells also compared with normal peripheral blood lymphocytes (Lagger et al., 2003; Wjst et al., 2010).

Methylation of *CYP27B1* in diseases might cause reduced local activation of 25-D_3 to 1,25-D_3, thus reducing local 1,25-D_3 levels and restricting its functions.

EPIGENETIC REGULATION OF CYP24A1

The 1,25-dihydroxyvitamin D_3 24-hydroxylase is an inner mitochondrial membrane P450 enzyme, which catalyzes both 25-D_3 and 1,25-D_3 (Kawashima et al., 1981; Pedersen et al., 1983; Sakaki et al., 2000). Its primary site of expression are the kidneys, where it plays a crucial role in regulating systemic vitamin D metabolite levels, however, expression is found in many other vitamin D target tissues.

The promoter of *CYP24A1* is spanned by a CpG island making it prone to regulation by DNA methylation. Several responsive elements are located within this area, including two VDREs, a vitamin stimulating element (VSE), and SP1 binding sites (see **Figure 2**).

In healthy kidney, skeletal muscle, whole blood, brain, skin fibroblasts, and sperm the *CYP24A1* promoter is not methylated (Novakovic et al., 2009), although the expression levels are highly variable. In peripheral blood lymphocytes methylation of *CYP24A1* was low (5%) (Wjst et al., 2010). Interestingly, in full term human placenta 56.5% of the *CYP24A1* promoter is methylated. *CYP24A1* methylation was also observed in the placenta of the marmoset and mouse, however, at a lower level.

In the choriocarcinoma cell lines JEG-3, BeWo, and JAR the promoter of *CYP24A1* was densely methylated and the methylation level correlated inversely with the low gene expression (Novakovic et al., 2009). Treatment of osteoblastic ROS cells with 1,25-D_3 did not induce CYP24A1 expression. Considering the strong methylation of the *CYP24A1* promoter region, epigenetic silencing of CYP24A1 may account for the unresponsiveness of this gene to 1,25-D_3 (Ohyama et al., 2002). In the human prostate cancer cell line PC3, methylation of the *CYP24A1*

promoter reduced reporter gene expression in a methylation-dependent manner (Luo et al., 2010). In prostate cancer cells, the methylation status of *CYP24A1* promoter inversely correlated with gene expression. Demethylating agents restored CYP24A1 expression only in cell lines where the *CYP24A1* promoter was methylated prior to treatment (Luo et al., 2010). Only DNA demethylation by DAC treatment permitted recruitment of VDR to the *CYP24A1* promoter (Luo et al., 2010). In patients, development from benign toward malignant prostate lesions was paralleled by increasing methylation levels of the *CYP24A1* promoter (Luo et al., 2010). Prostate tumor derived endothelial cells (TDEC) expressed less CYP24A1 compared with endothelial cells derived from normal cells or matrigel plugs, which may be attributed to increased *CYP24A1* promoter methylation in TDECs (Johnson et al., 2010). We have shown recently that in colon cancer cell lines DAC induced CYP24A1 expression in a cell line-specific manner, independent of the methylation level of the promoter. In these cells induction of CYP24A1 expression by DAC seems to be independent of *CYP24A1* promoter methylation (Höbaus et al., 2013a). Moreover, the methylation level of the *CYP24A1* promoter was comparably low both in colon adenocarcinomas and the adjacent mucosa, although the expression of CYP24A1 was significantly higher in the tumors (Höbaus et al., 2013b).

Taken together, the regulation of CYP24A1 by DNA methylation appears to be tissue-dependent, both in health and disease.

CONCLUSIONS

There is a strong reciprocity between the vitamin D system and epigenetic mechanisms. The vitamin D system is, on the one hand regulated by epigenetic mechanisms and, on the other hand, is involved in regulating epigenetic events. Critical vitamin D tool genes can be silenced by DNA methylation. The VDR protein interacts, directly or indirectly, with chromatin modifiers and remodelers. Liganded VDR regulates expression of several of these chromatin modifiers and remodelers, and it might even regulate DNA methylation.

Epigenetic regulation of gene expression is a fine-tuned mechanism and its deregulation can lead to pathological conditions. The impact of vitamin D in the maintenance of the normal epigenetic landscape underlines the central role of this hormone in physiology.

PERSPECTIVES

One of the most fundamental questions in the control of gene expression is the way how epigenetic marks are established, erased, and recognized. Regulating epigenetic events could be a further mechanism by which 1,25-D_3 may prevent or delay tumorigenesis and onset of chronic diseases. Therefore, we need to understand better the impact of vitamin D on the epigenome, and plan thorough and comprehensive studies to examine this interplay.

ACKNOWLEDGMENTS

We thank the Austrian Research Fund (FWF) Grant P22200-B11 and the European Union, FP7-PEOPLE-2010 Grant 264663 for support.

REFERENCES

Abedin, S. A., Banwell, C. M., Colston, K. W., Carlberg, C., and Campbell, M. J. (2006). Epigenetic corruption of VDR signalling in malignancy. *Anticancer Res.* 26, 2557–2566.

Aguilera, O., Pena, C., Garcia, J. M., Larriba, M. J., Ordonez-Moran, P., Navarro, D., et al. (2007). The Wnt antagonist DICKKOPF-1 gene is induced by 1alpha,25-dihydroxyvitamin D3 associated to the differentiation of human colon cancer cells. *Carcinogenesis* 28, 1877–1884. doi: 10.1093/carcin/bgm094

Andraos, C., Koorsen, G., Knight, J. C., and Bornman, L. (2011). Vitamin D receptor gene methylation is associated with ethnicity, tuberculosis, and TaqI polymorphism. *Hum. Immunol.* 72, 262–268. doi: 10.1016/j.humimm.2010.12.010

Arasaradnam, R. P., Commane, D. M., Bradburn, D., and Mathers, J. C. (2008). A review of dietary factors and its influence on DNA methylation in colorectal carcinogenesis. *Epigenetics* 3, 193–198. doi: 10.4161/epi.3.4.6508

Ballestar, E., and Esteller, M. (2005). Methyl-CpG-binding proteins in cancer: blaming the DNA methylation messenger. *Biochem. Cell Biol.* 83, 374–384. doi: 10.1139/o05-035

Barreto, G., Schafer, A., Marhold, J., Stach, D., Swaminathan, S. K., Handa, V., et al. (2007). Gadd45a promotes epigenetic gene activation by repair-mediated DNA demethylation. *Nature* 445, 671–675. doi: 10.1038/nature05515

Baylin, S. B., and Jones, P. A. (2011). A decade of exploring the cancer epigenome - biological and translational implications. *Nat. Rev. Cancer* 11, 726–734. doi: 10.1038/nrc3130

Bestor, T. H. (2005). Transposons reanimated in mice. *Cell* 122, 322–325. doi: 10.1016/j.cell.2005.07.024

Bird, A. (2002). DNA methylation patterns and epigenetic memory. *Genes Dev.* 16, 6–21. doi: 10.1101/gad.947102

Bird, A. P. (1980). DNA methylation and the frequency of CpG in animal DNA. *Nucleic Acids Res.* 8, 1499–1504. doi: 10.1093/nar/8.7.1499

Bjornsson, H. T., Brown, L. J., Fallin, M. D., Rongione, M. A., Bibikova, M., Wickham, E., et al. (2007). Epigenetic specificity of loss of imprinting of the IGF2 gene in Wilms tumors. *J. Natl. Cancer Inst.* 99, 1270–1273. doi: 10.1093/jnci/djm069

Bouillon, R., Carmeliet, G., Verlinden, L., Van Etten, E., Verstuyf, A., Luderer, H. F., et al. (2008). Vitamin D and human health: lessons from vitamin D receptor null mice. *Endocr. Rev.* 29, 726–776. doi: 10.1210/er.2008-0004

Bremmer, F., Thelen, P., Pottek, T., Behnes, C. L., Radzun, H. J., and Schweyer, S. (2012). Expression and function of the vitamin D receptor in malignant germ cell tumour of the testis. *Anticancer Res.* 32, 341–349.

Brock, M. V., Hooker, C. M., Ota-Machida, E., Han, Y., Guo, M., Ames, S., et al. (2008). DNA methylation markers and early recurrence in stage I lung cancer. *N. Engl. J. Med.* 358, 1118–1128. doi: 10.1056/NEJMoa0706550

Carling, T., Rastad, J., Szabó, E., Westin, G., and Akerström, G. (2000). Reduced parathyroid vitamin D receptor messenger ribonucleic acid levels in primary and secondary hyperparathyroidism. *J. Clin. Endocrinol. Metab.* 85, 2000–2003. doi: 10.1210/jc.85.5.2000

Chandel, N., Husain, M., Goel, H., Salhan, D., Lan, X., Malhotra, A., et al. (2013). VDR hypermethylation and HIV-induced T cell loss. *J. Leukoc. Biol.* 93, 623–631. doi: 10.1189/jlb.0812383

Choi, J. H., Kwon, H. J., Yoon, B. I., Kim, J. H., Han, S. U., Joo, H. J., et al. (2001). Expression profile of histone deacetylase 1 in gastric cancer tissues. *Jpn. J. Cancer Res.* 92, 1300–1304. doi: 10.1111/j.1349-7006.2001.tb02153.x

Christensen, J., Agger, K., Cloos, P. A., Pasini, D., Rose, S., Sennels, L., et al. (2007). RBP2 belongs to a family of demethylases, specific for tri-and dimethylated lysine 4 on histone 3. *Cell* 128, 1063–1076. doi: 10.1016/j.cell.2007.02.003

Cloos, P. A., Christensen, J., Agger, K., Maiolica, A., Rappsilber, J., Antal, T., et al. (2006). The putative oncogene GASC1 demethylates tri- and dimethylated lysine 9 on histone H3. *Nature* 442, 307–311. doi: 10.1038/nature04837

Cui, H., Cruz-Correa, M., Giardiello, F. M., Hutcheon, D. F., Kafonek, D. R., Brandenburg, S., et al. (2003). Loss of IGF2 imprinting: a potential marker of colorectal cancer risk. *Science* 299, 1753–1755. doi: 10.1126/science.1080902

Ding, J., Zhang, Z. M., Xia, Y., Liao, G. Q., Pan, Y., Liu, S., et al. (2013). LSD1-mediated epigenetic modification contributes to proliferation and metastasis of colon cancer. *Br. J. Cancer* 109, 994–1003. doi: 10.1038/bjc.2013.364

Doig, C. L., Singh, P. K., Dhiman, V. K., Thorne, J. L., Battaglia, S., Sobolewski, M., et al. (2013). Recruitment of NCOR1 to VDR target genes is enhanced in prostate cancer cells and associates with altered DNA methylation patterns. *Carcinogenesis* 34, 248–256. doi: 10.1093/carcin/bgs331

Eden, A., Gaudet, F., Waghmare, A., and Jaenisch, R. (2003). Chromosomal instability and tumors promoted by DNA hypomethylation. *Science* 300, 455. doi: 10.1126/science.1083557

Esteller, M. (2007a). Epigenetic gene silencing in cancer: the DNA hypermethylome. *Hum. Mol. Genet.* 16, Spec No 1, R50–R59. doi: 10.1093/hmg/ddm018

Esteller, M. (2007b). Epigenetics provides a new generation of oncogenes and tumour-suppressor genes. *Br. J. Cancer* 96(Suppl.), R26–R30. doi: 10.1038/sj.bjc.6602918

Esteller, M. (2008). Epigenetics in cancer. *N. Engl. J. Med.* 358, 1148–1159. doi: 10.1056/NEJMra072067

Esteller, M., Catasus, L., Matias-Guiu, X., Mutter, G. L., Prat, J., Baylin, S. B., et al. (1999). hMLH1 promoter hypermethylation is an early event in human endometrial tumorigenesis. *Am. J. Pathol.* 155, 1767–1772. doi: 10.1016/S0002-9440(10)65492-2

Feinberg, A. P., Cui, H., and Ohlsson, R. (2002). DNA methylation and genomic imprinting: insights from cancer into epigenetic mechanisms. *Semin. Cancer Biol.* 12, 389–398. doi: 10.1016/S1044-579X(02)00059-7

Ficz, G., Branco, M. R., Seisenberger, S., Santos, F., Krueger, F., Hore, T. A., et al. (2011). Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. *Nature* 473, 398–402. doi: 10.1038/nature10008

Flicek, P., Amode, M. R., Barrell, D., Beal, K., Billis, K., Brent, S., et al. (2014). Ensembl 2014. *Nucleic Acids Res.* 42, D749–D755. doi: 10.1093/nar/gkt1196

Fodor, B. D., Kubicek, S., Yonezawa, M., O'Sullivan, R. J., Sengupta, R., Perez-Burgos, L., et al. (2006). Jmjd2b antagonizes H3K9 trimethylation at pericentric heterochromatin in mammalian cells. *Genes Dev.* 20, 1557–1562. doi: 10.1101/gad.388206

Frescas, D., Guardavaccaro, D., Bassermann, F., Koyama-Nasu, R., and Pagano, M. (2007). JHDM1B/FBXL10 is a nucleolar protein that represses transcription of ribosomal RNA genes. *Nature* 450, 309–313. doi: 10.1038/nature06255

Frescas, D., Guardavaccaro, D., Kuchay, S. M., Kato, H., Poleshko, A., Basrur, V., et al. (2008). KDM2A represses transcription of centromeric satellite repeats and maintains the heterochromatic state. *Cell Cycle* 7, 3539–3547. doi: 10.4161/cc.7.22.7062

Fu, B., Wang, H., Wang, J., Barouhas, I., Liu, W., Shuboy, A., et al. (2013). Epigenetic regulation of BMP2 by 1,25-dihydroxyvitamin D3 through DNA methylation and histone modification. *PLoS ONE* 8:e61423. doi: 10.1371/journal.pone.0061423

Garcia-Bassets, I., Kwon, Y. S., Telese, F., Prefontaine, G. G., Hutt, K. R., Cheng, C. S., et al. (2007). Histone methylation-dependent mechanisms impose ligand dependency for gene activation by nuclear receptors. *Cell* 128, 505–518. doi: 10.1016/j.cell.2006.12.038

Gardiner-Garden, M., and Frommer, M. (1987). CpG islands in vertebrate genomes. *J. Mol. Biol.* 196, 261–282. doi: 10.1016/0022-2836(87)90689-9

Gaschott, T., and Stein, J. (2003). Short-chain fatty acids and colon cancer cells: the vitamin D receptor–butyrate connection. *Recent Results Cancer Res.* 164, 247–257. doi: 10.1007/978-3-642-55580-0_18

Godman, C. A., Joshi, R., Tierney, B. R., Greenspan, E., Rasmussen, T. P., Wang, H. W., et al. (2008). HDAC3 impacts multiple oncogenic pathways in colon cancer cells with effects on Wnt and vitamin D signaling. *Cancer Biol. Ther.* 7, 1570–1580. doi: 10.4161/cbt.7.10.6561

Gogusev, J., Duchambon, P., Hory, B., Giovannini, M., Goureau, Y., Sarfati, E., et al. (1997). Depressed expression of calcium receptor in parathyroid gland tissue of patients with hyperparathyroidism. *Kidney Int.* 51, 328–336. doi: 10.1038/ki.1997.41

Greer, E. L., and Shi, Y. (2012). Histone methylation: a dynamic mark in health, disease and inheritance. *Nat. Rev. Genet.* 13, 343–357. doi: 10.1038/nrg3173

Greger, V., Passarge, E., Höpping, W., Messmer, E., and Horsthemke, B. (1989). Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. *Hum. Genet.* 83, 155–158. doi: 10.1007/BF00286709

Gruenbaum, Y., Stein, R., Cedar, H., and Razin, A. (1981). Methylation of CpG sequences in eukaryotic DNA. *FEBS Lett.* 124, 67–71. doi: 10.1016/0014-5793(81)80055-5

Guarente, L. (2006). Sirtuins as potential targets for metabolic syndrome. *Nature* 444, 868–874. doi: 10.1038/nature05486

Habano, W., Gamo, T., Terashima, J., Sugai, T., Otsuka, K., Wakabayashi, G., et al. (2011). Involvement of promoter methylation in the regulation of Pregnane X receptor in colon cancer cells. *BMC Cancer* 11:81. doi: 10.1186/1471-2407-11-81

Haberland, M., Montgomery, R. L., and Olson, E. N. (2009). The many roles of histone deacetylases in development and physiology: implications for disease and therapy. *Nat. Rev. Genet.* 10, 32–42. doi: 10.1038/nrg2485

Hackett, J. A., Sengupta, R., Zylicz, J. J., Murakami, K., Lee, C., Down, T. A., et al. (2013). Germline DNA demethylation dynamics and imprint erasure through 5-hydroxymethylcytosine. *Science* 339, 448–452. doi: 10.1126/science.1229277

Halkidou, K., Gaughan, L., Cook, S., Leung, H. Y., Neal, D. E., and Robson, C. N. (2004). Upregulation and nuclear recruitment of HDAC1 in hormone refractory prostate cancer. *Prostate* 59, 177–189. doi: 10.1002/pros.20022

Halsall, J. A., Osborne, J. E., Hutchinson, P. E., and Pringle, J. H. (2007). *In silico* analysis of the 5' region of the Vitamin D receptor gene: functional implications of evolutionary conservation. *J. Steroid. Biochem. Mol. Biol.* 103, 352–356. doi: 10.1016/j.jsbmb.2006.12.046

Haussler, M. R., Whitfield, G. K., Kaneko, I., Haussler, C. A., Hsieh, D., Hsieh, J. C., et al. (2013). Molecular mechanisms of vitamin D action. *Calcif. Tissue Int.* 92, 77–98. doi: 10.1007/s00223-012-9619-0

Hebbes, T. R., Thorne, A. W., and Crane-Robinson, C. (1988). A direct link between core histone acetylation and transcriptionally active chromatin. *EMBO J.* 7, 1395–1402.

Helin, K., and Dhanak, D. (2013). Chromatin proteins and modifications as drug targets. *Nature* 502, 480–488. doi: 10.1038/nature12751

Hendrix, I., Anderson, P., May, B., and Morris, H. (2004). Regulation of gene expression by the CYP27B1 promoter-study of a transgenic mouse model. *J. Steroid Biochem. Mol. Biol.* 89–90, 139–142. doi: 10.1016/j.jsbmb.2004.03.093

Herman, J. G., and Baylin, S. B. (2003). Gene silencing in cancer in association with promoter hypermethylation. *N. Engl. J. Med.* 349, 2042–2054. doi: 10.1056/NEJMra023075

Höbaus, J., Fetahu, I. S. H., Khorchide, M., Manhardt, T., and Kallay, E. (2013a). Epigenetic regulation of the 1,25-dihydroxyvitamin D3 24-hydroxylase (CYP24A1) in colon cancer cells. *J. Steroid Biochem. Mol. Biol.* 136, 296–299. doi: 10.1016/j.jsbmb.2012.08.003

Höbaus, J., Hummel, D. M., Thiem, U., Fetahu, I. S., Aggarwal, A., Müllauer, L., et al. (2013b). Increased copy-number and not DNA hypomethylation causes overexpression of the candidate proto-oncogene CYP24A1 in colorectal cancer. *Int. J. Cancer* 133, 1380–1388. doi: 10.1002/ijc.28143

Hobaus, J., Thiem, U., Hummel, D. M., and Kallay, E. (2013). Role of calcium, vitamin d, and the extrarenal vitamin d hydroxylases in carcinogenesis. *Anticancer Agents Med. Chem.* 13, 20–35. doi: 10.2174/187152013804487434

Hofman-Bang, J., Gravesen, E., Olgaard, K., and Lewin, E. (2012). Epigenetic methylation of parathyroid CaR and VDR promoters in experimental secondary hyperparathyroidism. *Int. J. Nephrol.* 2012:123576. doi: 10.1155/2012/123576

Iwase, S., Lan, F., Bayliss, P., De La Torre-Ubieta, L., Huarte, M., Qi, H. H., et al. (2007). The X-linked mental retardation gene SMCX/JARID1C defines a family of histone H3 lysine 4 demethylases. *Cell* 128, 1077–1088. doi: 10.1016/j.cell.2007.02.017

Jiang, F., Li, P., Fornace, A. J., Nicosia, S. V., and Bai, W. (2003). G2/M arrest by 1,25-dihydroxyvitamin D3 in ovarian cancer cells mediated through the induction of GADD45 via an exonic enhancer. *J. Biol. Chem.* 278, 48030–48040. doi: 10.1074/jbc.M308430200

Jin, B., and Robertson, K. D. (2013). DNA methyltransferases, DNA damage repair, and cancer. *Adv. Exp. Med. Biol.* 754, 3–29. doi: 10.1007/978-1-4419-9967-2_1

Johnson, C. S., Chung, I., and Trump, D. L. (2010). Epigenetic silencing of CYP24 in the tumor microenvironment. *J. Steroid Biochem. Mol. Biol.* 121, 338–342. doi: 10.1016/j.jsbmb.2010.03.046

Jones, P. A., and Baylin, S. B. (2007). The epigenomics of cancer. *Cell* 128, 683–692. doi: 10.1016/j.cell.2007.01.029

Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., et al. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat. Genet.* 19, 187–191. doi: 10.1038/561

Jung-Hynes, B., Reiter, R. J., and Ahmad, N. (2010). Sirtuins, melatonin and circadian rhythms: building a bridge between aging and cancer. *J. Pineal Res.* 48, 9–19. doi: 10.1111/j.1600-079X.2009.00729.x

Kahl, P., Gullotti, L., Heukamp, L. C., Wolf, S., Friedrichs, N., Vorreuther, R., et al. (2006). Androgen receptor coactivators lysine-specific histone demethylase 1 and four and a half LIM domain protein 2 predict risk of prostate cancer recurrence. *Cancer Res.* 66, 11341–11347. doi: 10.1158/0008-5472.CAN-06-1570

Karlic, H., and Varga, F. (2011). Impact of vitamin D metabolism on clinical epigenetics. *Clin. Epigenetics* 2, 55–61. doi: 10.1007/s13148-011-0021-y

Karolchik, D., Barber, G. P., Casper, J., Clawson, H., Cline, M. S., Diekhans, M., et al. (2014). The UCSC Genome Browser database: 2014 update. *Nucleic Acids Res.* 42, D764–770. doi: 10.1093/nar/gkt1168

Kawashima, H., Torikai, S., and Kurokawa, K. (1981). Localization of 25-hydroxyvitamin D3 1 alpha-hydroxylase and 24-hydroxylase along the rat nephron. *Proc. Natl. Acad. Sci. U.S.A.* 78, 1199–1203. doi: 10.1073/pnas.78.2.1199

Khorchide, M., Lechner, D., and Cross, H. S. (2005). Epigenetic regulation of vitamin D hydroxylase expression and activity in normal and malignant human prostate cells. *J. Steroid Biochem. Mol. Biol.* 93, 167–172. doi: 10.1016/j.jsbmb.2004.12.022

Kim, G. D., Ni, J., Kelesoglu, N., Roberts, R. J., and Pradhan, S. (2002). Co-operation and communication between the human maintenance and de novo DNA (cytosine-5) methyltransferases. *EMBO J.* 21, 4183–4195. doi: 10.1093/emboj/cdf401

Kim, M. S., Fujiki, R., Kitagawa, H., and Kato, S. (2007a). 1alpha,25(OH)2D3-induced DNA methylation suppresses the human CYP27B1 gene. *Mol. Cell Endocrinol.* 265–266, 168–173. doi: 10.1016/j.mce.2006.12.014

Kim, M. S., Fujiki, R., Murayama, A., Kitagawa, H., Yamaoka, K., Yamamoto, Y., et al. (2007b). 1Alpha,25(OH)2D3-induced transrepression by vitamin D receptor through E-box-type elements in the human parathyroid hormone gene promoter. *Mol. Endocrinol.* 21, 334–342. doi: 10.1210/me.2006-0231

Kim, S., Li, M., Paik, H., Nephew, K., Shi, H., Kramer, R., et al. (2008). Predicting DNA methylation susceptibility using CpG flanking sequences. *Pac. Symp. Biocomput.* 13, 315–326.

Klose, R. J., Yamane, K., Bae, Y., Zhang, D., Erdjument-Bromage, H., Tempst, P., et al. (2006). The transcriptional repressor JHDM3A demethylates trimethyl histone H3 lysine 9 and lysine 36. *Nature* 442, 312–316. doi: 10.1038/nature04853

Klose, R. J., Yan, Q., Tothova, Z., Yamane, K., Erdjument-Bromage, H., Tempst, P., et al. (2007). The retinoblastoma binding protein RBP2 is an H3K4 demethylase. *Cell* 128, 889–900. doi: 10.1016/j.cell.2007.02.013

Kouzarides, T. (2007). Chromatin modifications and their function. *Cell* 128, 693–705. doi: 10.1016/j.cell.2007.02.005

Kriaucionis, S., and Heintz, N. (2009). The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. *Science* 324, 929–930. doi: 10.1126/science.1169786

Lagger, G., Doetzlhofer, A., Schuettengruber, B., Haidweger, E., Simboeck, E., Tischler, J., et al. (2003). The tumor suppressor p53 and histone deacetylase 1 are antagonistic regulators of the cyclin-dependent kinase inhibitor p21/WAF1/CIP1 gene. *Mol. Cell Biol.* 23, 2669–2679. doi: 10.1128/MCB.23.8.2669-2679.2003

Lee, J. W., Lee, Y. C., Na, S. Y., Jung, D. J., and Lee, S. K. (2001). Transcriptional coregulators of the nuclear receptor superfamily: coactivators and corepressors. *Cell Mol. Life Sci.* 58, 289–297. doi: 10.1007/PL00000856

Lewin, E., Garfia, B., Recio, F. L., Rodriguez, M., and Olgaard, K. (2002). Persistent downregulation of calcium-sensing receptor mRNA in rat parathyroids when severe secondary hyperparathyroidism is reversed by an isogenic kidney transplantation. *J. Am. Soc. Nephrol.* 13, 2110–2116. doi: 10.1097/01.ASN.0000024439.38838.03

Liau, J. Y., Liao, S. L., Hsiao, C. H., Lin, M. C., Chang, H. C., and Kuo, K. T. (2014). Hypermethylation of the CDKN2A gene promoter is a frequent epigenetic change in periocular sebaceous carcinoma and is associated with younger patient age. *Hum Pathol.* 45, 533–539. doi: 10.1016/j.humpath.2013.10.019

Lin, C. H., Li, B., Swanson, S., Zhang, Y., Florens, L., Washburn, M. P., et al. (2008). Heterochromatin protein 1a stimulates histone H3 lysine 36 demethylation by the Drosophila KDM4A demethylase. *Mol. Cell* 32, 696–706. doi: 10.1016/j.molcel.2008.11.008

Lin, Y. W., Ren, L. L., Xiong, H., Du, W., Yu, Y. N., Sun, T. T., et al. (2013). Role of STAT3 and vitamin D receptor in EZH2-mediated invasion of human colorectal cancer. *J. Pathol.* 230, 277–290. doi: 10.1002/path.4179

Longo, V. D., and Kennedy, B. K. (2006). Sirtuins in aging and age-related disease. *Cell* 126, 257–268. doi: 10.1016/j.cell.2006.07.002

Lopes, N., Carvalho, J., Duraes, C., Sousa, B., Gomes, M., Costa, J. L., et al. (2012). 1Alpha,25-dihydroxyvitamin D3 induces de novo E-cadherin expression in triple-negative breast cancer cells by CDH1-promoter demethylation. *Anticancer Res.* 32, 249–257.

Lu, P. J., Sundquist, K., Baeckstrom, D., Poulsom, R., Hanby, A., Meier-Ewert, S., et al. (1999). A novel gene (PLU-1) containing highly conserved putative DNA/chromatin binding motifs is specifically up-regulated in breast cancer. *J. Biol. Chem.* 274, 15633–15645. doi: 10.1074/jbc.274.22.15633

Luo, W., Karpf, A. R., Deeb, K. K., Muindi, J. R., Morrison, C. D., Johnson, C. S., et al. (2010). Epigenetic regulation of vitamin D 24-hydroxylase/CYP24A1 in human prostate cancer. *Cancer Res.* 70, 5953–5962. doi: 10.1158/0008-5472.CAN-10-0617

Marik, R., Fackler, M., Gabrielson, E., Zeiger, M. A., Sukumar, S., Stearns, V., et al. (2010). DNA methylation-related vitamin D receptor insensitivity in breast cancer. *Cancer Biol. Ther.* 10, 44–53. doi: 10.4161/cbt.10.1.11994

Marks, P., Rifkind, R. A., Richon, V. M., Breslow, R., Miller, T., and Kelly, W. K. (2001). Histone deacetylases and cancer: causes and therapies. *Nat. Rev. Cancer* 1, 194–202. doi: 10.1038/35106079

Meissner, A., Mikkelsen, T. S., Gu, H., Wernig, M., Hanna, J., Sivachenko, A., et al. (2008). Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature* 454, 766–770. doi: 10.1038/nature07107

Metzger, E., Wissmann, M., Yin, N., Müller, J. M., Schneider, R., Peters, A. H., et al. (2005). LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. *Nature* 437, 436–439. doi: 10.1038/nature04020

Meyer, M. B., Benkusky, N. A., and Pike, J. W. (2013). 1,25-Dihydroxyvitamin D3 induced histone profiles guide discovery of VDR action sites. *J. Steroid Biochem. Mol. Biol.* doi: 10.1016/j.jsbmb.2013.09.005. [Epub ahead of print].

Miller, C. W., Morosetti, R., Campbell, M. J., Mendoza, S., and Koeffler, H. P. (1997). Integrity of the 1,25-dihydroxyvitamin D3 receptor in bone, lung, and other cancers. *Mol. Carcinog.* 19, 254–257. doi: 10.1002/(SICI)1098-2744(199708)19:4<254::AID-MC6>3.0.CO;2-C

Mosammaparast, N., and Shi, Y. (2010). Reversal of histone methylation: biochemical and molecular mechanisms of histone demethylases. *Annu. Rev. Biochem.* 79, 155–179. doi: 10.1146/annurev.biochem.78.070907.103946

Mottet, D., Pirotte, S., Lamour, V., Hagedorn, M., Javerzat, S., Bikfalvi, A., et al. (2009). HDAC4 represses p21(WAF1/Cip1) expression in human cancer cells through a Sp1-dependent, p53-independent mechanism. *Oncogene* 28, 243–256. doi: 10.1038/onc.2008.371

Murayama, A., Kim, M. S., Yanagisawa, J., Takeyama, K., and Kato, S. (2004). Transrepression by a liganded nuclear receptor via a bHLH activator through co-regulator switching. *EMBO J.* 23, 1598–1608. doi: 10.1038/sj.emboj.7600157

Nagpal, S., Na, S., and Rathnachalam, R. (2005). Noncalcemic actions of vitamin D receptor ligands. *Endocr. Rev.* 26, 662–687. doi: 10.1210/er.2004-0002

Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N., et al. (1998). Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* 393, 386–389. doi: 10.1038/30764

Norman, A. W., and Bouillon, R. (2010). Vitamin D nutritional policy needs a vision for the future. *Exp. Biol. Med. (Maywood)* 235, 1034–1045. doi: 10.1258/ebm.2010.010014

Novakovic, B., Sibson, M., Ng, H. K., Manuelpillai, U., Rakyan, V., Down, T., et al. (2009). Placenta-specific methylation of the vitamin D 24-hydroxylase gene: implications for feedback autoregulation of active vitamin D levels at the fetomaternal interface. *J. Biol. Chem.* 284, 14838–14848. doi: 10.1074/jbc.M809542200

Ohyama, Y., Kusada, T., Yamasaki, T., and Ide, H. (2002). Extensive methylation of CpG island of CYP24 gene in osteoblastic ROS17/2.8 cells. *Nucleic Acids Res. Suppl.* 249–250. doi: 10.1093/nass/2.1.249

Okano, M., Bell, D. W., Haber, D. A., and Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 99, 247–257. doi: 10.1016/S0092-8674(00)81656-6

Okano, M., Xie, S., and Li, E. (1998). Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat. Genet.* 19, 219–220. doi: 10.1038/890

Padi, S. K., Zhang, Q., Rustum, Y. M., Morrison, C., and Guo, B. (2013). MicroRNA-627 mediates the epigenetic mechanisms of vitamin D to suppress proliferation of human colorectal cancer cells and growth of xenograft tumors in mice. *Gastroenterology* 145, 437–446. doi: 10.1053/j.gastro.2013.04.012

Pan, L., Matloob, A. F., Du, J., Pan, H., Dong, Z., Zhao, J., et al. (2010). Vitamin D stimulates apoptosis in gastric cancer cells in synergy with trichostatin A /sodium butyrate-induced and 5-aza-2′-deoxycytidine-induced PTEN upregulation. *FEBS J.* 277, 989–999. doi: 10.1111/j.1742-4658.2009.07542.x

Parbin, S., Kar, S., Shilpi, A., Sengupta, D., Deb, M., Rath, S. K., et al. (2014). Histone deacetylases: a saga of perturbed acetylation homeostasis in cancer. *J. Histochem. Cytochem.* 62, 11–33. doi: 10.1369/0022155413506582

Pedersen, J. I., Shobaki, H. H., Holmberg, I., Bergseth, S., and Björkhem, I. (1983). 25-Hydroxyvitamin D3-24-hydroxylase in rat kidney mitochondria. *J. Biol. Chem.* 258, 742–746.

Pedersen, M. T., and Helin, K. (2010). Histone demethylases in development and disease. *Trends Cell Biol.* 20, 662–671. doi: 10.1016/j.tcb.2010.08.011

Pendas-Franco, N., Aguilera, O., Pereira, F., Gonzalez-Sancho, J. M., and Munoz, A. (2008). Vitamin D and Wnt/beta-catenin pathway in colon cancer: role and regulation of DICKKOPF genes. *Anticancer Res.* 28, 2613–2623.

Pereira, F., Barbachano, A., Silva, J., Bonilla, F., Campbell, M. J., Munoz, A., et al. (2011). KDM6B/JMJD3 histone demethylase is induced by vitamin D and modulates its effects in colon cancer cells. *Hum. Mol. Genet.* 20, 4655–4665. doi: 10.1093/hmg/ddr399

Pereira, F., Barbachano, A., Singh, P. K., Campbell, M. J., Munoz, A., and Larriba, M. J. (2012). Vitamin D has wide regulatory effects on histone demethylase genes. *Cell Cycle* 11, 1081–1089. doi: 10.4161/cc.11.6.19508

Perillo, B., Ombra, M. N., Bertoni, A., Cuozzo, C., Sacchetti, S., Sasso, A., et al. (2008). DNA oxidation as triggered by H3K9me2 demethylation drives estrogen-induced gene expression. *Science* 319, 202–206. doi: 10.1126/science.1147674

Pfau, R., Tzatsos, A., Kampranis, S. C., Serebrennikova, O. B., Bear, S. E., and Tsichlis, P. N. (2008). Members of a family of JmjC domain-containing oncoproteins immortalize embryonic fibroblasts via a JmjC domain-dependent process. *Proc. Natl. Acad. Sci. U.SA.* 105, 1907–1912. doi: 10.1073/pnas.0711865105

Pike, J. W., and Meyer, M. B. (2013). Fundamentals of vitamin D hormone-regulated gene expression. *J. Steroid Biochem. Mol. Biol.* doi: 10.1016/j.jsbmb.2013.11.004. [Epub ahead of print].

Pike, J. W., Meyer, M. B., and Bishop, K. A. (2012). Regulation of target gene expression by the vitamin D receptor - an update on mechanisms. *Rev. Endocr. Metab. Disord.* 13, 45–55. doi: 10.1007/s11154-011-9198-9

Polly, P., Herdick, M., Moehren, U., Baniahmad, A., Heinzel, T., and Carlberg, C. (2000). VDR-Alien: a novel, DNA-selective vitamin D(3) receptor-corepressor partnership. *FASEB J.* 14, 1455–1463. doi: 10.1096/fj.14.10.1455

Rashid, S. F., Moore, J. S., Walker, E., Driver, P. M., Engel, J., Edwards, C. E., et al. (2001). Synergistic growth inhibition of prostate cancer cells by 1 alpha,25 Dihydroxyvitamin D(3) and its 19-nor-hexafluoride analogs in combination with either sodium butyrate or trichostatin A. *Oncogene* 20, 1860–1872. doi: 10.1038/sj.onc.1204269

Rawson, J. B., Sun, Z., Dicks, E., Daftary, D., Parfrey, P. S., Green, R. C., et al. (2012). Vitamin D intake is negatively associated with promoter methylation of the Wnt antagonist gene DKK1 in a large group of colorectal cancer patients. *Nutr. Cancer* 64, 919–928. doi: 10.1080/01635581.2012.711418

Reik, W., and Lewis, A. (2005). Co-evolution of X-chromosome inactivation and imprinting in mammals. *Nat. Rev. Genet.* 6, 403–410. doi: 10.1038/nrg1602

Rhee, I., Bachman, K. E., Park, B. H., Jair, K. W., Yen, R. W., Schuebel, K. E., et al. (2002). DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature* 416, 552–556. doi: 10.1038/416552a

Rhee, I., Jair, K. W., Yen, R. W., Lengauer, C., Herman, J. G., Kinzler, K. W., et al. (2000). CpG methylation is maintained in human cancer cells lacking DNMT1. *Nature* 404, 1003–1007. doi: 10.1038/35010000

Robertson, K. D. (2005). DNA methylation and human disease. *Nat. Rev. Genet.* 6, 597–610. doi: 10.1038/nrg1655

Saito, Y., Liang, G., Egger, G., Friedman, J. M., Chuang, J. C., Coetzee, G. A., et al. (2006). Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. *Cancer Cell* 9, 435–443. doi: 10.1016/j.ccr.2006.04.020

Sakai, T., Toguchida, J., Ohtani, N., Yandell, D. W., Rapaport, J. M., and Dryja, T. P. (1991). Allele-specific hypermethylation of the retinoblastoma tumor-suppressor gene. *Am. J. Hum. Genet.* 48, 880–888.

Sakaki, T., Sawada, N., Komai, K., Shiozawa, S., Yamada, S., Yamamoto, K., et al. (2000). Dual metabolic pathway of 25-hydroxyvitamin D3 catalyzed by human CYP24. *Eur. J. Biochem.* 267, 6158–6165. doi: 10.1046/j.1432-1327.2000.01680.x

Saramäki, A., Diermeier, S., Kellner, R., Laitinen, H., Vaïsänen, S., and Carlberg, C. (2009). Cyclical chromatin looping and transcription factor association on the regulatory regions of the p21 (CDKN1A) gene in response to 1alpha,25-dihydroxyvitamin D3. *J. Biol. Chem.* 284, 8073–8082. doi: 10.1074/jbc.M808090200

Schulte, J. H., Lim, S., Schramm, A., Friedrichs, N., Koster, J., Versteeg, R., et al. (2009). Lysine-specific demethylase 1 is strongly expressed in poorly differentiated neuroblastoma: implications for therapy. *Cancer Res.* 69, 2065–2071. doi: 10.1158/0008-5472.CAN-08-1735

Schwer, B., and Verdin, E. (2008). Conserved metabolic regulatory functions of sirtuins. *Cell Metab.* 7, 104–112. doi: 10.1016/j.cmet.2007.11.006

Seuter, S., Pehkonen, P., Heikkinen, S., and Carlberg, C. (2013). Dynamics of 1α,25-dihydroxyvitamin D3-dependent chromatin accessibility of early vitamin D receptor target genes. *Biochim. Biophys. Acta* 1829, 1266–1275. doi: 10.1016/j.bbagrm.2013.10.003

Shi, H., Guo, J., Duff, D. J., Rahmatpanah, F., Chitima-Matsiga, R., Al-Kuhlani, M., et al. (2007). Discovery of novel epigenetic markers in non-Hodgkin's lymphoma. *Carcinogenesis* 28, 60–70. doi: 10.1093/carcin/bgl092

Shi, H., Yan, P. S., Chen, C. M., Rahmatpanah, F., Lofton-Day, C., Caldwell, C. W., et al. (2002). Expressed CpG island sequence tag microarray for dual screening of DNA hypermethylation and gene silencing in cancer cells. *Cancer Res.* 62, 3214–3220.

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J. R., Cole, P. A., et al. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* 119, 941–953. doi: 10.1016/j.cell.2004.12.012

Shi, Y., and Whetstine, J. R. (2007). Dynamic regulation of histone lysine methylation by demethylases. *Mol. Cell* 25, 1–14. doi: 10.1016/j.molcel.2006.12.010

Singh, P. K., Doig, C. L., Dhiman, V. K., Turner, B. M., Smiraglia, D. J., and Campbell, M. J. (2013). Epigenetic distortion to VDR transcriptional regulation in prostate cancer cells. *J. Steroid Biochem. Mol. Biol.* 136, 258–263. doi: 10.1016/j.jsbmb.2012.10.002

Sulaiman, L., Juhlin, C. C., Nilsson, I. L., Fotouhi, O., Larsson, C., and Hashemi, J. (2013). Global and gene-specific promoter methylation analysis in primary hyperparathyroidism. *Epigenetics* 8, 646–655. doi: 10.4161/epi.24823

Tahiliani, M., Mei, P., Fang, R., Leonor, T., Rutenberg, M., Shimizu, F., et al. (2007). The histone H3K4 demethylase SMCX links REST target genes to X-linked mental retardation. *Nature* 447, 601–605. doi: 10.1038/nature05823

Takeyama, K., and Kato, S. (2011). The vitamin D3 1alpha-hydroxylase gene and its regulation by active vitamin D3. *Biosci. Biotechnol. Biochem.* 75, 208–213. doi: 10.1271/bbb.100684

Tan, J., Lu, J., Huang, W., Dong, Z., Kong, C., Li, L., et al. (2009). Genome-wide analysis of histone H3 lysine9 modifications in human mesenchymal stem cell osteogenic differentiation. *PLoS ONE* 4:e6792. doi: 10.1371/journal.pone.0006792

Tapp, H. S., Commane, D. M., Bradburn, D. M., Arasaradnam, R., Mathers, J. C., Johnson, I. T., et al. (2013). Nutritional factors and gender influence age-related DNA methylation in the human rectal mucosa. *Aging Cell* 12, 148–155. doi: 10.1111/acel.12030

Ting, A. H., McGarvey, K. M., and Baylin, S. B. (2006). The cancer epigenome–components and functional correlates. *Genes Dev.* 20, 3215–3231. doi: 10.1101/gad.1464906

Tsai, H. C., and Baylin, S. B. (2011). Cancer epigenetics: linking basic biology to clinical medicine. *Cell Res.* 21, 502–517. doi: 10.1038/cr.2011.24

Tsukada, Y., Fang, J., Erdjument-Bromage, H., Warren, M. E., Borchers, C. H., Tempst, P., et al. (2006). Histone demethylation by a family of JmjC domain-containing proteins. *Nature* 439, 811–816. doi: 10.1038/nature04433

Vanoirbeek, E., Eelen, G., Verlinden, L., Carmeliet, G., Mathieu, C., Bouillon, R., et al. (2014). PDLIM2 expression is driven by vitamin D and is involved in the pro-adhesion, and anti-migration and -invasion activity of vitamin D. *Oncogene* 33, 1904–1911. doi: 10.1038/onc.2013.123

Varshney, S., Bhadada, S. K., Sachdeva, N., Arya, A. K., Saikia, U. N., Behera, A., et al. (2013). Methylation status of the CpG islands in vitamin D and calcium-sensing receptor gene promoters does not explain the reduced gene expressions in parathyroid adenomas. *J. Clin. Endocrinol. Metab.* 98, E1631–E1635. doi: 10.1210/jc.2013-1699

Von Essen, M. R., Kongsbak, M., Schjerling, P., Olgaard, K., Odum, N., and Geisler, C. (2010). Vitamin D controls T cell antigen receptor signaling and activation of human T cells. *Nat. Immunol.* 11, 344–349. doi: 10.1038/ni.1851

Wang, Y., and Leung, F. C. (2004). An evaluation of new criteria for CpG islands in the human genome as gene markers. *Bioinformatics* 20, 1170–1177. doi: 10.1093/bioinformatics/bth059

Whetstine, J. R., Nottke, A., Lan, F., Huarte, M., Smolikov, S., Chen, Z., et al. (2006). Reversal of histone lysine trimethylation by the JMJD2 family of histone demethylases. *Cell* 125, 467–481. doi: 10.1016/j.cell.2006.03.028

Williams, K., Christensen, J., Pedersen, M. T., Johansen, J. V., Cloos, P. A., Rappsilber, J., et al. (2011). TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. *Nature* 473, 343–348. doi: 10.1038/nature10066

Wilson, A. J., Byun, D. S., Popova, N., Murray, L. B., L'Italien, K., Sowa, Y., et al. (2006). Histone deacetylase 3 (HDAC3) and other class I HDACs regulate colon cell maturation and p21 expression and are deregulated in human colon cancer. *J. Biol. Chem.* 281, 13548–13558. doi: 10.1074/jbc.M510023200

Wjst, M., Heimbeck, I., Kutschke, D., and Pukelsheim, K. (2010). Epigenetic regulation of vitamin D converting enzymes. *J. Steroid Biochem. Mol. Biol.* 121, 80–83. doi: 10.1016/j.jsbmb.2010.03.056

Xiang, Y., Zhu, Z., Han, G., Ye, X., Xu, B., Peng, Z., et al. (2007). JARID1B is a histone H3 lysine 4 demethylase up-regulated in prostate cancer. *Proc. Natl. Acad. Sci. U.S.A.* 104, 19226–19231. doi: 10.1073/pnas.0700735104

Yamaguchi, S., Hong, K., Liu, R., Shen, L., Inoue, A., Diep, D., et al. (2012). Tet1 controls meiosis by regulating meiotic gene expression. *Nature* 492, 443–447. doi: 10.1038/nature11709

Yamane, K., Tateishi, K., Klose, R. J., Fang, J., Fabrizio, L. A., Erdjument-Bromage, H., et al. (2007). PLU-1 is an H3K4 demethylase involved in transcriptional repression and breast cancer cell proliferation. *Mol. Cell* 25, 801–812. doi: 10.1016/j.molcel.2007.03.001

Yamshchikov, A. V., Desai, N. S., Blumberg, H. M., Ziegler, T. R., and Tangpricha, V. (2009). Vitamin D for treatment and prevention of infectious diseases: a systematic review of randomized controlled trials. *Endocr. Pract.* 15, 438–449. doi: 10.4158/EP09101.ORR

Yoneda, T., Aya, S., and Sakuda, M. (1984). Sodium butyrate (SB) augments the effects of 1,25 dihydroxyvitamin D3 (1,25(OH)2D3) on neoplastic and osteoblastic phenotype in clonal rat osteosarcoma cells. *Biochem. Biophys. Res. Commun.* 121, 796–801. doi: 10.1016/0006-291X(84)90748-4

Zhang, X., Nicosia, S. V., and Bai, W. (2006). Vitamin D receptor is a novel drug target for ovarian cancer treatment. *Curr. Cancer Drug. Targets* 6, 229–244. doi: 10.2174/156800906776842939

Zhang, Z., Yamashita, H., Toyama, T., Sugiura, H., Ando, Y., Mita, K., et al. (2005). Quantitation of HDAC1 mRNA expression in invasive carcinoma of the breast*. *Breast Cancer Res. Treat.* 94, 11–16. doi: 10.1007/s10549-005-6001-1

Zhu, H., Wang, X., Shi, H., Su, S., Harshfield, G. A., Gutin, B., et al. (2013). A genome-wide methylation study of severe vitamin D deficiency in African American adolescents. *J. Pediatr.* 162, 1004–1009.e1001. doi: 10.1016/j.jpeds.2012.10.059

Zhu, P., Martin, E., Mengwasser, J., Schlag, P., Janssen, K. P., and Göttlicher, M. (2004). Induction of HDAC2 expression upon loss of APC in colorectal tumorigenesis. *Cancer Cell* 5, 455–463. doi: 10.1016/S1535-6108(04)00114-X

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 09 February 2014; paper pending published: 07 March 2014; accepted: 09 April 2014; published online: 29 April 2014.

*Citation: Fetahu IS, Höbaus J and Kállay E (2014) Vitamin D and the epigenome. Front. Physiol. **5**:164. doi: 10.3389/fphys.2014.00164*

This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 03 June 2014
doi: 10.3389/fphys.2014.00166

Tumor suppression in skin and other tissues via cross-talk between vitamin D- and p53-signaling

Jörg Reichrath[1]*, Sandra Reichrath[1], Kristina Heyne[2], Thomas Vogt[1] and Klaus Roemer[2]

[1] *Department of Dermatology, The Saarland University Hospital, Homburg (Saar), Germany*
[2] *José Carreras Centre and Internal Medicine I, University of Saarland Medical Centre, Homburg (Saar), Germany*

Edited by:
Carsten Carlberg, University of Eastern Finland, Finland

Reviewed by:
Alberto Muñoz, Consejo Superior de Investigaciones Científicas, Spain
Moray J. Campbell, Roswell Park Cancer Institute, USA

****Correspondence:***
Jörg Reichrath, Department of Dermatology, The Saarland University Hospital, Kirrberger Str., 66421 Homburg, Germany
e-mail: joerg.reichrath@uks.eu

P53 and its family members have been implicated in the direct regulation of the vitamin D receptor (VDR). Vitamin D- and p53-signaling pathways have a significant impact on spontaneous or carcinogen-induced malignant transformation of cells, with VDR and p53 representing important tumor suppressors. VDR and the p53/p63/p73 proteins all function typically as receptors or sensors that turn into transcriptional regulators upon stimulus, with the main difference being that the nuclear VDR is activated as a transcription factor after binding its naturally occurring ligand 1,25-dihydroxyvitamin D with high affinity while the p53 family of transcription factors, mostly in the nucleoplasm, responds to a large number of alterations in cell homeostasis commonly referred to as stress. An increasing body of evidence now convincingly demonstrates a cross-talk between vitamin D- and p53-signaling that occurs at different levels, has genome-wide implications and that should be of high importance for many malignancies, including non-melanoma skin cancer. One interaction involves the ability of p53 to increase skin pigmentation via POMC derivatives including alpha-MSH and ACTH. Pigmentation protects the skin against UV-induced DNA damage and skin carcinogenesis, yet on the other hand reduces cutaneous synthesis of vitamin D. A second level of interaction may be through the ability of 1,25-dihydroxyvitamin D to increase the survival of skin cells after UV irradiation. UV irradiation-surviving cells show significant reductions in thymine dimers in the presence of 1,25-dihydroxyvitamin D that are associated with increased nuclear p53 protein expression, and significantly reduced NO products. A third level of interaction is documented by the ability of vitamin D compounds to regulate the expression of the *murine double minute 2* (*MDM2*) gene in dependence of the presence of wild-type p53. MDM2 has a well-established role as a key negative regulator of p53 activity. Finally, p53 and family members have been implicated in the direct regulation of VDR. This overview summarizes some of the implications of the cross-talk between vitamin D- and p53-signaling for carcinogenesis in the skin and other tissues.

Keywords: vitamin D, vitamin D receptor, p53, MDM2, cancer

SKIN, VDR AND THE VITAMIN D ENDOCRINE SYSTEM/REGULATORY NETWORK: AN INTRODUCTION

The skin is the largest organ of the human body, consisting of several compartments that are named epidermis, dermis and subcutis. The epidermis contains a basal layer (stratum basale), that is composed of self-renewing cells (keratinocytes) with limited proliferative capacity (transient amplifying cells), of stem cells with high proliferative capacity that need to be preserved, and of outwardly migrating layers (stratum spinosum, stratum granulosum and stratum corneum) of mostly resting keratinocytes at different stages of differentiation. The skin is one of the key tissues of the human body's vitamin D regulatory network (VDRN) (Lehmann et al., 2004; Holick, 2007; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). First, vitamin D is synthesized in the skin (**Figure 1**) by the action of solar or artificial ultraviolet B (UVB) radiation (under most living conditions, only a small amount of vitamin D is taken up by the diet) (Lehmann et al., 2004; Holick, 2007). Second, the skin represents an important target tissue for 1,25-dihydroxyvitamin D, the biologically active natural vitamin D metabolite, that is formed from vitamin D by consecutive hydroxylations at position 25 in the liver (mediated by CYP2R1 and by CYP27A1, resulting in 25-hydroxyvitamin D) and at position 1 in the kidneys and in many other tissues (mediated by CYP27B1) (Lehmann et al., 2004; Holick, 2007; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). 1,25-Dihydroxyvitamin D represents a potent seco-steroid hormone that regulates, via various independent mechanisms growth, many non-malignant and malignant cell types, including human keratinocytes (Lehmann et al., 2004; Holick, 2007; Haussler et al., 2012; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). It exerts its effects through the binding with high affinity to a corresponding receptor (VDR) that is located intranuclear in target tissues (Lehmann et al., 2004; Holick, 2007; Haussler et al., 2012; Reichrath and Reichrath,

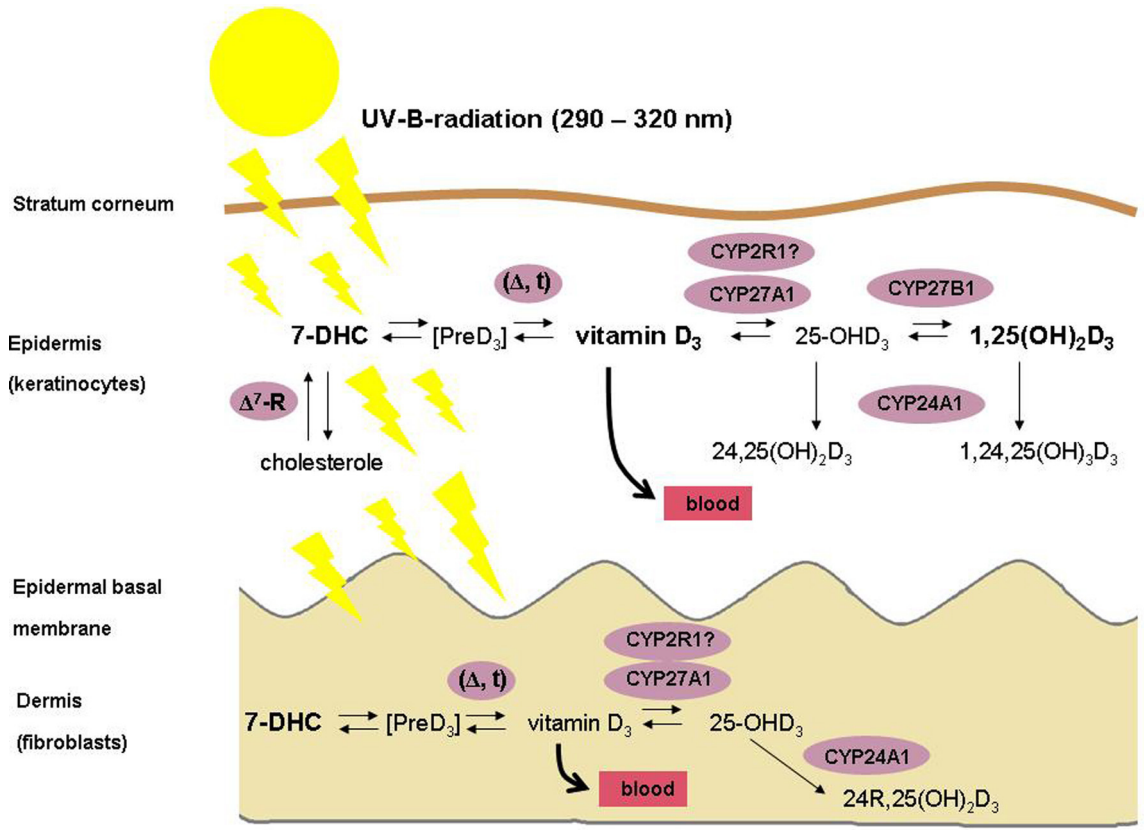

FIGURE 1 | Schematic illustration of the vitamin D metabolism in human skin. Please note that, in contrast to fibroblasts, keratinocytes possess the enzymatic machinery for the complete synthesis of 1,25-dihydroxyvitamin D from 7-dehydrocholesterole (7-DHC).

2012; Mason and Reichrath, 2013). VDR is a member of a superfamily named trans-acting transcriptional regulatory factors, that also contains the retinoic acid receptors (RARs) and the retinoid-X receptors (RXRs), as well as the thyroid and steroid hormone receptors (Lehmann et al., 2004; Holick, 2007; Haussler et al., 2012; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). The farnesoid-X receptor (FXR) that controls bile acid metabolism and the pregnane-X receptor (PXR) which regulates xenobiotic detoxification are evolutionarily most closely related to the VDR (Haussler et al., 2012). Binding of its ligand 1,25-dihydroxyvitamin D induces conformational changes of the VDR that lead to heterodimerization with RXR and to zinc finger-mediated binding to vitamin D response elements (VDREs) that are located in regulatory regions of target genes (Haussler et al., 2012). As a result, vitamin D activity in a particular cell largely depends upon sufficient expression of VDR and RXR proteins, the autocrine/paracrine production or the endocrine delivery of adequate amounts of the 1,25-dihydroxyvitamin D ligand, and of cell-specific programming of gene transcription to regulate expression of distinctive genes that encode proteins that finally exert the vitamin D effect (Haussler et al., 2012). cDNA microarray analyses of mRNAs and other investigations suggest that as many as 500–1000 coding genes may be regulated by the VDR, which may contact up to ∼8000 loci in the human genome (Haussler et al., 2012). 1,25-Dihydroxyvitamin D-mediated transcriptional regulation of many genes involved in cellular growth and differentiation has been demonstrated, including the genes for ß$_3$-integrin, fibronectin, and cell cycle regulatory proteins such as p21/WAF-1 (CDKN1A) (Lehmann et al., 2004; Holick, 2007; Haussler et al., 2012). Like most other skin cells, keratinocytes express VDR (Lehmann et al., 2004; Holick, 2007); in these cells, 1,25-dihydroxyvitamin D, blocks proliferation and promotes differentiation *in vitro* (Lehmann et al., 2004; Holick, 2007; Haussler et al., 2012). Interestingly, it has been reported that the combination of 1,25-dihydroxyvitamin D and the retinoic acid metabolite isotretinoin is efficient in the therapy of precancerous skin lesions and of non-melanoma skin cancer (cutaneous squamous and basal cell carcinomas) (Tang et al., 2012a,b; Mason and Reichrath, 2013). Moreover, it has been demonstrated that VDR ablation promotes chemically induced skin carcinogenesis (Tang et al., 2012a,b; Mason and Reichrath, 2013).

VDR-signaling comprises much more than just ligand/receptor triggering of gene expression. Distinct and fine-tuned responses indicate a complex regulation of this signaling pathway. Moreover, chemical and other modifications of the VDR signaling pathway govern such important parameters as intracellular trafficking, duration of interaction between the receptor and

cofactors, the receptor and ligand, as well as turnover and stability of other relevant proteins (Haussler et al., 2012). Not least, regulation of VDR target genes is controlled by stability and turnover of relevant microRNAs and RNAs (Haussler et al., 2012).

Depending on cell type and context, both VDR- and p53-signaling regulate many cellular functions that are of relevance for cancer development, including proliferation, differentiation, apoptosis and cell survival (Murray-Zmijewski et al., 2006; Holick, 2007; McKeon and Melino, 2007; Vousden and Lane, 2007; Vousden and Prives, 2009; Haussler et al., 2012; Mason and Reichrath, 2013). Consequently, vitamin D- and p53-signaling pathways have a significant impact on spontaneous or carcinogen-induced malignant transformation of cells, with vitamin D receptor (VDR) and p53 representing important tumor suppressors (Murray-Zmijewski et al., 2006; Holick, 2007; McKeon and Melino, 2007; Vousden and Lane, 2007; Vousden and Prives, 2009; Haussler et al., 2012; Mason and Reichrath, 2013). Mutations in genes encoding for proteins of the p53 pathway represent a hallmark of many if not all types of cancer (Vousden and Lane, 2007; Vousden and Prives, 2009). Low serum 25(OH)D concentrations and distinct polymorphisms (SNPs) in the *VDR* gene and other vitamin D-related genes, on the other hand, are associated with an increased incidence and an unfavorable outcome of various malignancies (Mason and Reichrath, 2013). The VDR and the p53 family all function typically as activatable transcriptional regulators, with the main difference being that VDR is activated after binding its naturally occurring ligand 1,25-dihydroxyvitamin D ($1,25(OH)_2D$ or calcitriol) with high affinity (Haussler et al., 2012) while p53, mostly in the nucleoplasm, responds to a large and still growing number of alterations in cell homeostasis (Murray-Zmijewski et al., 2006; McKeon and Melino, 2007; Vousden and Lane, 2007; Vousden and Prives, 2009). In any event is the result of such activation—manifested by conformational changes and heterodimerization with retinoid X receptor (RXR) of VDR and by chemical modifications and oligomerization of the p53 family—the direct contact with regulatory DNA. In both pathways the cell type- and context-dependent recruitment of nuclear co-regulators entails the stimulation or repression of a very large number, typically hundreds, of genes (Lin et al., 2005; Holick, 2007; Perez and Pietenpol, 2007; Sbisa et al., 2007; Riley et al., 2008; Haussler et al., 2012). Several of these code themselves for transcriptional regulators, adding a further level of complexity to the networks. It is obvious that transcription factor pathways may cross-talk, for instance, through the sharing of target genes or co-regulators, and through the engagement in interdependent regulatory loops. Indeed, all of these mechanisms, plus several others, seem to have been realized in the cross-talk of VDR and the p53 family (**Table 1**).

Intriguingly, both pathways are critically involved in cellular processes that are important for carcinogenesis such as cell differentiation/proliferation, in the regulation of stem cell maintenance, and in cell homeostasis. While VDR controls proliferation/differentiation of many cell types (Holick, 2007; Haussler et al., 2012), some members and isoforms of the p53 family, and in particular p53 itself, reduce the stem cell potential and stimulate differentiation (Lin et al., 2005). Interestingly, on the side of the p53 family, all three members (p53/p63/p73) can be expressed as truncated isoforms capable of counteracting their siblings' transactivating effects (Murray-Zmijewski et al., 2006). Not too surprising, VDR and p53 have been linked to many malignancies, including non-melanoma skin cancer (Mason and Reichrath, 2013). The present review aims at providing an overview on this interesting signaling network, with a focus on non-melanoma skin cancer. Future genome-wide analyses of the target genes will shed further light on the interaction of these pleiotropic regulators. Before the cross-talk is discussed, the p53 pathway shall be briefly outlined.

THE p53 FAMILY OF TRANSCRIPTIONAL REGULATORS

p53, p63, and p73 (the p53 family hereafter) are homotetrameric transcriptional regulators that bind to very closely related DNA motifs, consisting of two consecutive 10-mers (half-sites),

Table 1 | Overview of the cross talk between vitamin D- and p53 signaling.

Crooss talk/interaction	Mechanism	References
p53 modulates cutaneous vitamin D synthesis	p53 upregulates skin pigmentation via POMC derivatives including alpha-MSH and ACTH.	Rev. in Yamaguchi and Hearing, 2009 (77)
p53 regulates VDR expression	p53 and its family members have been implicated in the direct regulation of the VDR.	Maruyama et al., 2006 (97)
	p53 protein binds to highly conserved intron-sequences of the VDR gene.	Kommagani et al., 2007 (96)
1,25-D increases survival of UV-irradiated skin cells	Significant reductions in thymine dimers in the presence of 1,25-D in UV-irradiated, surviving cells that are associated with increased nuclear p53 protein expression.	Gupta et al., 2007 (78)
1,25-D regulates MDM2 expression	Dependent on presence of wild type p53, 1,25-D regulates expression of the MDM2 gene.	Chen et al., 2013 (79)
	Interaction between VDRE and p53Res in the P2 promoter region of the MDM2 gene.	

1,25-D, 1,25-dihydroxyvitamin D; MDM2, murine double minute 2.

preferentially spaced by no more than zero to 2 base pairs, with the consensus r,r,r,C,A/T,T,G,y,c,y (p53); r,r,r,C,G,T,G,y,y,y; t/a,a/t,a,C,A/T,T,G,t,t/a,t or r,r,r,C,A/G,T/A,G,y,y,y (p63), and a/c/g,g/a,g,C,A,T,G,c/t,c,c/t (p73; r = purines; y = pyrimidines) (Osada et al., 2005; Riley et al., 2008; Brandt et al., 2009; Roemer, 2012). They share a large number of target sequences, as expected given the high degree of homology within the DNA binding domains, among the consensus sequence motifs, and the degeneracy of the individual binding sites (Roemer, 2012). It is therefore perhaps no surprise that the regulation of a defined sequence by any of these transcription factors is controlled at several levels including posttranslational modifications and protein/protein interactions. Many of these are specific for each family paralog. Moreover, the binding of p53, p63, and p73 to DNA is affected by additional parameters such as the number of the half-sites, their orientation, their position relative to the target gene, and their overlap with binding sites for other transcription factors. Finally, differential recruitment of co-activator/co-repressor complexes to promoters has been documented. These may be coded, for example, by specific spacings between the 10-mers of the DNA binding motifs (Riley et al., 2008). Epigenetic CpG methylation does not seem to affect the binding to DNA significantly (Brandt et al., 2009; Roemer, 2012).

The p53 family proteins display a modular organization that is quite different from that of the VDR (see above). Typically, an N-terminal transactivation domain (TD), a central DNA binding domain (DBD) and C-terminal regulatory and protein/protein interaction domain is present. The DBDs are the most highly conserved regions among the paralogs, sharing ∼60% homology (Murray-Zmijewski et al., 2006; Roemer, 2012). In addition to the full-length variants, a large number of isoforms exists, owing to transcription initiation from internal promoters, alternative splicing and the use of alternative translation initiation sites; however, in most cases the DBD is maintained. More than 10 different isoforms of p53, more than six of p63 and at least 29 of p73 are currently known (Murray-Zmijewski et al., 2006; Hollstein and Hainaut, 2010; Roemer, 2012). In most cases, their biological functions are not fully understood. Furthermore, an arsenal of posttranslational modifications that are in part interdependent has evolved. These include phosphorylations, acetylations, ubiquitinations, sumoylations, neddylations, methylations, glycosylations, and oxidation/reduction, and they control the proteins' abundance, DNA binding, level of activity as transcription factor, cross-talk with other proteins and subcellular localization (Murray-Zmijewski et al., 2006; Toledo and Wahl, 2006; Kruse and Gu, 2009). All these levels of regulation are best studied in p53 and have revealed an enormous degree of complexity (Vousden and Prives, 2009; Roemer, 2012) which may be exemplified by the chemical modification "code" that seems to regulate p53 function in a tissue-specific manner through the sequential build-up of poly-phosphorylation patterns at different sites and that may even be accompanied by other chemical changes such as acetylations (Gu and Roeder, 1997; Ashcroft et al., 2000; Wang et al., 2004; Roemer, 2012). At the level of the cell, p53 is involved in the regulation of the cell cycle (Wang and El-Deiry, 2006), cell survival and autophagy, DNA repair, respiration, oxidative stress protection, glucose metabolism, cell adhesion/motility, the cytoskeleton and endo/exosome compartments, and of angiogenesis. At the organismal level, p53 is involved in tumor suppression and maintenance of genome stability, and the control of stem cell compartments, female fertility and ageing (Riley et al., 2008; Roemer, 2012).

The complexity of the regulation of the p53 family is further highlighted by the antagonistic partnership between p53 and its central negative regulators, the E3 ubiquitin ligases murine double minute 2 (MDM2) and MDM4 (Roemer, 2012). Activation of p53 is almost always involving inhibition of MDM2/4. For example, acetylation of p53 and MDM2 overcomes the inhibitory ubiquitination of p53 by MDM2 through the blocking of MDM2 enzymatic function, the dissociation of the p53/MDM2 complex and thereby, the stimulation of p53's interaction with DNA as well as the recruitment of co-activators (Gu and Roeder, 1997; Ashcroft et al., 2000; Wang et al., 2004; Roemer, 2012). MDM4 is not functioning as a ubiquitin ligase for p53 but can inhibit p53's transcriptional activity and modulate the p53/MDM2 interaction (Toledo and Wahl, 2006; Roemer, 2012). Since p53 can transactivate the *MDM2* gene, a negative feedback loop is formed (Toledo and Wahl, 2006; Kruse and Gu, 2009; Roemer, 2012). Such a loop is also established with p63 and p73; however, MDM2 inhibits these transcription factors at promoters yet in contrast to p53 cannot ubiquitin-mark them for degradation (Murray-Zmijewski et al., 2006; Roemer, 2012).

Cell context determines the respective function of individual p53 family members. In the absence of extra stress, i.e., under physiological background stress induced, for instance, by reactive oxygen species (ROS) as a by-product of respiration, p53, p63, and p73 primarily control cell fate, differentiation and development. Intriguingly, these functions seem to be predominantly mediated by the DNA binding competent yet transactivation impaired delta-N isoforms of the proteins (ΔNp63, ΔNp73). In cells or tissue that have been challenged by further stresses, as for example by overt ROS production, radiation, hypoxia, hypo/hyperthermia, metabolite shortages and imbalances, oncogene dysregulation, and virus/bacterial/parasite infections, the p53 family members, and in particular p53 itself, seem to mainly control repair, proliferative capacity and survival. Central to these functions are the transactivation-proficient isoforms (p53, TAp63, TAp73). Since many of the damaging stresses can support cell transformation, the p53 family, and here again, mostly p53 itself, thus act as tumor suppressors by inducing cell cycle arrest, temporary or permanent senescence, apoptosis, and differentiation (Vousden and Lane, 2007; Levine and Oren, 2009; Vousden and Prives, 2009; Roemer, 2012). Conversely, lack of proper function of p53 or p73, or overproduction of dominant-negative ΔNp63, support tumor formation in animals and humans. Along the same line, tumor-inducing viruses encode proteins that target p53, and perhaps there is no tumor in which the p53 pathway itself plus all ascending/descending pathways are fully intact (Gatza et al., 2007; Vousden and Lane, 2007; Feng et al., 2008; Hu et al., 2008; Roemer, 2012).

Like p53, p63, and p73 can act as tumor suppressors, although this does not seem to be their primary functions (Murray-Zmijewski et al., 2006; Roemer, 2012). For example, p63 and p73 are not as frequently mutated in human cancers as is p53.

Rather, p63 is often overproduced in tumors (Park et al., 2000), which seems to contradict its function as a tumor suppressor, yet as mentioned above, this is often due to p63 isoforms that lack the transactivation domain but not their ability to bind to DNA and that thereby may act dominant-negatively (Candi et al., 2007; Roemer, 2012). In contrast and as expected from a tumor suppressor, transactivation competent p63 (TAp63) can sensitize cells to apoptosis in response to DNA damaging stress (Gressner et al., 2005; Roemer, 2012). Moreover, some p63± mice are tumor-prone, and the resulting tumors often display loss of the remaining wild-type allele (Flores, 2007; Roemer, 2012). Mice with a specific deficiency for TAp73 show genomic instability and a higher tumor incidence (Tomasini et al., 2008; Roemer, 2012). Furthermore, p63 and p73 seem to have p53-independent roles in DNA repair (Talos et al., 2007; Lin et al., 2009; Roemer, 2012).

p63 and p73, but not p53, are crucial for embryonic development in all organisms examined so far (Danilova et al., 2008; Roemer, 2012). Although p53-deficiency interferes with mesoderm/endoderm fate determination in the frog *Xenopus* (Wallingford et al., 1997; Roemer, 2012), this condition fails to generate significant early phenotypes in mice or humans (Choi and Donehower, 1999; Varley, 2003; Roemer, 2012). However, at a more subtle level, and since p53 can induce stem cell differentiation, lack of p53 function may cause unrestrained stem cell proliferation (Gil-Perotin et al., 2006; Dumble et al., 2007; Roemer, 2012). Other more subtle functions of p53 are in mitochondrial respiration and glucose metabolism (Matoba et al., 2006; Roemer, 2012). Overactivity of p53, by contrast, does indeed entail immediate and dramatic consequences in the development of the early mouse embryo—its apoptotic loss—and one of the most striking functions of the p53 inhibitors MDM2 and MDM4 during embryonic development is the prevention of this consequence (Marine et al., 2006; Roemer, 2012). Later in embryonal development, for example during neurogenesis, the DNA binding-proficient yet transactivation-incompetent dominant-negative isoform of p73, ΔNp73, may serve as a p53 and p63 restraining factor to inhibit apoptosis (Jacobs et al., 2004; Roemer, 2012). p53 and possibly p63, but most importantly p73, help shape the nervous system during life, perhaps primarily by controlling apoptosis (Jacobs et al., 2005; Miller and Kaplan, 2007; Roemer, 2012).

p63 function during development is critical for epithelial stem cell maintenance (Yi et al., 2008; Roemer, 2012), squamous epithelial differentiation and skin renewal (Truong et al., 2006; Koster et al., 2007; Mikkola, 2007; Roemer, 2012). The ΔNp63 isoform acts mainly through controlling the expansion of epithelial layers while TAp63 seems to support differentiation, and it functions as the guardian of the female germ line by inducing apoptosis in damaged resting oocytes (Suh et al., 2006; Roemer, 2012). p73 deficiency in mice results in neuronal and olfactory dysfunctions as well as in chronic infection and inflammation (Murray-Zmijewski et al., 2006; Roemer, 2012). Collectively, the stem cell/differentiated cell decision seem to be regulated in part by the balance between the ΔNp63/TAp63 antagonists in the skin and - in an analogous manner - by the balance between the ΔNp73/TAp73 antagonists in the developing nervous and immune systems. Thus, p73 may be to neuronal development and homeostasis what p63 is to the development and homeostasis of the skin (De Laurenzi et al., 2000; Jacobs et al., 2004; Roemer, 2012). Since the pleiotropic VDR is important for tumor suppression as well as skin development and differentiation, it is perhaps no surprise that both transcriptional regulator pathways talk to each other.

CROSS-TALK BETWEEN THE VDR AND THE p53 FAMILY IN CANCER

An increasing body of evidence points to a cross-talk between vitamin D- and p53-signaling occuring at different levels that might be of great importance for many malignancies, including non-melanoma skin cancer (**Table 1**). Both p53 and VDR act as tumor suppressors in several tissues, including skin. Much of the tumor suppressor function in the skin may be mediated through the interaction of the VDR and p53 pathways—either by mutual activation or inhibition. What is known about this interaction, in particular in non-melanoma skin cancer? DNA damage induced by solar or artificial ultraviolet (UV) radiation represents the most important environmental risk factor for carcinogenesis of cutaneous squamous cell carcinoma (SCC) (Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). The predominant types of DNA damage which are directly induced by UV are promutagenic pyrimidine dimers (Wikonkal and Brash, 1999; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). Thymine-thymine dimers, which represent cys-syn cyclobutane pyrimidine dimers (CPDs), are the major form of pyrimidine dimers that are identified in human skin following UV-B exposure. In contrast, other types of DNA damage, including cytosine-cytosine, thymine-cytosine bipyrimidines, and 6–4 photoproducts are less frequently detected (Douki et al., 2000; Cooke et al., 2003; Courdavault et al., 2004a; Mouret et al., 2006; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). CPDs are caused via disruption of the 5–6 double bonds in two adjacent pyrimidine bases, thereby inducing atypical covalent binding which connects the 2 bases by a stable ring configuration, resulting in a bipyrimidine (Ravanat et al., 2001; Pattison and Davies, 2006; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). In general, CPDs are induced by UV-B (290–320 nm) (Reichrath and Reichrath, 2012; Mason and Reichrath, 2013), although the production of thymine dimer by UV-A (320–400 nm) wavelengths below 330 nm has also been reported (Applegate et al., 1999; Jiang et al., 1999; Rochette et al., 2003; Courdavault et al., 2004b; Mouret et al., 2006; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). UV-radiation induces gene mutations which may result in photocarcinogenesis (Hart et al., 1977; Sutherland et al., 1985; Brash et al., 1991; Agar et al., 2004; Besaratinia et al., 2008; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). Moreover, it has been shown that DNA damage initiates and promotes cellular mechanisms which block the detection and elimination of transformed cells by immune surveillance (Applegate et al., 1989; Kripke et al., 1992; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). UV radiation induces different forms of DNA lesions which are generated either photochemically and directly or indirectly by UV activation of several photoreceptors which have the capacity to modulate the cellular redox equilibrium, thereby generating reactive oxygen species (ROS) (Reichrath and Reichrath, 2012;

Mason and Reichrath, 2013). ROS induced cellular damage then leads both to oxidative DNA damage, and to lipid peroxidation (Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). Additionally, UV-induced increased levels of nitric oxide synthase (Deliconstantinos et al., 1995; Bruch-Gerharz et al., 1998; Cals-Grierson and Ormerod, 2004; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013) cause excess levels of nitric oxide (NO) (Paunel et al., 2005; Mowbray et al., 2009; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). It has been shown that these pathophysiologically increased concentrations of NO and ROS combine to generate genotoxic NO products, including peroxynitrite, which modify the bases and the sugar-phosphate scaffold of DNA via nitrosative and oxidative damage (Reichrath and Reichrath, 2012; Mason and Reichrath, 2013) Both UV- and ROS-induced damages activate p53.

UV-irradiation induces p53 to stimulate skin pigmentation via POMC derivatives including alpha-MSH and ACTH (Yamaguchi and Hearing, 2009), thereby protecting the skin against further UV-induced DNA damage and skin carcinogenesis. However, this reduces cutaneous synthesis of vitamin D. This may be important because, on a second level, 1,25-dihydroxyvitamin D can increase the survival of UV-irradiated keratinocytes and prevent further accumulation of DNA damage in these surviving skin cells (Gupta et al., 2007). Following UVR, the survival of 1,25-dihydroxyvitamin D-treated skin cells was significantly higher as compared to vehicle-treated cells ($P < 0.01$) (Gupta et al., 2007). In that study, UVR-surviving and 1,25-dihydroxyvitamin D-treated keratinocytes showed significantly reduced levels of thymine dimers (TDs) as compared to vehicle-treated cells ($P < 0.001$) (Gupta et al., 2007). Following UVR, nuclear p53 protein levels were elevated and, notably, became elevated to significantly higher levels in the presence of 1,25-dihydroxyvitamin D ($P < 0.01$). In contrast, NO derivatives were significantly decreased in 1,25-dihydroxyvitamin D-treated keratinocytes ($P < 0.05$) (Gupta et al., 2007). Both the elevated levels of nuclear p53 protein and the decreased production of nitric oxide products were suggested to be responsible at least in part for the decrease in TDs seen with 1,25-dihydroxyvitamin D-treatment after UVR (Gupta et al., 2007). In addition, a reduction in the number of TDs ($P < 0.05$) and in sunburn cells ($P < 0.01$) were demonstrated in skin sections from Skh:hr1 mice that had been treated with 1,25-dihydroxyvitamin D, at 24 h after UVR (Gupta et al., 2007). It was concluded that the vitamin D system in skin, in combination with p53, may represent an intrinsic mechanism that protects against UV damage (Gupta et al., 2007).

As a further molecular level of interaction it has been demonstrated that vitamin D compounds can regulate the expression of the MDM2 gene in dependence of p53 (Chen et al., 2013). As outlined above, *MDM2* represents a p53-inducible gene that encodes an E3 ubiquitin ligase mainly responsible for the degradation of p53 by the 26S proteasome (Roemer, 2012). A major function of MDM2 is its role as a key negative feed-back regulator of p53 activity (Bond et al., 2004), p53 activates *MDM2* expression via binding to corresponding p53 response elements (p53REs) in the P2 promoter of the *MDM2* gene. The increase of MDM2 protein then leads to its binding to p53 (primarily at the N-terminal 1–52 residues), which causes degradation of p53 or inhibition of p53 activity as a transcription factor (Chen et al., 1993). Vitamin D may thus prevent a lasting and overt p53 response in the face of damage and may thereby protect reparable cells from p53-induced apoptosis. However, MDM2 also exerts many p53-independent functions, and interacts with a broad variety of other proteins (including insulin like growth factor receptor, androgen receptor, estrogen receptor, Numb, RB, p300, etc.) that are of importance for various cellular functions including proliferation/differentiation, cell fate determination, and signaling (Ries et al., 2000; Ganguli and Wasylyk, 2003; Steinman et al., 2004; Zhang and Zhang, 2005; Lengner et al., 2006; Araki et al., 2010). VDR may activate the *MDM2* gene directly, through a VDR-response element in the promoter P2 of the *MDM2* gene (Barak et al., 1994; Zauberman et al., 1995; Roemer, 2012; Chen et al., 2013) However, even with this direct binding to *MDM2* sequences, p53 seems to be required for the induction of *MDM2* expression by VDR (Chen et al., 2013). Perhaps this is reflecting a cross-talk between the VDR and p53 bound to DNA since the p53 response element in the *MDM2* gene is also located in promoter P2.

VDR and p53 family members act first and foremost as transcription factors, and accordingly, much of the highly complex cross-regulation between them seems to happen at this level. For example, members of the p53 family including ΔNp63 can modulate VDR signaling through competitive binding to various VDR target genes including *p21Waf1/Cip (CDKN1A)*. Multiple VDREs have recently been identified in the promoter region of the *CDKN1A* gene, which is a transcriptional target of p53 and encodes a powerful blocker of the cell cycle in G1 and G2 phases (Saramaki et al., 2006). Notably, like with the *MDM2* gene, the VDR and p53 binding sites are in close proximity in the *CDKN1A* promoter (Saramaki et al., 2006). A much more detailed and unbiased (pathway-independent) genome-wide analysis of the VDR:p53 family interactions bound to DNA is in need. To this end, it shall be useful to employ chromatin immunoprecipitations (ChIPs) with either ChIPping with p53-antibodies first and re-ChIPping with VDR antibodies, or *vice versa*. Also, knock-in studies in which VDR response elements or p53 response elements in close proximity are deleted, should provide a deeper insight into the cooperativity or antagonism between these important tumor-suppressing transcription factors.

In the skin, p53/p63 play an important regulatory role in the maintenance of the stem cells as well as in the establishment of the differentiation gradient. In the undifferentiated proliferating basal layer of the skin, the dominant negatively acting, because DNA binding but transactivation impaired, ΔNp63 rules. Most effects exerted by the transactivation-competent p53 family members are inhibited by it (Yang et al., 1998; Lee and Kimelman, 2002; Roemer, 2012) In addition, ΔNp63 may inhibit differentiation by the blunting of VDR signaling through binding to various VDR target genes including *CDKN1A* (Pellegrini et al., 2001; Westfall et al., 2003; Roemer, 2012) TAp63 that is minor to ΔNp63 in this proliferating compartment of the skin, may become more dominant as ΔNp63 levels decrease in the course of differentiation (Nylander et al., 2002; Roemer, 2012).

Finally, p53 family members may regulate VDR directly (Maruyama et al., 2006; Kommagani et al., 2007). In a

comparative genomics investigation in the human and mouse genome designed to locate conserved p53 binding sites, the VDR and 31 other genes were newly described as putative p53 targets. Reverse transcription-PCR and real-time PCR confirmed the responsiveness of these genes to p53 in human cancer cell lines (Maruyama et al., 2006). It was shown that VDR is upregulated by p53 and some other members of the p53 family. For example, an isoform of p63 (p63 gamma) can specifically upregulate VDR by directly associating with the VDR promoter *in vivo* (Kommagani et al., 2007). Moreover, ChIP analysis demonstrated that wild-type p53 protein binds to a conserved intronic site of the VDR gene (Maruyama et al., 2006). Conversely, transfection of VDR into cells resulted in upregulation of several p53 target genes and in growth suppression of colorectal cancer cells. In addition and as discussed above, p53 stimulated several VDR target genes in a 1,25-dihydroxyvitamin D-dependent manner, that is, in cooperation with VDR (Maruyama et al., 2006). Future, whole transcriptome-including studies will identify new transcripts that are initiated by VDR and p53 in concert.

An increasing body of evidence highlights the relevance of the cross-talk between VDR- and p53-signaling under various physiological and pathophysiological conditions. One study identified the VDRE as overrepresented in promoter sequences bound by mutated p53 (mutp53), and showed that mutp53 can interact functionally and physically with VDR (Stambolsky et al., 2010). In that investigation, mutp53 was recruited to VDR target genes and modulated their expression (increasing transactivation or relieving repression) (Stambolsky et al., 2010). Moreover, mutp53 promoted the nuclear accumulation of VDR and converted 1,25-dihydroxyvitamin D into an anti-apoptotic agent (Stambolsky et al., 2010).

Several investigations analyzed the cross-talk between VDR- and p53-signaling in bone. It was demonstrated that hepatocyte growth factor (HGF) and 1,25-dihydroxyvitamin D act together to induce osteogenic differentiation of human bone marrow stem cells (hMSC) potentially through elevating p53 (Chen et al., 2012). The authors of this study hypothesized that the combination of HGF and 1,25-dihydroxyvitamin D can promote hMSC differentiation by up-regulation of 1,25-dihydroxyvitamin D and/or VDR expression to booster cell response(s) to 1,25-dihydroxyvitamin D. In line with this hypothesis, it was shown that HGF up-regulated gene expression of VDR and p63 and that p63 gene knockdown by siRNA eliminated the effects of HGF on VDR gene expression (Chen et al., 2012). Moreover, recent findings suggest that the cross-talk of VDR and p53 may directly target the human osteocalcin gene and positively affect osteocalcin gene expression. It was reported that osteocalcin promoter activity can be up-regulated both by exogenous and endogenous p53 and downregulated by p53-specific siRNA (Chen et al., 2011). It was shown that p53 binds to the human osteocalcin promoter *in vitro* and a p53 response element within the osteocalcin promoter region was identified (Chen et al., 2011). In this investigation, an additive effect of p53 and VDR on the regulation of osteocalcin promoter activity was observed. Another study demonstrated that p73 acts as an upstream regulator of 1,25-dihydroxyvitamin D-induced osteoblastic differentiation (Kommagani et al., 2010). In that investigation, silencing p73 significantly decreased 1,25-dihydroxyvitamin D-mediated osteoblastic differentiation; although p73 induced by DNA-damage increased 1,25-dihydroxyvitamin D-mediated differentiation of osteosarcoma cells (Kommagani et al., 2010).

CONCLUSIONS AND PERSPECTIVES

VDR and the members of the p53 family are activatable transcriptional regulators that are at the hub of a common molecular network to control cell homeostasis, proliferation, differentiation and survival, and that way, act as classical tumor suppressors. Malfunction of either entails elevated susceptibility to transformation. A tissue archetypical of this interaction is the skin. Here, VDR as well as p53 and p63 control differentiation and the maintenance of the stem cell compartment. Accordingly, damage to skin cells such as induced by UV irradiation, or transformation of skin cells, typically come with characteristic responses of these proteins in the form of specific gene expression profiles to control differentiation, proliferation and survival. Since both classes of nuclear transcription factors act primarily through the regulation of genes, it is thus no great surprise to find functional interaction at several levels. Future, ChIP- and transcriptome analysis-based genome-wide studies of the DNA sequences that are targeted individually or by both factors together should provide us with new insights into this fascinating network.

REFERENCES

Agar, N. S., Halliday, G. M., Barnetson, R. S., Ananthaswamy, H. N., Wheeler, M., and Jones, A. M. (2004). The basal layer in human squamous tumours harbors more UVA than UVB fingerprint mutations: a role for UVA in human skin carcinogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 101, 4954–4959. doi: 10.1073/pnas.0401141101

Applegate, L. A., Ley, R. D., Alcalay, J., and Kripke, M. L. (1989). Identification of the molecular target for the suppression of contact hypersensitivity by ultraviolet radiation. *J. Exp. Med.* 170, 1117–1131. doi: 10.1084/jem.170.4.1117

Applegate, L. A., Scaletta, C., Panizzon, R., Niggli, H., and Frenk, E. (1999). *In vivo* induction of pyrimidine dimers in human skin by UVA radiation: initiation of cell damage and/or intercellular communication? *Int. J. Mol. Med.* 3, 467–472. doi: 10.3892/ijmm.3.5.467

Araki, S., Eitel, J. A., Batuello, C. N., Bijangi-Vishehsaraei, K., Xie, X. J., Danielpour, D., et al. (2010). TGF-beta1-induced expression of human Mdm2 correlates with late-stage metastatic breast cancer. *J. Clin. Invest.* 120, 290–302. doi: 10.1172/JCI39194

Ashcroft, M., Taya, Y., and Vousden, K. H. (2000). Stress signals utilize multiple pathways to stabilize p53. *Mol. Cell. Biol.* 20, 3224–3233. doi: 10.1128/MCB.20.9.3224-3233.2000

Barak, Y., Gottlieb, E., Juven-Gershon, T., and Oren, M. (1994). Regulation of mdm2 expression by p53: alternative promoters produce transcripts with nonidentical translation potential. *Genes Dev.* 8, 1739–1749. doi: 10.1101/gad.8.15.1739

Besaratinia, A., Kim, S. I., and Pfeifer, G. P. (2008). Rapid repair of UVA-induced oxidized purines and persistence of UVB-induced dipyrimidine lesions determine the mutagenicity of sunlight in mouse cells. *FASEB J.* 22, 2379–2392. doi: 10.1096/fj.07-105437

Bond, G. L., Hu, W., Bond, E. E., Robins, H., Lutzker, S. G., Arva, N. C., et al. (2004). A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumour suppressor pathway and accelerates tumour formation in humans. *Cell* 119, 591–602. doi: 10.1016/j.cell.2004.11.022

Brandt, T., Petrovich, M., Joerger, A. C., and Veprintsev, D. B. (2009). Conservation of DNA-binding specificity and oligomerisation properties within the p53 family. *BMC Genomics* 10:628. doi: 10.1186/1471-2164-10-628

Brash, D. E., Rudolph, J. A., Simon, J. A., Lin, A., McKenna, G. J., Baden, H. P., et al. (1991). A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proc. Natl. Acad. Sci. U.S.A.* 88, 10124–10128. doi: 10.1073/pnas.88.22.10124

Bruch-Gerharz, D., Ruzicka, T., and Kolb-Bachofen, V. (1998). Nitric oxide in human skin: current status and future prospects. *J. Invest. Dermatol.* 110, 1–7. doi: 10.1046/j.1523-1747.1998.00084.x

Cals-Grierson, M. M., and Ormerod, A. D. (2004). Nitric oxide function in the skin. *Nitric Oxide* 10, 179–193. doi: 10.1016/j.niox.2004.04.005

Candi, E., Dinsdale, D., Rufini, A., Salomoni, P., Knight, R. A., Mueller, M., et al. (2007). TAp63 and DeltaNp63 in cancer and epidermal development. *Cell Cycle* 6, 274–285. doi: 10.4161/cc.6.3.3797

Chen, H., Hays, E., Liboon, J., Neely, C., Kolman, K., and Chandar, N. (2011). Osteocalcin gene expression is regulated by wild-type p53. *Calcif. Tissue Int.* 89, 411–418. doi: 10.1007/s00223-011-9533-x

Chen, H., Reed, G., Guardia, J., Lakhan, S., Couture, O., Hays, E., et al. (2013). Vitamin D directly regulates Mdm2 gene expression in osteoblasts. *Biochem. Biophys. Res. Commun.* 430, 370–374. doi: 10.1016/j.bbrc.2012.11.003

Chen, J., Marechal, V., and Levine, A. J. (1993). Mapping of the p53 and mdm-2 interaction domains. *Mol. Cell. Biol.* 13, 4107–4114.

Chen, K., Aenlle, K. K., Curtis, K. M., Roos, B. A., and Howard, G. A. (2012). Hepatocyte growth factor (HGF) and 1,25-dihydroxyvitamin D together stimulate human bone marrow-derived stem cells toward the osteogenic phenotype by HGF-induced up-regulation of VDR. *Bone* 51, 69–77. doi: 10.1016/j.bone.2012.04.002

Choi, J., and Donehower, L. A. (1999). p53 in embryonic development: maintaining a fine balance. *Cell. Mol. Life Sci.* 55, 38–47. doi: 10.1007/s000180050268

Cooke, M. S., Podmore, I. D., Mistry, N., Evans, M. D., Herbert, K. E., Griffiths, H. R. et al. (2003). Immunochemical detection of UV-induced DNA damage and repair. *J Immunol Methods.* 280, 125–133. doi: 10.1016/S0022-1759(03)00269-2

Courdavault, S., Baudouin, C., Charveron, M., Favier, A., Cadet, J., and Douki, T. (2004a). Larger yield of cyclobutane dimers than 8-oxo-7,8-dihydroguanine in the DNA of UVA-irradiated human skin cells. *Mutat. Res.* 556, 135–142. doi: 10.1016/j.mrfmmm.2004.07.011

Courdavault, S., Baudouin, C., Sauvaigo, S., Mouret, S., Candéias, S., Charveron, M., et al. (2004b). Unrepaired cyclobutane pyrimidine dimers do not prevent proliferation of UV-B-irradiated cultured human fibroblasts. *Photochem. Photobiol.* 79, 145–151. doi: 10.1562/0031-8655(2004)079<0145:UCPDDN>2.0.CO;2

Danilova, N., Sakamoto, K. M., and Lin, S. (2008). p53 family in development. *Mech. Dev.* 125, 919–931. doi: 10.1016/j.mod.2008.09.003

De Laurenzi, V., Raschella, G., Barcaroli, D., Annicchiarico-Petruzzelli, M., Ranalli M, Catani, M. V., et al. (2000). Induction of neuronal differentiation by p73 in a neuroblastoma cell line. *J. Biol. Chem.* 275, 15226–15231. doi: 10.1074/jbc.275.20.15226

Deliconstantinos, G., Villiotou, V., and Stravrides, J. C. (1995). Release by ultraviolet B (u.v.B) radiation of nitric oxide (NO) from human keratinocytes: a potential role for nitric oxide in erythema production. *Br. J. Pharmacol.* 114, 1257–1265. doi: 10.1111/j.1476-5381.1995.tb13341.x

Douki, T., Court, M., Sauvaigo, S., Odin, F., and Cadet, J. (2000). Formation of the main UV-induced thymine dimeric lesions within isolated and cellular DNA as measured by high performance liquid chromatography-tandem mass spectrometry. *J. Biol. Chem.* 275, 11678–11685. doi: 10.1074/jbc.275.16.11678

Dumble, M., Moore, L., Chambers, S. M., Geiger, H., Van Zant, G., Goodell, M. A., et al. (2007). The impact of altered p53 dosage on hematopoietic stem cell dynamics during aging. *Blood* 109, 1736–1742. doi: 10.1182/blood-2006-03-010413

Feng, Z., Hu, W., Rajagopal, G., and Levine, A. J. (2008). The tumour suppressor p53: cancer and aging. *Cell Cycle* 7, 842–847. doi: 10.4161/cc.7.7.5657

Flores, E. R. (2007). The roles of p63 in cancer. *Cell Cycle* 6, 300–304. doi: 10.4161/cc.6.3.3793

Ganguli, G., and Wasylyk, B. (2003). p53-independent functions of MDM2. *Mol. Cancer Res.* 1, 1027–1035.

Gatza, C., Moore, L., Dumble, M., and Donehower, L. A. (2007). Tumour suppressor dosage regulates stem cell dynamics during aging. *Cell Cycle* 6, 52–55. doi: 10.4161/cc.6.1.3667

Gil-Perotin, S., Marin-Husstege, M., Li, J., Soriano-Navarro, M., Zindy, F., Roussel, M. F., et al. (2006). Loss of p53 induces changes in the behavior of subventricular zone cells: implication for the genesis of glial tumours. *J. Neurosci.* 26, 1107–1116. doi: 10.1523/JNEUROSCI.3970-05.2006

Gressner, O., Schilling, T., Lorenz, K., Schulze Schleithoff, E., Koch, A., Schulze-Bergkamen, H., et al. (2005). TAp63alpha induces apoptosis by activating signaling via death receptors and mitochondria. *EMBO J.* 24, 2458–2471. doi: 10.1038/sj.emboj.7600708

Gu, W., and Roeder, R. G. (1997). Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* 90, 595–606. doi: 10.1016/S0092-8674(00)80521-8

Gupta, R., Dixon, K. M., Deo, S. S., Holliday, C. J., Slater, M., Halliday, G. M., et al. (2007). Photoprotection by 1,25 dihydroxyvitamin D3 is associated with an increase in p53 and a decrease in nitric oxide products. *J. Invest. Dermatol.* 27, 707–715. doi: 10.1038/sj.jid.5700597

Hart, R. W., Setlow, R. B., and Woodhead, A. D. (1977). Evidence that pyrimidine dimers in DNA can give rise to tumours. *Proc. Natl. Acad. Sci. U.S.A.* 74, 5574–5578. doi: 10.1073/pnas.74.12.5574

Haussler, M. R., Whitfield, G. K., Kaneko, I., Haussler, C. A., Hsieh, D., Hsieh, J. C., et al. (2012). Molecular mechanisms of vitamin D action. *Calcif. Tissue Int.* 92, 77–98. doi: 10.1007/s00223-012-9619-0

Holick, M. F. (2007). Vitamin D deficiency. *N. Engl. J. Med.* 357, 266–281. doi: 10.1056/NEJMra070553

Hollstein, M., and Hainaut, P. (2010). Massively regulated genes: the example of TP53. *J. Pathol.* 220, 164–173. doi: 10.1002/path.2637

Hu, W., Feng, Z., Atwal, G. S., and Levine, A. J. (2008). p53: a new player in reproduction. *Cell Cycle* 7, 848–852. doi: 10.4161/cc.7.7.5658

Jacobs, W. B., Govoni, G., Ho, D., Atwal, J. K., Barnabe-Heider, F., Keyes, W. M., et al. (2005). p63 is an essential proapoptotic protein during neural development. *Neuron*. 48, 743–756. doi: 10.1016/j.neuron.2005.10.027

Jacobs, W. B., Walsh, G. S., and Miller, F. D. (2004). Neuronal survival and p73/p63/p53: a family affair. *Neuroscientist* 10, 443–455. doi: 10.1177/1073858404263456

Jiang, W., Ananthaswamy, H. N., Muller, H. K., and Kripke, M. L. (1999). p53 protects against skin cancer induction by UV-B radiation. *Oncogene* 18, 4247–4253. doi: 10.1038/sj.onc.1202789

Kommagani, R., Payal, V., and Kadakia, M. P. (2007). Differential regulation of vitamin D receptor (VDR) by the p53 Family: p73-dependent induction of VDR upon DNA damage. *J. Biol. Chem.* 282, 29847–29854. doi: 10.1074/jbc.M703641200

Kommagani, R., Whitlatch, A., Leonard, M. K., and Kadakia, M. P. (2010). p73 is essential for vitamin D-mediated osteoblastic differentiation. *Cell Death Differ.* 17, 398–407. doi: 10.1038/cdd.2009.135

Koster, M. I., Dai, D., and Roop, D. R. (2007). Conflicting roles for p63 in skin development and carcinogenesis. *Cell Cycle* 6, 269–273. doi: 10.4161/cc.6.3.3792

Kripke, M. L., Cox, P. A., Alas, L. G., and Yarosh, D. B. (1992). Pyrimidine dimers in DNA initiate systemic immunosuppression in UV-irradiated mice. *Proc. Natl. Acad. Sci. U.S.A.* 89, 7516–7520. doi: 10.1073/pnas.89.16.7516

Kruse, J. P., and Gu, W. (2009). Modes of p53 regulation. *Cell* 137, 609–622. doi: 10.1016/j.cell.2009.04.050

Lee, H., and Kimelman, D. (2002). A dominant-negative form of p63 is required for epidermal proliferation in zebrafish. *Dev. Cell.* 2, 607–616. doi: 10.1016/S1534-5807(02)00166-1

Lehmann, B., Querings, K., and Reichrath, J. (2004). Vitamin D and skin: new aspects for dermatology. *Exp. Dermatol.* 13, 11–15. doi: 10.1111/j.1600-0625.2004.00257.x

Lengner, C. J., Steinman, H. A., Gagnon, J., Smith, T. W., Henderson, J. E., Kream, B. E., et al. (2006). Osteoblast differentiation and skeletal development are regulated by Mdm2-p53 signaling. *J. Cell Biol.* 172, 909–921. doi: 10.1083/jcb.200508130

Levine, A. J., and Oren, M. (2009). The first 30 years of p53: growing ever more complex. *Nat. Rev. Cancer*. 9, 749–758. doi: 10.1038/nrc2723

Lin, T., Chao, C., Saito, S., Mazur, S. J., Murphy, M. E., Appella, E., et al. (2005). p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. *Nat. Cell Biol.* 7, 165–171. doi: 10.1038/ncb1211

Lin, Y. L., Sengupta, S., Gurdziel, K., Bell, G. W., Jacks, T., and Flores, E. R. (2009). p63 and p73 transcriptionally regulate genes involved in DNA repair. *PLoS Genet*. 5:e1000680. doi: 10.1371/journal.pgen.1000680

Marine, J. C., Francoz, S., Maetens, M., Wahl, G., Toledo, F., and Lozano, G. (2006). Keeping p53 in check: essential and synergistic functions of Mdm2 and Mdm4. *Cell Death Differ.* 13, 927–934. doi: 10.1038/sj.cdd.4401912

Maruyama, R., Aoki, F., Toyota, M., Sasaki, Y., Akashi, H., Mita, H., et al. (2006). Comparative genome analysis identifies the vitamin D receptor gene as a direct target of p53-mediated transcriptional activation. *Cancer Res.* 66, 4574–4583. doi: 10.1158/0008-5472.CAN-05-2562

Mason, R. S., and Reichrath, J. (2013). Sunlight vitamin D and skin cancer. *Anticancer Agents Med. Chem.* 13, 83–97. doi: 10.2174/187152013804487272

Matoba, S., Kang, J. G., Patino, W. D., Wragg, A., Boehm, M., Gavrilova, O., et al. (2006). p53 regulates mitochondrial respiration. *Science* 312, 1650–1653. doi: 10.1126/science.1126863

McKeon, F., and Melino, G. (2007). Fog of war: the emerging p53 family. *Cell Cycle* 6, 229–232. doi: 10.4161/cc.6.3.3876

Mikkola, M. L. (2007). p63 in skin appendage development. *Cell Cycle* 6, 285–290. doi: 10.4161/cc.6.3.3798

Miller, F. D., and Kaplan, D. R. (2007). To die or not to die: neurons and p63. *Cell Cycle* 6, 312–317. doi: 10.4161/cc.6.3.3795

Mouret, S., Baudouin, C., Charveron, M., Favier, A., Cadet, J., and Douki, T. (2006). Cyclobutane pyrimidine dimers are predominant DNA lesions in whole human skin exposed to UVA radiation. *Proc. Natl. Acad. Sci. U.S.A.* 103, 13765–13770. doi: 10.1073/pnas.0604213103

Mowbray, M., McLintock, S., Weerakoon, R., Lomatschinsky, N., Jones, S., Rossi, A. G., et al. (2009). Enzyme-independent NO stores in human skin: quantification and influence of UV radiation. *J. Invest. Dermatol.* 129, 834–842. doi: 10.1038/jid.2008.296

Murray-Zmijewski, F., Lane, D. P., and Bourdon, J. C. (2006). p53/p63/p73 isoforms: an orchestra of isoforms to harmonise cell differentiation and response to stress. *Cell Death Differ.* 13, 962–972. doi: 10.1038/sj.cdd.4401914

Nylander, K., Vojtesek, B., Nenutil, R., Lindgren, B., Roos, G., Zhanxiang, W., et al. (2002). Differential expression of p63 isoforms in normal tissues and neoplastic cells. *J. Pathol.* 198, 417–427. doi: 10.1002/path.1231

Osada, M., Park, H. L., Nagakawa, Y., Yamashita, K., Fomenkov, A., Kim, M. S., et al. (2005). Differential recognition of response elements determines target gene specificity for p53 and p63. *Mol. Cell. Biol.* 25, 6077–6089. doi: 10.1128/MCB.25.14.6077-6089.2005

Park, B. J., Lee, S. J., Kim, J. I., Lee, S. J., Lee, C. H., Chang, S. G., et al. (2000). Frequent alteration of p63 expression in human primary bladder carcinomas. *Cancer Res.* 60, 3370–3374.

Pattison, D. I., and Davies, M. J. (2006). Actions of ultraviolet light on cellular structures. *EXS* 96, 131–157. doi: 10.1007/3-7643-7378-4_6

Paunel, A., Dejam, A., Thelen, S., Kirsch, M., Horstjann, M., Gharini, P., et al. (2005). UVA induces immediate and enzyme-independent nitric oxide formation in healthy human skin leading to NO-specific signalling. *J. Invest. Dermatol.* 125:A3.

Pellegrini, G., Dellambra, E., Golisano, O., Martinelli, E., Fantozzi, I., Bondanza, S., et al. (2001). p63 identifies keratinocyte stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 98, 3156–3161. doi: 10.1073/pnas.061032098

Perez, C. A., and Pietenpol, J. A. (2007). Transcriptional programs regulated by p63 in normal epithelium and tumours. *Cell Cycle* 6, 246–254. doi: 10.4161/cc.6.3.3801

Ravanat, J. L., Douki, T., and Cadet, J. (2001). Direct and indirect effects of UV radiation on DNA and its components. *J. Photochem. Photobiol. B.* 63, 88–102. doi: 10.1016/S1011-1344(01)00206-8

Reichrath, J., and Reichrath, S. (2012). Hope and challenge: the importance of ultraviolet (UV) radiation for cutaneous vitamin D synthesis and skin cancer. *Scand. J. Clin. Lab. Invest. Suppl.* 243, 112–119. doi: 10.3109/00365513.2012.682876

Ries, S., Biederer, C., Woods, D., Shifman, O., Shirasawa, S., Sasazuki, T., et al. (2000). Opposing effects of Ras on p53: transcriptional activation of mdm2 and induction of p19ARF. *Cell* 103, 321–330. doi: 10.1016/S0092-8674(00)00123-9

Riley, T., Sontag, E., Chen, P., and Levine, A. (2008). Transcriptional control of human p53-regulated genes. *Nat. Rev. Mol. Cell Biol.* 9, 402–412. doi: 10.1038/nrm2395

Rochette, P. J., Therrien, J., Drouin, R., Perdiz, D., Bastien, N., and Drobetsky, E. A., et al. (2003). UVA-induced cyclobutane pyrimidine dimers form predominantly at thymine-thymine dipyrimidines and correlate with the mutation spectrum in rodent cells. *Nucleic Acids Res.* 31, 2786–2794. doi: 10.1093/nar/gkg402

Roemer, K. (2012). Notch and the p53 clan of transcription factors. *Adv. Exp. Med. Biol.* 727, 223–240. doi: 10.1007/978-1-4614-0899-4_17

Saramaki, A., Banwell, C. M., Campbell, M. J., and Carlberg, C. (2006). Regulation of the human p21(waf1/cip1) gene promoter via multiple binding sites for p53 and the vitamin D3 receptor. *Nucleic Acids Res.* 34, 543–554. doi: 10.1093/nar/gkj460

Sbisa, E., Catalano, D., Grillo, G., Licciulli, F., Turi, A., Liuni, S., et al. (2007). p53FamTaG: a database resource of human p53, p63 and p73 direct target genes combining *in silico* prediction and microarray data. *BMC Bioinformatics* 8(Suppl. 1):S20. doi: 10.1186/1471-2105-8-S1-S20

Stambolsky, P., Tabach, Y., Fontemaggi, G., Weisz, L., Maor-Aloni, R., Siegfried, Z., et al. (2010). Modulation of the vitamin D3 response by cancer-associated mutant p53. *Cancer Cell.* 17, 273–285. doi: 10.1016/j.ccr.2009.11.025

Steinman, H. A., Burstein, E., Lengner, C., Gosselin, J., Pihan, G., Duckett, C. S., et al. (2004). An alternative splice form of Mdm2 induces p53-independent cell growth and tumourigenesis. *J. Biol. Chem.* 279, 4877–4886. doi: 10.1074/jbc.M305966200

Suh, E. K., Yang, A., Kettenbach, A., Bamberger, C., Michaelis, A. H., Zhu, Z., et al. (2006). p63 protects the female germ line during meiotic arrest. *Nature* 444, 624–628. doi: 10.1038/nature05337

Sutherland, B. M., Blackett, A. D., Feng, N. I., Freeman, S. E., Ogut, E. S., Gange, R. W., et al. (1985). Photoreactivation and other ultraviolet/visible light effects on DNA in human skin. *Ann. N.Y. Acad. Sci.* 453, 73–79. doi: 10.1111/j.1749-6632.1985.tb11799.x

Talos, F., Nemajerova, A., Flores, E. R., Petrenko, O., and Moll, U. M. (2007). p73 suppresses polyploidy and aneuploidy in the absence of functional p53. *Mol. Cell.* 27, 647–659. doi: 10.1016/j.molcel.2007.06.036

Tang, J. Y., Fu, T., Lau, C., Oh, D. H., Bikle, D. D., and Asgari, M. M. (2012a). Vitamin D in cutaneous carcinogenesis: Part I. *J. Am. Acad. Dermatol.* 67, 803.e1–803.e12. doi: 10.1016/j.jaad.2012.05.044

Tang, J. Y., Fu, T., Lau, C., Oh, D. H., Bikle, D. D., and Asgari, M. M. (2012b). Vitamin D in cutaneous carcinogenesis: Part II. *J. Am. Acad. Dermatol.* 67, 817.e1–817.e11. doi: 10.1016/j.jaad.2012.07.022

Toledo, F., and Wahl, G. M. (2006). Regulating the p53 pathway: *in vitro* hypotheses, *in vivo* veritas. *Nat. Rev. Cancer*. 6, 909–923. doi: 10.1038/nrc2012

Tomasini, R., Tsuchihara, K., Wilhelm, M., Fujitani, M., Rufini, A., Cheung, C. C., et al. (2008). TAp73 knockout shows genomic instability with infertility and tumour suppressor functions. *Genes Dev.* 22, 2677–2691. doi: 10.1101/gad.1695308

Truong, A. B., Kretz, M., Ridky, T. W., Kimmel, R., and Khavari, P. A. (2006). p63 regulates proliferation and differentiation of developmentally mature keratinocytes. *Genes Dev.* 20, 3185–3197. doi: 10.1101/gad.1463206

Varley, J. M. (2003). Germline TP53 mutations and Li-Fraumeni syndrome. *Hum. Mutat.* 21, 313–320. doi: 10.1002/humu.10185

Vousden, K. H., and Lane, D. P. (2007). p53 in health and disease. *Nat. Rev. Mol. Cell Biol.* 8, 275–283. doi: 10.1038/nrm2147

Vousden, K. H., and Prives, C. (2009). Blinded by the light: the growing complexity of p53. *Cell.* 137, 413–431. doi: 10.1016/j.cell.2009.04.037

Wallingford, J. B., Seufert, D. W., Virta, V. C., and Vize, P. D. (1997). p53 activity is essential for normal development in Xenopus. *Curr. Biol.* 7, 747–757. doi: 10.1016/S0960-9822(06)00333-2

Wang, S., and El-Deiry, W. S. (2006). p73 or p53 directly regulates human p53 transcription to maintain cell cycle checkpoints. *Cancer Res.* 66, 6982–6989. doi: 10.1158/0008-5472.CAN-06-0511

Wang, X., Taplick, J., Geva, N., and Oren, M. (2004). Inhibition of p53 degradation by Mdm2 acetylation. *FEBS Lett.* 561, 195–201. doi: 10.1016/S0014-5793(04)00168-1

Westfall, M. D., Mays, D. J., Sniezek, J. C., and Pietenpol, J. A. (2003). The Delta Np63 alpha phosphoprotein binds the p21 and 14-3-3 sigma promoters *in vivo* and has transcriptional repressor activity that is reduced by Hay-Wells syndrome-derived mutations. *Mol. Cell. Biol.* 23, 2264–2276. doi: 10.1128/MCB.23.7.2264-2276.2003

Wikonkal, N. M., and Brash, D. E. (1999). Ultraviolet radiation induced signature mutations in photocarcinogenesis. *J. Invest. Dermatol. Symp. Proc.* 4, 6–10. doi: 10.1038/sj.jidsp.5640173

Yamaguchi, Y., and Hearing, V. J. (2009). Physiological factors that regulate skin pigmentation. *Biofactors* 35, 193–199. doi: 10.1002/biof.29

Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M. D., Dötsch, V., et al. (1998). p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol. Cell.* 2, 305–316. doi: 10.1016/S1097-2765(00)80275-0

Yi, R., Poy, M. N., Stoffel, M., and Fuchs, E. (2008). A skin microRNA promotes differentiation by repressing "stemness." *Nature* 452, 225–229. doi: 10.1038/nature06642

Zauberman, A., Flusberg, D., Haupt, Y., Barak, Y., and Oren, M. (1995). A functional p53-responsive intronic promoter is contained within the human mdm2 gene. *Nucleic Acids Res.* 23, 2584–2592. doi: 10.1093/nar/23.14.2584

Zhang, Z., and Zhang, R. (2005). p53-independent activities of MDM2 and their relevance to cancer therapy. *Curr. Cancer Drug Targets*. 5, 9–20. doi: 10.2174/1568009053332618

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 28 February 2014; accepted: 10 April 2014; published online: 03 June 2014.
*Citation: Reichrath J, Reichrath S, Heyne K, Vogt T and Roemer K (2014) Tumor suppression in skin and other tissues via cross-talk between vitamin D- and p53-signaling. Front. Physiol. **5**:166. doi: 10.3389/fphys.2014.00166*
This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 11 July 2014
doi: 10.3389/fphys.2014.00248

Vitamin D: a critical and essential micronutrient for human health

Igor Bendik*, Angelika Friedel, Franz F. Roos, Peter Weber and Manfred Eggersdorfer

Human Nutrition and Health (IB, AF, FFR), and Nutrition Science & Advocacy (PW, ME), DSM Nutritional Products Ltd., Basel, Switzerland

Edited by:
Carsten Carlberg, University of Eastern Finland, Finland

Reviewed by:
Katie Marie Dixon, The University of Sydney, Australia
Miguel A. Maestro, University of A Coruña, Spain

****Correspondence:***
Igor Bendik, Department of Human Nutrition and Health, DSM Nutritional Products Ltd., Bldg. 201/219A, PO Box 2676, CH-4002 Kaiseraugst, Basel, Switzerland
e-mail: igor.bendik@dsm.com

Vitamin D is a micronutrient that is needed for optimal health throughout the whole life. Vitamin D_3 (cholecalciferol) can be either synthesized in the human skin upon exposure to the UV light of the sun, or it is obtained from the diet. If the photoconversion in the skin due to reduced sun exposure (e.g., in wintertime) is insufficient, intake of adequate vitamin D from the diet is essential to health. Severe vitamin D deficiency can lead to a multitude of avoidable illnesses; among them are well-known bone diseases like osteoporosis, a number of autoimmune diseases, many different cancers, and some cardiovascular diseases like hypertension are being discussed. Vitamin D is found naturally in only very few foods. Foods containing vitamin D include some fatty fish, fish liver oils, and eggs from hens that have been fed vitamin D and some fortified foods in countries with respective regulations. Based on geographic location or food availability adequate vitamin D intake might not be sufficient on a global scale. The International Osteoporosis Foundation (IOF) has collected the 25-hydroxy-vitamin D plasma levels in populations of different countries using published data and developed a global vitamin D map. This map illustrates the parts of the world, where vitamin D did not reach adequate 25-hydroxyvitamin D plasma levels: 6.7% of the papers report 25-hydroxyvitamin D plasma levels below 25 nmol/L, which indicates vitamin D deficiency, 37.3% are below 50 nmol/Land only 11.9% found 25-hydroxyvitamin D plasma levels above 75 nmol/L target as suggested by vitamin D experts. The vitamin D map is adding further evidence to the vitamin D insufficiency pandemic debate, which is also an issue in the developed world. Besides malnutrition, a condition where the diet does not match to provide the adequate levels of nutrients including micronutrients for growth and maintenance, we obviously have a situation where enough nutrients were consumed, but lacked to reach sufficient vitamin D micronutrient levels. The latter situation is known as hidden hunger. The inadequate vitamin D status impacts on health care costs, which in turn could result in significant savings, if corrected. Since little is known about the effects on the molecular level that accompany the pandemic like epigenetic imprinting, the insufficiency-triggered gene regulations or the genetic background influence on the body to maintain metabolic resilience, future research will be needed. The nutrition community is highly interested in the molecular mechanism that underlies the vitamin D insufficiency caused effect. In recent years, novel large scale technologies have become available that allow the simultaneous acquisition of transcriptome, epigenome, proteome, or metabolome data in cells of organs. These important methods are now used for nutritional approaches summarized in emerging scientific fields of nutrigenomics, nutrigenetics, or nutriepigenetics. It is believed that with the help of these novel concepts further understanding can be generated to develop future sustainable nutrition solutions to safeguard nutrition security.

Keywords: vitamin D, 25-hydroxyvitamin D, nutrition, micronutrients, hidden hunger, nutrition security, nutritional pathways, nutrigenomics

INTRODUCTION

Vitamin D is needed to maintain calcium concentrations within a narrow physiological range. This function is vital as the calcium ion is essential for a large variety of cellular and metabolic processes in the body (Berridge, 2012). To secure the calcium supplies besides intestinal absorption, calcium is stored in the skeleton and acts as a large calcium reservoir that is mainly controlled by PTH and vitamin D (Bouillon et al., 2014). Humans produce vitamin D by exposure to sunlight that includes ultraviolet B radiation; if ultraviolet B radiation is not available in sufficient amounts, vitamin D needs to be obtained from the diet or dietary supplements (Holick, 2007). The start of the vitamin D endocrine system is believed to have been initiated before the start of vertebrates and evolved over millions of years

(Bouillon and Suda, 2014). Therefore, the vitamin D micronutrient either synthesized through the sun by the skin or through dietary uptake is well-adapted to the human body. The endogenously conjugated vitamin D metabolites have taken over many important roles in the maintenance of human health, of which many still await to be discovered.

In this paper, we summarize the knowledge on vitamin D as an essential micronutrient important for human health and discuss the new nutritional research on its way to gain further knowledge on the function of vitamin D for nutrition.

VITAMIN D PART OF NUTRITION AND CONTENT IN FOODS

The history of vitamin D is linked to first scientific description of the classic bone disease rickets by Whistler in 1645 (Norman, 2012). Two centuries later it was Schütte who observed the usefulness of cod liver oil in the treatment of rickets and osteomalacia in 1824. The hunt for the anti-rachitic factor ended in early twentieth century, when Mellanby could demonstrate in a series of hallmark studies (1919–1924) that a nutritional component in the diet was the anti-rachitic factor to prevent rickets (Mellanby, 1919, 1976; Platt, 1956). Shortly after, vitamin D was inaugurated without the characterization of the chemical structure. In 1919, Hudschinsky showed in parallel that UV light was able to ameliorate rickets by increasing calcification in rachitic children (Huldschinsky, 1919, 1926). Both findings of the cod liver oil and the UV light preventing rickets remained independent observations until Hess and Weinstock elegantly could demonstrate that the anti-rachitic vitamin D was produce by UV irradiation in skin (Hess and Weinstock, 1925a,b). In 1936, Windaus and colleagues determined the chemical structure of the fat-soluble seco-steroid vitamin D (Windaus et al., 1936).

The vitamin D definition comprises a group of molecules called the calciferols. The main forms present in foods are cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2), whereas the metabolite 25-hydroxycholecalciferol (25-hydroxyvitamin D_3) is a natural part of the food chain by its occurrence in animal products. Vitamin D_3 is unique by the fact that the same nutrient can be synthesized in the skin through the action of sunlight or being taken up by diet. This dual source of intake secures the body to maintain sufficient vitamin D levels in the body. The production in skin is usually the major vitamin D_3 source for the body. However, in countries that receive insufficient sun exposure, people rely on dietary vitamin D as a major source. Exposure of the precursor 7-dehydrocholesterol in the basal and suprabasal layers of the epidermis to ultraviolet B (UVB) light with a wavelength of 290–315 nm is needed for the formation of the previtamin D_3. The subsequent conversion is a non-enzymatic process that includes a thermal isomerization of the previtamin D_3 to produce vitamin D_3 (Collins and Norman, 2001; Holick, 2011). This vitamin D_3 is rapidly converted to 25-hydroxyvitamin D_3 in the liver. The vitamin D status is evaluated by measuring the circulating levels of serum 25-hydroxyvitamin D, which is the sum of cutaneous synthesis (vitamin D_3) or dietary contribution (vitamin D_3 and vitamin D_2). The 25-hydroxyvitamin D_3 needs to be further hydroxylated in the kidney (or locally in other organs Lehmann et al., 2001) to form 1,25-dihydroxyvitamin D_3, the active endogenous hormone, which is responsible for most of the physiological actions of vitamin D through the binding to the vitamin D receptor (VDR). The plant-derived vitamin D_2 is processed in the same way. For both vitamers, vitamin D_2, and vitamin D_3, the consecutive molecular action is believed to be identical, whereas only 1,25-dihydroxy vitamin D_3 is the endogenous hormone, the activated vitamer 1,25-dihydroxyvitamin D_2 is hormone mimetic. Therefore, it was not surprising that vitamin D_3 has been reported to be superior to vitamin D_2 in terms of bioavailability and maintaining the vitamin D status by the majority of studies (Trang et al., 1998; Armas et al., 2004; Romagnoli et al., 2008; Glendenning et al., 2009; Heaney et al., 2011; Lehmann et al., 2013). Only one study reported that the two vitamers were essentially equipotent (Holick et al., 2008).

The level of cutaneous vitamin D_3 synthesis is mainly affected by the amount of solar UVB radiation reaching the human skin, which is a function that needs to take into account the wavelength, thickness of the ozone layer in the atmosphere and solar zenith angle. Furthermore, the geographic latitude, season of the year and time of day influence and restrict the skin-borne synthesis of vitamin D_3 (Webb et al., 1988; Holick, 2011). It was described that vitamin D_3 synthesis in the skin declines with age, which is due in part to a fall of 7-dehydrocholesterol and the morphological changes due to biological aging (MacLaughlin and Holick, 1985; Holick et al., 1989). Matsuoka et al. (1991) have shown that in Caucasians and Asian subjects having a lighter skin pigmentation UVB radiation produce significantly higher vitamin D_3 serum levels than in African American and East Indian groups. It is not of a surprise that skin pigmentation reduces vitamin D_3 formation. This skin tone dependent down regulation is easily overcome by increased sun exposures (Armas et al., 2007). Apart to darker pigmented skin, cutaneous vitamin D_3 production can be reduced for many other reasons like severe air pollution in large cities, less outdoor activity as a consequence of an unhealthy lifestyle change, immobility of institutionalized elderly populations, topical application of sunscreens with a high sun protection factors or cultural dress codes (e.g., veiling). Therefore, dietary intake of vitamin D through foods or supplements plays a vital part to maintain healthy vitamin D levels.

Through nutrition, vitamin D intake is limited. There are few naturally-occurring food sources containing relevant levels of vitamin D. **Table 1** summarizes the vitamin D content in selected foods. Vegetarian diets are limited to the plant vitamin D_2 that is only present in some mushrooms. Commercially dark cultivated white button mushrooms contain low amounts of vitamin D_2, only wild mushrooms or sun-dried mushrooms contain elevated amounts of ergocalciferol (Mattila et al., 1994, 1999b, 2001; Teichmann et al., 2007). Some commercial producers include an UVB radiation step to increase the vitamin D_2 content in their products (Mau et al., 1998; Roberts et al., 2008). Vitamin D_2 is formed out from ergosterol in the mushrooms. Some plants that are used as foods however can contain ergosterol, but this provitamin form is not converted to vitamin D_2. Vitamin D_3 is not found in food-borne plants. In plants, the occurrence of vitamin D_3-related compounds is scarce. Interestingly, species belonging to the botanical *Solanaceae* family, like *Solanum malacoxylon* (*Solanum glaucophyllum* and *Solanum glaucum*), contain a glycoside of the active 1,25-dihydroxyvitamin D_3 hormone

Table 1 | Vitamin D content in raw products, processed foods, and fortified foods.

Category	Foodstuff	Range		References
		(μg vitamin D per 100 g)	(IU vitamin D per 100 g)	
RAW PRODUCTS				
Fish	Herring	2.2–38.0	88–1,520	Kobayashi et al., 1995; Mattila et al., 1995a, 1997; Ostermeyer and Schmidt, 2006; Byrdwell et al., 2013
	Salmon	4.2–34.5	168–1,380	Kobayashi et al., 1995; Ostermeyer and Schmidt, 2006; Lu et al., 2007; Byrdwell et al., 2013
	Halibut	4.7–27.4	188–1,094	Ostermeyer and Schmidt, 2006; Byrdwell et al., 2013
	Perch	0.3–25.2	12–1,012	Mattila et al., 1995a, 1997; Ostermeyer and Schmidt, 2006; Byrdwell et al., 2013
	Trout	3.8–19.0	152–760	Mattila et al., 1995a; Ostermeyer and Schmidt, 2006; Byrdwell et al., 2013
	Tuna	1.7–18.7	68–748	Takeuchi et al., 1984, 1986; Kobayashi et al., 1995; Byrdwell et al., 2013
	Mackerel	0.5–15.5	20–620	Egaas and Lambertsen, 1979; Aminullah Bhuiyan et al., 1993; Kobayashi et al., 1995; Ostermeyer and Schmidt, 2006; Lu et al., 2007
	Cod	0.5–6.9	20–276	Kobayashi et al., 1995; Mattila et al., 1995a; Ostermeyer and Schmidt, 2006; Byrdwell et al., 2013
Mushrooms	Morel	4.2–6.3	168–252	Phillips et al., 2011
	Dark cultivated white bottom mushrooms	0–0.2	0–8	Mattila et al., 2001; Teichmann et al., 2007; Phillips et al., 2011
	Wild grown mushrooms	0.3–29.8	10–1,192	Mattila et al., 1994, 1999b, 2001; Kobayashi et al., 1995; Teichmann et al., 2007
Animal products	Pork meat	0.1–0.7	4–28	Kobayashi et al., 1995; Bilodeau et al., 2011; Strobel et al., 2013
	Beef meat	0–0.95	0–38	Kobayashi et al., 1995; Montgomery et al., 2000, 2002; Bilodeau et al., 2011; Strobel et al., 2013
	Chicken meat	0–0.3	0–12	Kobayashi et al., 1995; Mattila et al., 1995b; Bilodeau et al., 2011; Strobel et al., 2013
	Beef liver	0–14.1	0–560	Kobayashi et al., 1995; Mattila et al., 1995b; Montgomery et al., 2000, 2002
	Eggs	0.4–12.1	28–480	Mattila et al., 1992, 1999a; Kobayashi et al., 1995; Bilodeau et al., 2011; Exler et al., 2013
PROCESSED FOODS				
Fish	Tuna (skipjack) liver oil	144,400	5,776,000	Takeuchi et al., 1984
	Halibut liver oil	13,400	536,000	Egaas and Lambertsen, 1979
	Cod liver oil	137.5–575.0	5,500–23,000	Egaas and Lambertsen, 1979; Takeuchi et al., 1984
	Canned pink salmon	12.7–43.5	508–1,740	Bilodeau et al., 2011
	Canned sardines	3.2–10	128–400	Mattila et al., 1995a
	Smoked salmon	4.9–27.2	196–1,088	Ostermeyer and Schmidt, 2006
Mushrooms	Irradiated mushrooms	6.6–77.4	264–3,094	Mau et al., 1998; Roberts et al., 2008
Dairy	Butter	0.2–2.0	8–80	Kobayashi et al., 1995; Mattila et al., 1995b; Jakobsen and Saxholt, 2009
	Cheese	0–0.1	0–4	Mattila et al., 1995b; Wagner et al., 2008
FORTIFIED FOODS				
Cereals	Corn flakes	2–4.7	87–189	Haytowitz et al., 2009; U.S. Department of Agriculture, 2013
Beverages	Orange juice	1.1	44	Wacker and Holick, 2013
	Malted drink mix, powder	3	123	Haytowitz et al., 2009; U.S. Department of Agriculture, 2013
Dairy	Milk	1.1–2.0	42–79	Calvo et al., 2004; Haytowitz et al., 2009; U.S. Department of Agriculture, 2013
	Cheese	2.6–25.0	102–1,000	Haytowitz et al., 2009; Tippetts et al., 2012; U.S. Department of Agriculture, 2013

(Boland, 1986; Boland et al., 2003; Japelt et al., 2013). This deciduous shrub (1.5–3.0 m stem length) is widely distributed in the provinces of Buenos Aires in Argentina and in Brazil and is responsible for the calcinotic disease in cattle and other grazing animal.

Animal food products are the main dietary source for naturally occurring vitamin D_3 (Schmid and Walther, 2013). Since the discovery of vitamin D, vitamin D was associated with oily fish products. It was driven by the early observation that the amount of vitamin D in a teaspoon of cod liver oil was sufficient to prevent rickets in infants. It is still the fish liver oil that contains the highest amounts of vitamin D_3. The highest reported concentration was found in skipjack liver oil 144,400 μg/100 g (Takeuchi et al., 1984). The fish liver oils besides other nutritional ingredients might contain high levels of vitamin A. The vitamin A to vitamin D ratio in the fish liver oils is species and fishing area dependent. The ratio range starts with a factor of 0.5 for skipjack liver oil and can even reach an extreme ratio of 119 (pollack liver oil) (Takeuchi et al., 1984). This wide vitamin A to vitamin D ratio range is the reason why fish liver oils often need further processing. In fresh fish products we observe a huge variation in the vitamin D_3 content per 100 g wet weight (Egaas and Lambertsen, 1979; Takeuchi et al., 1984, 1986; Kobayashi et al., 1995; Mattila et al., 1995a, 1997; Ostermeyer and Schmidt, 2006; Lu et al., 2007; Byrdwell et al., 2013) (**Table 1**). Large variations in vitamin D_3 content were found within the same species, but also between the different fish species. Fish obtain their vitamin D_3 requirements through their diet (Holick, 2003). Therefore, the vitamin D_3 levels in the zooplankton, the primary food source of fish, or seasonal changes in the zooplankton reservoirs in the different habitats, might be the reasons for the observed fluctuation in the fish product. Interestingly, the weight, the sex, or the age of the fish could not be correlated to the vitamin D_3 content. Furthermore, no significant correlation between the tissue fat content and vitamin D levels was detected (Mattila et al., 1995a, 1997). Significant differences in vitamin D_3 content were found between muscle and skin tissues and even more pronounced between muscle and liver tissues (Takeuchi et al., 1986). The 25-hydroxyvitamin D_3 compound was also detected, though at low concentrations (Takeuchi et al., 1986; Mattila et al., 1995a; Ovesen et al., 2003; Bilodeau et al., 2011).

Wild and sun-dried mushrooms can be a good dietary source of vitamin D_2 (Mattila et al., 1994, 1999b, 2001; Kobayashi et al., 1995; Teichmann et al., 2007; Phillips et al., 2011). However, the commercially produced mushrooms, e.g., the white button mushroom, do not contain or contain only very low amounts of vitamin D_2 (Mattila et al., 2001; Teichmann et al., 2007; Phillips et al., 2011). The vitamin D_2 content in commercially produced mushrooms can be increased by UVB exposure during the culturing or the postharvest process (Mau et al., 1998; Roberts et al., 2008). The concentration of vitamin D in eggs can vary from 0.4 to 12.1 μg (Parrish, 1979; Mattila et al., 1992, 1999a; Bilodeau et al., 2011; Exler et al., 2013), it is in a similar range like offal (Mattila et al., 1995b; Montgomery et al., 2000, 2002). Other animal products like pork, beef, and chicken muscle meat are low in vitamin D content (Mattila et al., 1995b; Montgomery et al., 2000, 2002; Bilodeau et al., 2011; Strobel et al., 2013). By adding vitamin D_3 into the feed, the vitamin D_3 content can be increased in muscle and liver of cattle, to 4.6 μg per 100 g of tissue and 99.6 μg per 100 g of tissue, respectively (Montgomery et al., 2004). Milk, unless fortified, has been shown to contain no or very little amounts of vitamin D, whereas in dairy products like butter and cheese the vitamin D content is higher, but in serving size amounts still very low (Kobayashi et al., 1995; Mattila et al., 1995b; Jakobsen and Saxholt, 2009; Trenerry et al., 2011). In general, household cooking seems to have some effect on vitamin D stability depending on the actual foodstuffs and the heating process used (Mattila et al., 1999b; Jakobsen and Knuthsen, 2014).

To meet the vitamin D needs in the countries some states fortify foods. Dairy products are ideal for vitamin D fortification. In Canada vitamin D fortification is mandatory for milk (1 μg/100 ml) and margarine (13.3 μg/100 g) (Health Canada, 2014). In other countries, like the United States, vitamin D fortification is optional for products like milk, breakfast cereals, and fruit juices (Calvo et al., 2004). In the U.S. Department of Agriculture (2013) of the US Department of Agriculture (USDA)'s Nutrient Databank System (Haytowitz et al., 2009), 5036 foods have been determined for their vitamin D content, of which only 259 food items had detectable vitamin D levels. The data showed that per serving only seven fish products had >15 μg vitamin D. All 29 foods that contained between 2.5 μg 15 μg vitamin D per serving were either fortified foods (21) or fish produce (8). Two-thirds of all vitamin D containing foods were far below the 1.0 μg level, whereas 20 percent had even negligible vitamin D content per serving (below 0.1 μg).

Despite the fact that moderate sun exposure of arms and legs in summer for 5–30 min between the hours of 10 a.m. and 3 p.m. twice a week is enough to produce sufficient vitamin D_3 in the body (Holick, 2007), it is astonishing that many populations that live at these privileged latitudes fail to achieve this goal (Holick and Chen, 2008; Lips, 2010; Wahl et al., 2012; Hilger et al., 2014). During winter time, when vitamin D_3 production by the sun ceased, adequate vitamin D levels can only be achieved by UVB exposure from indoor tanning units, or by a daily diet of fortified foods or a few selected food items. This restricted list of options to achieve sufficient levels is one of the reasons, why the use of dietary vitamin D supplements has become so popular. It is currently the most applied and secure option to reach adequate vitamin D intake levels (Holick, 2007).

VITAMIN D MAP, MALNUTRITION, HIDDEN HUNGER, AND NUTRITION SECURITY

An accepted biomarker for the vitamin D status in the general population is to measure the serum concentration of 25-hydroxyvitamin D levels, which is the major circulating form of vitamin D and reflects both dietary vitamin D intake and the endogenous vitamin D production (Lips, 2001, 2007). The serum concentration of 25-hydroxyvitamin D is linked to the serum level of the active hormone 1,25-dihydroxyvitamin D and also to the clinical relevant parathyroid hormone level. Lips has classified the 25-hydroxyvitamin D levels into four stages (Lips, 2001; Lips et al., 2013): severe deficiency (<12.5 nmol/L), deficiency (12.5–25 nmol/L), insufficiency (25–50 nmol/L), repletion

(>50 nmol/L). The thresholds for severe deficiency and deficiency are undisputed; however, a controversy has arisen for defining the border between insufficiency and repletion. In 2011, the Institute of Medicine (IOM) suggested a serum level of 50 nmol/L as the value at which 97.5% of the vitamin D needs of the population would be covered (Institute of Medicine, 2011; Ross et al., 2011), whereas, the Endocrine Society (ES) defined it to be higher: 75 nmol/L (Holick et al., 2011). All deficiency levels including insufficiency, as so-called mild deficiency, must be prevented through focused supplementation.

In 2010, the Institute of Medicine (IOM) introduced new dietary reference intake (DRI) values for vitamin D after comprehensive reviewing of more than 1000 high quality research articles to renew thereby their first settings from 1997 (Institute of Medicine, 2011). The DRIs address an adequate nutritional intake of all sources. The IOM has set the dietary allowance (RDA) to 600 IU per day for the general population and at 800 IU per day for persons 70 years and older, whereas 1 IU is the biological equivalent of 0.025 μg vitamin D_3. The tolerable upper intake level or UL (Upper Level of Intake), which represents the safe upper limit, was set to 4000 IU per day for vitamin D intake (Ross et al., 2011). The new RDAs reflect the scientific outcome from large dietary studies that revealed vitamin D insufficiency (Looker et al., 2002; Zadshir et al., 2005). In 2012, Troesch et al. analyzed the vitamin intake from different dietary surveys that included the German Nutritional Intake Study (Nationale Verzehrstudie II) 2008 (Max Rubner-Institut, 2008), the US National Health and Nutrition Examination Survey (NHANES) from 2003 to 2008 (Centers for Disease Control and Prevention & National Center for Health Statistics, 2009), the UK (The British National Diet and Nutrition Survey, 2003) (Henderson et al., 2003) and the Netherlands (van Rossum et al., 2011), and could confirm that vitamin D is one of the critical vitamins, which intake is below the recommendation (Troesch et al., 2012).

A gap exists between the intake and the recommendation of vitamin D. The chronic insufficient intake of micronutrients like vitamin D without seeing immediate clinical signs is called Hidden Hunger. Hidden Hunger, in particular for vitamin D, is more prevalent in the populations of the developed countries as anticipated (Biesalski, 2013). Hidden Hunger is a threat for the nutrition security for a given country. Nutrition security mandates sufficient micronutrients in an adequate food supply and is required to safeguard an optimal nutritional status of a population.

Many groups have identified vitamin D deficiency or insufficiency to become a public health problem worldwide (Holick, 2007; Holick and Chen, 2008; Mithal et al., 2009; Lips and Van Schoor, 2011; Wahl et al., 2012; Hilger et al., 2014). Mithal et al. (2009) described in their global report that most populations do not achieve a desirable vitamin D status and particular people at risk and elderly people suffer from vitamin D deficiency. In two reports, the International Osteoporosis Foundation (IOF) and its partners published the global vitamin D status map (Wahl et al., 2012; Hilger et al., 2014). The vitamin D map was based on a systematic review of the worldwide vitamin D levels, using all available publications published between 1990 and February 2011 (Hilger et al., 2014). Eligible studies include 168,389 participants from the general populations throughout the world where the mean or median serum 25-hydroxyvitamin D levels were measured. Studies included had a cross-sectional design or were based on a population based cohorts. The analysis identified nearly 200 studies from 44 countries, whereas only half of the studies were included in the global vitamin D status map as 50.2% of the studies were not representative for the target populations. **Figure 1** shows the global vitamin D status map listed by countries and by continents. The largest numbers of studies were performed in Europe, followed by North America and Asia-Pacific. Available data from Latin America and even more from Africa are limited. Results of this review showed that 6.7% of the population were vitamin D deficient (mean 25-hydroxyvitamin D values <25 nmol/L), 37.3% were vitamin D insufficient according to IOM (mean values below 50 nmol/L) and 88.1% of the population showed an insufficient vitamin D status according to the ES (mean values below 75 nmol/L). No significant differences were found for gender or age, when looking at the worldwide data, but some regional differences could be identified (Hilger et al., 2014). The 25-hydroxyvitamin D serum levels were higher in Europe and the US, when compared to Middle East and Africa. This might be due to the vitamin D food fortification programs in North America (Prentice, 2008). Furthermore, the systematic analysis revealed that institutionalized elderly were more at risk to have low 25-hydroxyvitamin D levels in Europe and Asia/Pacific. The compared non-institutionalized elderly group showed higher levels, possibly due to spending more of time outdoors. The group of institutionalized elderly is therefore at high risk to become vitamin D deficient. Further research is needed to inform public health policy makers to reduce the risk for potential health consequences of low vitamin D status.

In the past few years the national recommendations for dietary vitamin D were adjusted in several countries; they are not harmonized across the European Union yet and vary from 200 to 800 IU. The higher recommendations for dietary vitamin D intake are increasingly being suggested in government documents, position statements and clinical practice guidelines for bone health. In 2008, the US Food and Drug Administration updated the health claim for the prevention of osteoporosis by including vitamin D to the consumption of calcium (Food and Drug Administration, 2008). In 2008, the American Academy of Pediatrics also reacted and issued an update of their guidelines for vitamin D intake and rickets prevention (Perrine et al., 2010). They doubled the recommended dose of vitamin D for children to 400 IU per day, beginning in the first few days of life and continuing throughout adolescence. In 2010, the Institute of Medicine (IOM) released the revised Dietary Reference Intakes (DRI's) for calcium and vitamin D and tripled the recommendations for vitamin D intakes to 600 IU per day for children and all adults up to age 69 years (Institute of Medicine, 2011). The IOM stated that there was insufficient evidence to make recommendations for non-skeletal benefits.

In 2012, the German, Austrian, and Swiss Nutrition Societies raised the recommended vitamin D intake to 800 IU per day, in case of absent UVB exposure, for all age groups starting from 1 year of age (German Nutrition Society, 2012). Furthermore, key opinion leaders are increasingly recommending higher daily intakes for vitamin D, between 800 and 1000 IU or even higher for people at risk or older adults. The recent statement by the IOF and the guidelines by the US ES suggest that higher vitamin D doses

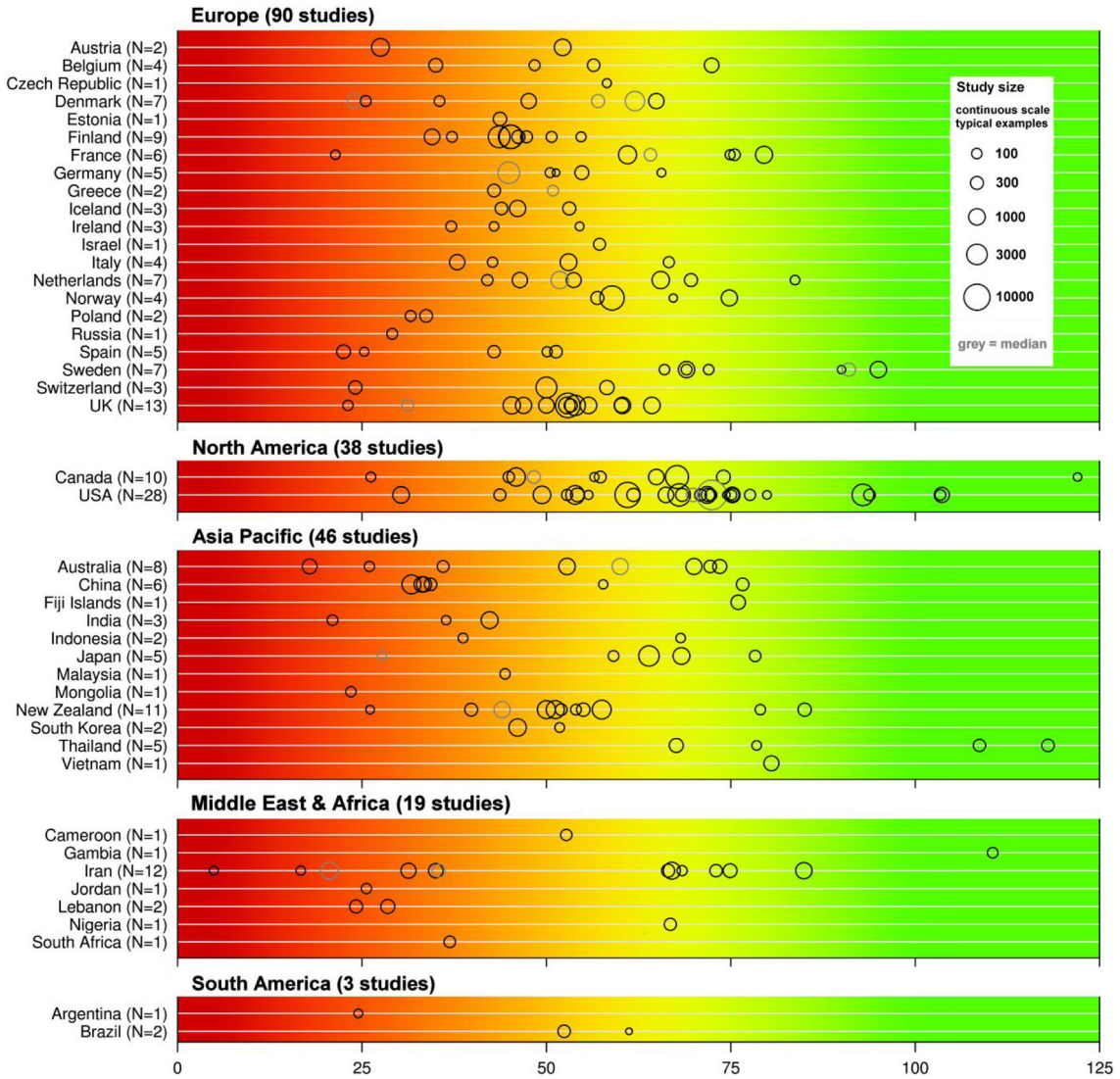

FIGURE 1 | Overview of published 25-hydroxyvitamin D mean/median values by countries (modified from Hilger et al., 2014). The color trend from red, yellow to green shown above the graphical diagram represents the current uncertainty around the definition of 25-hydroxyvitamin D_3 serum thresholds starting from severe deficiency (red), deficiency, insufficiency to total repletion (green). The reported means are shown as black circles, studies that reported medians are given in gray circle. The study size is indicated by the circle size. Mean/median values falling within the intensely red zone are most consistent with severe vitamin D deficiency.

would be needed to achieve the desirable 25-hydroxyvitamin D serum level of 75 nmol/L for people at risk or older individuals.

Increasing the vitamin D levels in the population would also ameliorate health economics. Grant and colleagues calculated the benefit of increasing vitamin D levels to reduce the economic burden of diseases (Grant et al., 2009). A rise in the vitamin D serum level of all Europeans to 40 nmol/L would reduce the economic burden of different diseases and could save health care costs of up to 16.7%. Besides reducing the economic costs, vitamin D intake could in addition also reduce mortality rates and maintain a longer healthy life style.

NUTRITIONAL RESEARCH TO ADDRESS AND UNDERSTAND VITAMIN D INSUFFICIENCY

Vitamin D deficiency is undoubtedly linked to severe consequences in the growing child by causing incomplete mineralization of the bone and in the adult accounting to wasteful osteomalacia. In the vitamin D insufficiency stage, this severity gets gradually less, but the outcome remains unchanged. Besides the established and accepted functional skeletal health relationship, more and more evidence is accumulating for falls (Pfeifer et al., 2000, 2009; Bischoff et al., 2003; Flicker et al., 2005; Broe et al., 2007; Prince et al., 2008; Bischoff-Ferrari et al., 2009) and physical performance (Bischoff-Ferrari et al., 2004; Houston et al., 2011; Ceglia et al., 2013; Redzic et al., 2013; Sohl et al., 2013;

Tieland et al., 2013), which has been recognized by a health claim of the European Food and Safety Authority in 2011: "Vitamin D may reduce the risk of falling. Falling is a risk factor for bone fractures." This health claim is targeting men and women 60 years of age and older and the dose required is a daily consumption of 800 IU vitamin D, which can come from all sources. Further emerging vitamin D health relationships include physiological parameters like improved immune response (Baeke et al., 2010; Schwalfenberg, 2011; Hewison, 2012; White, 2012), improved respiratory health(Berry et al., 2011; Charan et al., 2012; Choi et al., 2013; Hirani, 2013) possibly also relate to reduced tuberculosis incidence (Nnoaham and Clarke, 2008; Martineau et al., 2011; Mitchell et al., 2011; Coussens et al., 2012; Salahuddin et al., 2013; Huaman et al., 2014); and reduced risk to develop autoimmune diseases like multiple sclerosis (Solomon and Whitham, 2010; Cantorna, 2012; Dobson et al., 2013) or type 1 diabetes (Hypponen et al., 2001; Holick, 2003; Ramos-Lopez et al., 2006; Baeke et al., 2010; De Boer et al., 2012; Dong et al., 2013; Van Belle et al., 2013). In chronic, non-communicable diseases, vitamin D deficiency is being discussed to possibly ameliorate the incidence of some neoplastic diseases like colorectal, lung, prostate, and breast cancers (Ng et al., 2008; Rosen et al., 2012; Welsh, 2012; Cheng et al., 2013); cardiovascular diseases (CVDs) including hypertension, myocardial infarction, stroke (Forman et al., 2007; Giovannucci et al., 2008; Gardner et al., 2011; Bischoff-Ferrari et al., 2012; Tamez and Thadhani, 2012; Karakas et al., 2013; Pilz et al., 2013a; Schroten et al., 2013); life-style diseases like obesity and type 2 diabetes (Pittas et al., 2007; González-Molero et al., 2012; Khan et al., 2013; Pilz et al., 2013b; Schottker et al., 2013; Tsur et al., 2013; Van Belle et al., 2013; Bouillon et al., 2014); diseases related to the decline in sight function including age-related macular degeneration (Parekh et al., 2007; Millen et al., 2011; Lee et al., 2012); and neurological disorders including Alzheimer and Parkinson disease (Buell and Dawson-Hughes, 2008; Annweiler et al., 2012; Eyles et al., 2013; Zhao et al., 2013). One may wonder about the width of possible implications being looked at, but considering the more than 1000 genes which vitamin D is regulating through the VDR (Carlberg and Campbell, 2013), this may actually not be a surprise. To determine the potential role of vitamin D supplementation in the prevention or treatment of chronic non-skeletal diseases notwithstanding, large-scale clinical trials are demanded. In this respect for the nutrition field, four new large-scale ongoing long-term supplementation studies are expected to deliver results in near future (**Table 2**). The two very large studies, VITAL trial ($n = 20,000$) and FIND study ($n = 18,000$), are meant to deliver clinical evidence for the effect of vitamin D_3 on cancer, CVD and diabetes outcomes. The two smaller trials, CAPS and DO-HEALTH, each having more than 2,000 participants are including cancer, infections, fractures, hypertension, cognitive function, and physical performance outcomes. In all four studies the placebo group will produce vitamin D_3 in the skin and will possibly consume vitamin D through food, and therefore this will narrow the vitamin D serum level gap between the placebo and treatment groups. It remains to be seen whether the applied supplementation doses (2000 IU and 1600 IU, 3200 IU) will be sufficient to see a clear difference between the treatment and the control groups. An open likelihood will remain for the placebo group potentially obtaining sufficient vitamin D_3 (600–800 IU) levels that are considered to be sufficient for skeletal effects. In such a case only an incremental increase of an additional ∼1000 IU can be considered as the effective dose, for which no power calculation was available at the time before study begun. In light of such a situation, it will be of interest whether the micronutrient triage theory of Bruce Ames can be validated with vitamin D_3 (Ames, 2006; McCann and Ames, 2009). The triage theory postulates, as a result of recurrent shortages of micronutrients during evolution, that the body has selected and developed a metabolic rebalancing response to shortage. These rebalancing favored micronutrient-needs for short term survival, while those only required for long-term health were starved. In the case of the micronutrient vitamin D_3, calcium and bone metabolism can be considered to be secured with highest priority, therefore, it might be speculated that the 600–800 IU intake would satisfy this vitamin D_3 serum level threshold. For the chronic non-skeletal diseases however, which have only secondary priority in an evolutionary perspective, higher serum vitamin D_3 levels would be required. The ongoing four vitamin D_3 studies that have chronic diseases as their main outcomes and use nutritionally relevant ∼2000 IU are therefore well-suited to address whether the triage theory holds also true for the micronutrient vitamin D_3.

Vitamin D_3 once in the blood immediately binds to the vitamin D-binding protein (DBP) and gets transported into the liver (Holick, 2007). The first hydroxylation at position 25 generates the major circulating metabolites 25-hydroxyvitamin D_3. This metabolite circulates throughout all organs and undergoes hydroxylation at position 1, which occurs mainly in the kidney, but also in other organs, to form 1,25-dihydroxyvitamin D_3, the active hormone. Besides the major circulating metabolite 25-hydroxyvitamin D_3 and the hormonally active metabolite 1,25-dihydroxyvitamin D_3, more than 35 additional vitamin D_3 metabolites are formed by the body (Bouillon et al., 1995; Norman et al., 2001). It is speculated that they might be intermediates in the catabolism of 1,25-dihydroxyvitamin D_3. The human body has evolved many CYP enzymes and invests energy to form these additional 35 vitamin D_3 metabolites, whether this is for the purpose to catabolize 1,25-dihydroxyvitamin D_3, remains still to be answered. More appealing is the theory that these metabolites are formed to fulfill yet unknown functions of vitamin D_3. This perspective could potentially also account to the pleiotropic non-skeletal health benefits reported by the many vitamin D intake studies. For some of the vitamin D_3 metabolites like the 24*R*,25-dihydroxyvitamin D_3 potential function was explored *in vitro* (Norman et al., 2002).

The 24*R*,25-dihydroxyvitamin D_3 has been shown to be an essential hormone in the process of bone fracture healing. The 24*R*,25-dihydroxyvitamin D_3 most likely initiates its biological responses via binding to the ligand binding domain of a postulated cell membrane receptor $VDR_{mem24,25}$, similar to the better studied, but still not cloned cell membrane receptor for 1,25-dihydroxyvitamin D_3, $VDR_{mem1,25}$ (Norman et al., 2002). From the nutritional point of view, it will be of interest to investigate the function of the all vitamin D_3 metabolites and relate the function to the level of vitamin D_3 intake to secure the health benefit according to the triage theory.

Table 2 | List of ongoing large nutritional vitamin D_3 supplementation trials (>2,000 subjects) using nutrition-related daily vitamin D_3 doses (1,600–3,200 IU).

Acronym	Name, clinical trial identifier	Principal investigator	Place	Participants	Dose	Duration	Main outcomes	Results expected	Web link
CAPS	Clinical Trial of Vitamin D_3 to Reduce Cancer Risk in Postmenopausal Women NCT01052051	Joan Lappe, Creighton University	USA	2,332, healthy postmenopausal women: 55+	2,000 IU D_3 (and 1,500 mg calcium) daily	5 years	All cancers	2015	http://clinicaltrials.gov/ct2/show/NCT01052051?term=NCT01052051&rank=1
VITAL	Vitamin D and Omega-3 Trial NCT01169259	JoAnn E. Manson, Brigham and Women's Hospital	USA	20,000, men: 50+ women: 55+	2,000 IU D_3, daily omega-3 fatty acids	5 years	Cancer, Cardiovascular disease	2017	http://clinicaltrials.gov/show/NCT01169259
DO-HEALTH	Vitamin D3—Omega3—Home Exercise—Healthy Ageing and Longevity Trial NCT01745263	Heike Bischoff-Ferrari, University Zürich	8 European Cities	2,152, 70+	2,000 IU D_3 daily omega-3 fatty acids	3 years	Infections, Fractures, Blood pressure, Cognitive function, Lower extremity function	2017	http://clinicaltrials.gov/ct2/show/NCT01745263?term=bischoff-ferrari&rank=1;
FIND	Finnish Vitamin D Trial NCT01463813	Tomi-Pekka Tuomainen, University of Eastern Finland	Finland	18,000 men: 60+, women: 65+	1,600 IU D_3 daily or 3,200 IU D_3 daily	5 years	Cancer, Cardiovascular disease Diabetes	2020	http://clinicaltrials.gov/show/NCT01463813

According to the current knowledge, the vitamin D endocrine system is funneled through the biologically most active metabolite 1,25-dihydroxyvitamin D_3 that is mainly produced in the kidney, but also in other organs (Bouillon et al., 2013). Mechanistically 1,25-dihydroxyvitamin D_3 binds the VDR directly on a DNA sequence, the 1,25-dihydroxyvitamin D_3 response element (VDRE), in the regulatory region of primary 1,25-dihydroxyvitamin D_3 target genes (Carlberg and Campbell, 2013). The VDR forms together with the retinoid X receptor or putative other transcription factors a heterodimer on the VDRE, recruiting tissue-specific transcriptional co-activators and regulates through a conformational change upon 1,25-dihydroxyvitamin D_3 binding the downstream gene. The VDR is widespread in more than 30 tissues (Bouillon et al., 1995) and may trigger expression of more than 1000 genes through 1,25-dihydroxyvitamin (Carlberg et al., 2013; Hossein-Nezhad et al., 2013). The regulation of tissue-specific gene expression by 1,25-dihydroxyvitamin D_3 is of high interest, as it guides us toward the better understanding of the mechanistic action of vitamin D_3 in the different tissues. The gained knowledge from the mechanistic studies can help to design smaller and more focused nutritional intervention RCTs to answer whether vitamin D contributes to a specific health benefit of interest. In this respect the GeneChip-based transcriptomics methodology using high-density microarrays demonstrated the expression of genes in a variety of important functions of more than 100 different pathways that could be linked to vitamin D deficiency (Bossé et al., 2007; Tarroni et al., 2012; Hossein-Nezhad et al., 2013). The development of chromatin immunoprecipitation (ChiP) methodology linked to site-specific PCR amplification of the VDR bound genomic DNA fragment, and later the methods using tiled microarrays (ChiP-chip) applying the first unbiased genome-wide approach, which then was followed by the massive parallel NGS sequencing approach of the immunoprecipitated DNA segments, opened up new avenues to investigate 1,25-dihydroxyvitamin D_3 target genes in selected tissues (Ramagopalan et al., 2010; Heikkinen et al., 2011; Carlberg et al., 2012, 2013; Pike et al., 2014). In an elegant study, Carlberg et al. identified in samples of 71 pre-diabetic individuals of the VitDmet study changes in serum 25-hydroxyvitamin D_3 concentrations that were associated to primary vitamin D target genes (Carlberg et al., 2013). Based on their finding the authors proposed the genes CD14 and THBD as transcriptomics biomarkers, from which the effects of a successful vitamin D_3 supplementation can be evaluated. These biomarkers are potentially suitable for displaying the transcriptomics response of human tissues to vitamin D_3 supplementation.

Epigenetic alterations of the genome refer to heritable and modifiable changes in gene expression that are not affecting the DNA sequence. They may be inherited as Mendelian, non-Mendelian, or environmentally caused traits. One of the 1,25-dihydroxyvitamin D_3 induced epigenetic modification was shown for the hypo-methylating effect on the osteocalcin promoter (Haslberger et al., 2006). 1,25-Dihydroxyvitamin D_3 was associated with the demethylation of the osteocalcin promoter and induced the osteocalcin gene expression. The activity of VDR can be modulated by epigenetic histone acetylation. The VDR alone or in concert with other transcription factors can recruit histone-modifying enzymes like histone acetyl transferases (HATs) or histone deacetylases (HDACs) and epigenically direct transcriptional expression of downstream genes (Burrell et al., 2011; Karlic and Varga, 2011; Sundar and Rahman, 2011; Hossein-Nezhad et al., 2013). The trans-generational epigenetic inheritance of vitamin D_3 triggered epigenome modification is not fully explored, however maternal vitamin D deficiency has been discussed with adverse pregnancy outcomes or potential susceptibility for diseases (Burrell et al., 2011; Hossein-Nezhad and Holick, 2012). For future nutritional research it would be of great value to identify and validate epigenetic biomarkers that could serve as risk assessment tool for vitamin D insufficiency related susceptibility to develop a disease later in life.

Variations in vitamin D status have been shown to be related to inheritance. The disparity of vitamin D levels according to ethnicity given skin pigmentation is well-established (Cashman, 2014; Ng et al., 2014). Dark skinned population individuals have compared to Caucasian descendants almost one-half the serum concentrations of 25-hydroxyvitamin D (Nesby-O'dell et al., 2002). From twin studies it has been estimated that the heritability of genetic regulation of vitamin D levels to be between 23 and 80% (Dastani et al., 2013). In addition, large-scale genetic association studies using linkage disequilibrium analysis have identified genetic loci correlating with serum vitamin D level within five candidate genes (Dastani et al., 2013). The identified SNPs are within the 1alpha-hydroxylase of 25-hydroxyvitamin D (CYP27B1) gene, the 25-hydroxylase of vitamin D (CYP2R1) gene, the vitamin D carrier protein (GC) gene, the VDR gene, and the cytochrome P-450 (CYP24A1) gene coding for an enzyme that inactivates 1,25-dihydroxyvitamin D. It is important to note that replication studies in separate populations have to follow to verify the validity of the identified SNPs. The SNP information will provide the additional guidance toward a personalized nutritional advice to reach a sufficient vitamin D status.

CONCLUSION AND FUTURE PERSPECTIVES

In the recent years the knowledge about vitamin D and its implications have extended far beyond its classical role in bone health in either fields of basic research as well as in human trials. In particular, the evidence for the role of vitamin D in reducing the risk of fractures as well as decreasing the risk for falling is convincing and authorities have responded to it. Besides a health claim issued by the EFSA on the risk reduction for falling the dietary intake recommendations have been significantly increased in several countries such as the US and in Europe (Austria, Germany, Switzerland). A number of other countries around the globe are in the process of establishing new dietary intake recommendations as well. It turns out that on average a daily intake of 600–800 IU vitamin D appears to be required to meet fundamental needs of the human body, for specific applications higher daily intakes may be necessary, which will become clearer as the results of a number of ongoing clinical studies will become available.

The obvious question to answer is: do people obtain the recommended amounts of vitamin D? The diet is typically only a minor vitamin D source as only few food items contain relevant amounts of vitamin D, such as fatty sea fish. The primary vitamin

D source for humans is the vitamin D synthesis in the skin from vitamin D precursors by the sunlight—provided the skin is sufficiently exposed to strong enough sun radiation. Several groups have reviewed the published results on 25-hydroxyvitamin D serum levels the established marker of the vitamin D status, showing that low 25-hydroxyvitamin D levels are found in many cohorts around the world. A recent systematic review of the global vitamin D status (Hilger et al., 2014) showed that 6.7% of the overall populations reported deficient 25-hydroxyvitamin D levels below 25 nmol/L, 37% had 25-hydroxyvitamin D levels below 50 nmol/L, and only 11% were above 75 nmol/L, which is considered an adequate status by the IOF and the ES. So a very important task ahead of us is to find efficient ways to improve the vitamin D status on the population level, be it by dietary means, food fortification, or dietary supplements.

In addition, it will be very important to gather sound and convincing evidence for the many additional implicated health benefits of vitamin D besides the ones that already reached a health claim status and to see which of them will actually hold up. This will require appropriate human studies on the one hand, and also involve the appropriate use of the novel experimental approaches like nutrigenomics, nutrigenetics, and nutriepigenetics on the other hand. In conclusion, the evidence we have for vitamin D in human health is exciting, however we have to make sure that appropriate measures are taken to improve the vitamin D status to the levels required to be beneficial for human health. In future, we will also need to further apply, exploit and invest in novel, innovative and break-through technologies in the vitamin D research to understand the underlying mechanisms by which vitamin D is exerting so many effects in the human body, which is knowledge needed to the purpose to obtain and secure optimal public health through nutrition.

REFERENCES

Ames, B. N. (2006). Low micronutrient intake may accelerate the degenerative diseases of aging through allocation of scarce micronutrients by triage. *Proc. Natl. Acad. Sci. U.S.A.* 103, 17589–17594. doi: 10.1073/pnas.0608757103

Aminullah Bhuiyan, A. K. M., Ratnayake, W. M. N., and Ackman, R. G. (1993). Nutritional composition of raw and smoked atlantic mackerel (scomber scombrus): oil- and water-soluble vitamins. *J. Food Compos. Anal.* 6, 172–184. doi: 10.1006/jfca.1993.1019

Annweiler, C., Rolland, Y., Schott, A. M., Blain, H., Vellas, B., Herrmann, F. R., et al. (2012). Higher vitamin D dietary intake is associated with lower risk of Alzheimer's disease: a 7-year follow-up. *J. Gerontol. A Biol. Sci. Med. Sci.* 67, 1205–1211. doi: 10.1093/gerona/gls107

Armas, L. A., Dowell, S., Akhter, M., Duthuluru, S., Huerter, C., Hollis, B. W., et al. (2007). Ultraviolet-B radiation increases serum 25-hydroxyvitamin D levels: the effect of UVB dose and skin color. *J. Am. Acad. Dermatol.* 57, 588–593. doi: 10.1016/j.jaad.2007.03.004

Armas, L. A., Hollis, B. W., and Heaney, R. P. (2004). Vitamin D_2 is much less effective than vitamin D_3 in humans. *J. Clin. Endocrinol. Metab.* 89, 5387–5391. doi: 10.1210/jc.2004-0360

Baeke, F., Takiishi, T., Korf, H., Gysemans, C., and Mathieu, C. (2010). Vitamin D: modulator of the immune system. *Curr. Opin. Pharmacol.* 10, 482–496. doi: 10.1016/j.coph.2010.04.001

Berridge, M. J. (2012). Calcium signalling remodelling and disease. *Biochem. Soc. Trans.* 40, 297–309. doi: 10.1042/BST20110766

Berry, D. J., Hesketh, K., Power, C., and Hyppönen, E. (2011). Vitamin D status has a linear association with seasonal infections and lung function in British adults. *Br. J. Nutr.* 106, 1433–1440. doi: 10.1017/S0007114511001991

Biesalski, H. K. (2013). "Hidden hunger in the developed world," in *The Road to Good Nutrition, A Global Perspective, 1st Edn.*, eds M. Eggersdorfer, K. Kraemer, M. Ruel, M. Van Ameringen, H. K. Biesalski, M. Bloem, et al. (Basel: Karger), 39–50.

Bilodeau, L., Dufresne, G., Deeks, J., Clément, G., Bertrand, J., Turcotte, S., et al. (2011). Determination of vitamin D_3 and 25-hydroxyvitamin D_3 in foodstuffs by HPLC UV-DAD and LC–MS/MS. *J. Food Compos. Anal.* 24, 441–448. doi: 10.1016/j.jfca.2010.08.002

Bischoff, H. A., Stahelin, H. B., Dick, W., Akos, R., Knecht, M., Salis, C., et al. (2003). Effects of vitamin D and calcium supplementation on falls: a randomized controlled trial. *J. Bone Miner. Res.* 18, 343–351. doi: 10.1359/jbmr.2003.18.2.343

Bischoff-Ferrari, H. A., Dawson-Hughes, B., Staehelin, H. B., Orav, J. E., Stuck, A. E., Theiler, R., et al. (2009). Fall prevention with supplemental and active forms of vitamin D: a meta-analysis of randomised controlled trials. *BMJ* 339:b3692. doi: 10.1136/bmj.b3692

Bischoff-Ferrari, H. A., Dawson-Hughes, B., Stocklin, E., Sidelnikov, E., Willett, W. C., Edel, J. O., et al. (2012). Oral supplementation with 25(OH)D_3 versus vitamin D_3: effects on 25(OH)D levels, lower extremity function, blood pressure, and markers of innate immunity. *J. Bone Miner. Res.* 27, 160–169. doi: 10.1002/jbmr.551

Bischoff-Ferrari, H. A., Dietrich, T., Orav, E. J., Hu, F. B., Zhang, Y., Karlson, E. W., et al. (2004). Higher 25-hydroxyvitamin D concentrations are associated with better lower-extremity function in both active and inactive persons aged $\geq$60 y. *Am. J. Clin. Nutr.* 80, 752–758.

Boland, R., Skliar, M., Curino, A., and Milanesi, L. (2003). Vitamin D compounds in plants. *Plant Sci.* 164, 357–369. doi: 10.1016/S0168-9452(02)00420-X

Boland, R. L. (1986). Plants as a source of vitamin D_3 metabolites. *Nutr. Rev.* 44, 1–8. doi: 10.1111/j.1753-4887.1986.tb07543.x

Bossé, Y., Maghni, K., and Hudson, T. J. (2007). 1α,25-dihydroxy-vitamin D_3 stimulation of bronchial smooth muscle cells induces autocrine, contractility, and remodeling processes. *Physiol. Genomics* 29, 161–168. doi: 10.1152/physiolgenomics.00134.2006

Bouillon, R., Carmeliet, G., Lieben, L., Watanabe, M., Perino, A., Auwerx, J., et al. (2014). Vitamin D and energy homeostasis—Of mice and men. *Nat. Rev. Endocrinol.* 10, 79–87. doi: 10.1038/nrendo.2013.226

Bouillon, R., Lieben, L., Mathieu, C., Verstuyf, A., and Carmeliet, G. (2013). Vitamin D action: lessons from VDR and Cyp27b1 null mice. *Pediatr. Endocrinol. Rev.* 10(Suppl. 2), 354–366.

Bouillon, R., Okamura, W. H., and Norman, A. W. (1995). Structure-function relationships in the vitamin D endocrine system. *Endocr. Rev.* 16, 200–257.

Bouillon, R., and Suda, T. (2014). Vitamin D: calcium and bone homeostasis during evolution. *Bonekey Rep.* 3:480. doi: 10.1038/bonekey.2013.214

Broe, K. E., Chen, T. C., Weinberg, J., Bischoff-Ferrari, H. A., Holick, M. F., and Kiel, D. P. (2007). A higher dose of vitamin D reduces the risk of falls in nursing home residents: a randomized, multiple-dose study. *J. Am. Geriatr. Soc.* 55, 234–239. doi: 10.1111/j.1532-5415.2007.01048.x

Buell, J. S., and Dawson-Hughes, B. (2008). Vitamin D and neurocognitive dysfunction: preventing "D"ecline? *Mol. Aspects Med.* 29, 415–422. doi: 10.1016/j.mam.2008.05.001

Burrell, A. M., Handel, A. E., Ramagopalan, S. V., Ebers, G. C., and Morahan, J. M. (2011). Epigenetic mechanisms in multiple sclerosis and the major histocompatibility complex (MHC). *Discov. Med.* 11, 187–196.

Byrdwell, W. C., Horst, R. L., Phillips, K. M., Holden, J. M., Patterson, K. Y., Harnly, J. M., et al. (2013). Vitamin D levels in fish and shellfish determined by liquid chromatography with ultraviolet detection and mass spectrometry. *J. Food Compos. Anal.* 30, 109–119. doi: 10.1016/j.jfca.2013.01.005

Calvo, M. S., Whiting, S. J., and Barton, C. N. (2004). Vitamin D fortification in the United States and Canada: current status and data needs. *Am. J. Clin. Nutr.* 80, 1710S–1716S.

Cantorna, M. T. (2012). Vitamin D, multiple sclerosis and inflammatory bowel disease. *Arch. Biochem. Biophys.* 523, 103–106. doi: 10.1016/j.abb.2011.11.001

Carlberg, C., and Campbell, M. J. (2013). Vitamin D receptor signaling mechanisms: integrated actions of a well-defined transcription factor. *Steroids* 78, 127–136. doi: 10.1016/j.steroids.2012.10.019

Carlberg, C., Seuter, S., De Mello, V. D., Schwab, U., Voutilainen, S., Pulkki, K., et al. (2013). Primary vitamin d target genes allow a categorization of possible benefits of vitamin D_3 supplementation. *PLoS ONE* 8:e71042. doi: 10.1371/journal.pone.0071042

Carlberg, C., Seuter, S., and Heikkinen, S. (2012). The first genome-wide view of vitamin D receptor locations and their mechanistic implications. *Anticancer Res.* 32, 271–282.

Cashman, K. D. (2014). The vitamin D RDA for African American adults: higher than that for white persons? *Am. J. Clin. Nutr.* 99, 427–428. doi: 10.3945/ajcn.113.082271

Ceglia, L., Niramitmahapanya, S., Da Silva Morais, M., Rivas, D. A., Harris, S. S., Bischoff-Ferrari, H., et al. (2013). A randomized study on the effect of vitamin D_3 supplementation on skeletal muscle morphology and vitamin d receptor concentration in older women. *J. Clin. Endocrinol. Metab.* 98, E1927–E1935. doi: 10.1210/jc.2013-2820

Centers for Disease Control and Prevention & National Center for Health Statistics. (2009). *NHANES 2003-2004, NHANES 2005-2006, NHANES 2007-2008. Data, Documentation, Codebooks, SAS Code. Dietary Interview. Individual Foods, Total Nutrient Intakes First and Second Day*. Hyattsville, MD: U.S. Department of Health and Human Services & Centers for Disease Control and Prevention. Available online at: http://www.cdc.gov/nchs/nhanes/nhanes_questionnaires.htm

Charan, J., Goyal, J. P., Saxena, D., and Yadav, P. (2012). Vitamin D for prevention of respiratory tract infections: a systematic review and meta-analysis. *J. Pharmacol. Pharmacother.* 3, 300–303. doi: 10.4103/0976-500X.103685

Cheng, T. Y., Lacroix, A. Z., Beresford, S. A., Goodman, G. E., Thornquist, M. D., Zheng, Y., et al. (2013). Vitamin D intake and lung cancer risk in the Women's Health Initiative. *Am. J. Clin. Nutr.* 98, 1002–1011. doi: 10.3945/ajcn.112.055905

Choi, C. J., Seo, M., Choi, W. S., Kim, K. S., Youn, S. A., Lindsey, T., et al. (2013). Relationship between serum 25-hydroxyvitamin D and lung function among Korean adults in Korea National Health and Nutrition Examination Survey, 2008–2010. *J. Clin. Endocrinol. Metab.* 98, 1703–1710. doi: 10.1210/jc.2012-3901

Collins, E. D., and Norman, A. W. (2001). "Vitamin D," in *Handbook of Vitamins*, eds R. B. Rucker, J. W. Suttie, D. B. Mccormick, and L. J. Machlin (New York, NY: Marcel Dekker Inc.), 51–113.

Coussens, A. K., Wilkinson, R. J., Hanifa, Y., Nikolayevskyy, V., Elkington, P. T., Islam, K., et al. (2012). Vitamin D accelerates resolution of inflammatory responses during tuberculosis treatment. *Proc. Natl. Acad. Sci. U.S.A.* 109, 15449–15454. doi: 10.1073/pnas.1200072109

Dastani, Z., Li, R., and Richards, B. (2013). Genetic regulation of vitamin D levels. *Calcif. Tissue Int.* 92, 106–117. doi: 10.1007/s00223-012-9660-z

De Boer, I. H., Sachs, M. C., Cleary, P. A., Hoofnagle, A. N., Lachin, J. M., Molitch, M. E., et al. (2012). Circulating Vitamin D metabolites and kidney disease in type 1 diabetes. *J. Clin. Endocrinol. Metab.* 97, 4780–4788. doi: 10.1210/jc.2012-2852

Dobson, R., Giovannoni, G., and Ramagopalan, S. (2013). The month of birth effect in multiple sclerosis: systematic review, meta-analysis and effect of latitude. *J. Neurol. Neurosurg. Psychiatry* 84, 427–432. doi: 10.1136/jnnp-2012-303934

Dong, J. Y., Zhang, W. G., Chen, J. J., Zhang, Z. L., Han, S. F., and Qin, L. Q. (2013). Vitamin D intake and risk of type 1 diabetes: a meta-analysis of observational studies. *Nutrients* 5, 3551–3562. doi: 10.3390/nu5093551

Egaas, E., and Lambertsen, G. (1979). Naturally occurring vitamin D_3 in fish products analysed by HPLC, using vitamin D2 as an international standard. *Int. J. Vitam. Nutr. Res.* 49, 35–42.

Exler, J., Phillips, K. M., Patterson, K. Y., and Holden, J. M. (2013). Cholesterol and vitamin D content of eggs in the U.S. retail market. *J. Food Compos. Anal.* 29, 110–116. doi: 10.1016/j.jfca.2012.11.001

Eyles, D. W., Burne, T. H. J., and McGrath, J. J. (2013). Vitamin D, effects on brain development, adult brain function and the links between low levels of vitamin D and neuropsychiatric disease. *Front. Neuroendocrinol.* 34, 47–64. doi: 10.1016/j.yfrne.2012.07.001

Flicker, L., Macinnis, R. J., Stein, M. S., Scherer, S. C., Mead, K. E., Nowson, C. A., et al. (2005). Should older people in residential care receive vitamin D to prevent falls? Results of a randomized trial. *J. Am. Geriatr. Soc.* 53, 1881–1888. doi: 10.1111/j.1532-5415.2005.00468.x

Food and Drug Administration. (2008). Food labeling: health claims; calcium and osteoporosis, and calcium, vitamin D, and osteoporosis. *Fed. Regist*. 73, 56477–56487.

Forman, J. P., Giovannucci, E., Holmes, M. D., Bischoff-Ferrari, H. A., Tworoger, S. S., Willett, W. C., et al. (2007). Plasma 25-hydroxyvitamin D levels and risk of incident hypertension. *Hypertension* 49, 1063–1069. doi: 10.1161/HYPERTENSIONAHA.107.087288

Gardner, D. G., Chen, S., Glenn, D. J., and Ni, W. (2011). *Vitamin D and The Cardiovascular System*. Amsterdam: Elsevier Inc.

German Nutrition Society. (2012). New reference values for vitamin D. *Ann. Nutr. Metab.* 60, 241–246. doi: 10.1159/000337547

Giovannucci, E., Liu, Y., Hollis, B. W., and Rimm, E. B. (2008). 25-hydroxyvitamin d and risk of myocardial infarction in men: a prospective study. *Arch. Intern. Med.* 168, 1174–1180. doi: 10.1001/archinte.168.11.1174

Glendenning, P., Chew, G. T., Seymour, H. M., Gillett, M. J., Goldswain, P. R., Inderjeeth, C. A., et al. (2009). Serum 25-hydroxyvitamin D levels in vitamin D-insufficient hip fracture patients after supplementation with ergocalciferol and cholecalciferol. *Bone* 45, 870–875. doi: 10.1016/j.bone.2009.07.015

González-Molero, I., Rojo-Martínez, G., Morcillo, S., Gutiérrez-Repiso, C., Rubio-Martín, E., Almaraz, M. C., et al. (2012). Vitamin D and incidence of diabetes: a prospective cohort study. *Clin. Nutr.* 31, 571–573. doi: 10.1016/j.clnu.2011.12.001

Grant, W. B., Cross, H. S., Garland, C. F., Gorham, E. D., Moan, J., Peterlik, M., et al. (2009). Estimated benefit of increased vitamin D status in reducing the economic burden of disease in western Europe. *Prog. Biophys. Mol. Biol.* 99, 104–113. doi: 10.1016/j.pbiomolbio.2009.02.003

Haslberger, A., Varga, F., and Karlic, H. (2006). Recursive causality in evolution: a model for epigenetic mechanisms in cancer development. *Med. Hypotheses* 67, 1448–1454. doi: 10.1016/j.mehy.2006.05.047

Haytowitz, D. B., Lemar, L. E., and Pehrsson, P. R. (2009). USDA's Nutrient Databank System -a tool for handling data from diverse sources. *J. Food Compos. Anal.* 22, 433–441. doi: 10.1016/j.jfca.2009.01.003

Health Canada. (2014). *Consolidation Food and Drugs Regulation.* Ottawa, ON: The Minister of Justice Canada.

Heaney, R. P., Recker, R. R., Grote, J., Horst, R. L., and Armas, L. A. (2011). Vitamin D_3 is more potent than vitamin D_2 in humans. *J. Clin. Endocrinol. Metab.* 96, E447–E452. doi: 10.1210/jc.2010-2230

Heikkinen, S., Vaisanen, S., Pehkonen, P., Seuter, S., Benes, V., and Carlberg, C. (2011). Nuclear hormone 1alpha,25-dihydroxyvitamin D_3 elicits a genome-wide shift in the locations of VDR chromatin occupancy. *Nucleic Acids Res.* 39, 9181–9193. doi: 10.1093/nar/gkr654

Henderson, L., Irving, K., Gregory, J. F., Bates, C. J., Prentice, A., Perks, J., et al. (2003). *The National Diet and Nutrition Survey: Adults Aged 19–64 Years. Vol. 3: Vitamin and Mineral Intake and Urinary Analytes*. London: The Stationery Office.

Hess, A. F., and Weinstock, M. (1925a). The antirachitic value of irradiated cholesterol an phytosterol. II. Further evidence of change in biological activity. *Methods Enzymol.* 64, 181–191.

Hess, A. F., and Weinstock, M. (1925b). The antirachitic value of irradiated cholesterol an phytosterol. III. Evidence of chemical change as shown by absorption spectra. *Methods Enzymol.* 64, 193–201.

Hewison, M. (2012). An update on vitamin D and human immunity. *Clin. Endocrinol.* 76, 315–325. doi: 10.1111/j.1365-2265.2011.04261.x

Hilger, J., Friedel, A., Herr, R., Rausch, T., Roos, F., Wahl, D. A., et al. (2014). A systematic review of vitamin D status in populations worldwide. *Br. J. Nutr.* 111, 23–45. doi: 10.1017/S0007114513001840

Hirani, V. (2013). Associations between vitamin D and self-reported respiratory disease in older people from a nationally representative population survey. *J. Am. Geriatr. Soc.* 61, 969–973. doi: 10.1111/jgs.12254

Holick, M. F. (2003). Vitamin D: a millenium perspective. *J. Cell. Biochem.* 88, 296–307. doi: 10.1002/jcb.10338

Holick, M. F. (2007). Vitamin D deficiency. *N. Engl J. Med* 357, 266–281. doi: 10.1056/NEJMra070553

Holick, M. F. (2011). "Photobiology of Vitamin D," in *Vitamin D, 3rd Edn.,* eds D. Feldman, J. W. Pike, and J. S. Adams (London, GB: Elsevier), 13–22.

Holick, M. F., Biancuzzo, R. M., Chen, T. C., Klein, E. K., Young, A., Bibuld, D., et al. (2008). Vitamin D_2 is as effective as vitamin D_3 in maintaining circulating concentrations of 25-hydroxyvitamin D. *J. Clin. Endocrinol. Metab.* 93, 677–681. doi: 10.1210/jc.2007-2308

Holick, M. F., Binkley, N. C., Bischoff-Ferrari, H. A., Gordon, C. M., Hanley, D. A., Heaney, R. P., et al. (2011). Evaluation, treatment, and prevention of vitamin d deficiency: an endocrine society clinical practice guideline. *J. Clin. Endocrinol. Metab.* 96, 1911–1930. doi: 10.1210/jc.2011-0385

Holick, M. F., and Chen, T. C. (2008). Vitamin D deficiency: a worldwide problem with health consequences. *Am. J. Clin. Nutr.* 87, 1080S–1086S.

Holick, M. F., Matsuoka, L. Y., and Wortsman, J. (1989). Age, vitamin D, and solar ultraviolet. *Lancet* 2, 1104–1105. doi: 10.1016/S0140-6736(89)91124-0

Hossein-Nezhad, A., and Holick, M. F. (2012). Optimize dietary intake of vitamin D: an epigenetic perspective. *Curr. Opin. Clin. Nutr. Metab. Care* 15, 567–579. doi: 10.1097/MCO.0b013e3283594978

Hossein-Nezhad, A., Spira, A., and Holick, M. F. (2013). Influence of vitamin D status and vitamin D_3 supplementation on genome wide expression of white blood cells: a randomized double-blind clinical trial. *PLoS ONE* 8:e58725. doi: 10.1371/journal.pone.0058725

Houston, D. K., Tooze, J. A., Hausman, D. B., Johnson, M. A., Nicklas, B. J., Miller, M. E., et al. (2011). Change in 25-hydroxyvitamin D and physical performance in older adults. *J. Gerontol. A Biol. Sci. Med. Sci.* 66, 430–436. doi: 10.1093/gerona/glq235

Huaman, M. A., Sterling, T. R., Shepherd, B. E., and Fiske, C. T. (2014). 25-Hydroxyvitamin D levels after recovery from tuberculosis: insights into pathogenesis. *Tuberculosis (Edinb)* 94, 51–54. doi: 10.1016/j.tube.2013.10.009

Huldschinsky, K. (1919). Heilung von Rachitis durch künstliche Höhensonne. *Dtsch. Med. Wochenschr.* 45, 712–713. doi: 10.1055/s-0028-1137830

Huldschinsky, K. (1926). The antirachitic: zone of the ultra-violet. *Klinische Wochenschrift* 5, 1972–1973. doi: 10.1007/BF01710213

Hypponen, E., Laara, E., Reunanen, A., Jarvelin, M. R., and Virtanen, S. M. (2001). Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study. *Lancet* 358, 1500–1503. doi: 10.1016/S0140-6736(01)06580-1

Institute of Medicine, F. A. N. B. (2011). *Dietary Reference Intakes for Calcium and Vitamin D.* Washington, DC: The National Academies Press.

Jakobsen, J., and Knuthsen, P. (2014). Stability of vitamin D in foodstuffs during cooking. *Food Chem.* 148, 170–175. doi: 10.1016/j.foodchem.2013.10.043

Jakobsen, J., and Saxholt, E. (2009). Vitamin D metabolites in bovine milk and butter. *J. Food Compos. Anal.* 22, 472–478. doi: 10.1016/j.jfca.2009.01.010

Japelt, R. B., Silvestro, D., Smedsgaard, J., Jensen, P. E., and Jakobsen, J. (2013). Quantification of vitamin D_3 and its hydroxylated metabolites in waxy leaf nightshade (*Solanum glaucophyllum* Desf.), tomato (*Solanum lycopersicum* L.) and bell pepper (*Capsicum annuum* L.). *Food Chem.* 138, 1206–1211. doi: 10.1016/j.foodchem.2012.11.064

Karakas, M., Thorand, B., Zierer, A., Huth, C., Meisinger, C., Roden, M., et al. (2013). Low levels of serum 25-hydroxyvitamin d are associated with increased risk of myocardial infarction, especially in women: results from the MONICA/KORA augsburg case-cohort study. *J. Clin. Endocrinol. Metab.* 98, 272–280. doi: 10.1210/jc.2012-2368

Karlic, H., and Varga, F. (2011). Impact of vitamin D metabolism on clinical epigenetics. *Clin. Epigenetics* 2, 55–61. doi: 10.1007/s13148-011-0021-y

Khan, H., Kunutsor, S., Franco, O. H., and Chowdhury, R. (2013). Vitamin D, type 2 diabetes and other metabolic outcomes: a systematic review and meta-analysis of prospective studies. *Proc. Nutr. Soc.* 72, 89–97. doi: 10.1017/S0029665112002765

Kobayashi, T., Takeuchi, A., and Okano, T. (1995). "Vitamin D contents in various kinds of Japanese foods," in *Challenges of Modern Medicine* (Rome: Ares-Serono Symposia), 345–350.

Lee, V., Rekhi, E., Hoh Kam, J., and Jeffery, G. (2012). Vitamin D rejuvenates aging eyes by reducing inflammation, clearing amyloid beta and improving visual function. *Neurobiol. Aging* 33, 2382–2389. doi: 10.1016/j.neurobiolaging.2011.12.002

Lehmann, B., Genehr, T., Knuschke, P., Pietzsch, J., and Meurer, M. (2001). UVB-induced conversion of 7-dehydrocholesterol to 1alpha,25-dihydroxyvitamin D_3 in an *in vitro* human skin equivalent model. *J. Invest. Dermatol.* 117, 1179–1185. doi: 10.1046/j.0022-202x.2001.01538.x

Lehmann, U., Hirche, F., Stangl, G. I., Hinz, K., Westphal, S., and Dierkes, J. (2013). Bioavailability of vitamin D_2 and D_3 in healthy volunteers, a randomized placebo-controlled trial. *J. Clin. Endocrinol. Metab.* 98, 4339–4345. doi: 10.1210/jc.2012-4287

Lips, P. (2001). Vitamin D deficiency and secondary hyperparathyroidism in the elderly: consequences for bone loss and fractures and therapeutic implications. *Endocr. Rev.* 22, 477–501. doi: 10.1210/edrv.22.4.0437

Lips, P. (2007). Relative value of 25(OH)D and 1,25(OH)2D measurements. *J. Bone Miner. Res.* 22, 1668–1671. doi: 10.1359/jbmr.070716

Lips, P. (2010). Worldwide status of vitamin D nutrition. *J. Steroid Biochem. Mol. Biol.* 121, 297–300. doi: 10.1016/j.jsbmb.2010.02.021

Lips, P., and Van Schoor, N. (2011). "Worldwide vitamin D status," in *Vitamin D, 3rd Edn.,* eds D. Feldman, J. W. Pike, and J. S. Adams (London, GB: Elsevier Inc.), 947–963.

Lips, P., Van Schoor, N. M., and Bravenboer, N. (2013). "Vitamin D-related disorders," in *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism 8th Edn.,* eds R. B. Clifford J. Rosen, J. E. Compston, V. Rosen (Singapore: John Wiley & Sons, Inc.), 613–623.

Looker, A. C., Dawson-Hughes, B., Calvo, M. S., Gunter, E. W., and Sahyoun, N. R. (2002). Serum 25-hydroxyvitamin D status of adolescents and adults in two seasonal subpopulations from NHANES III. *Bone* 30, 771–777. doi: 10.1016/S8756-3282(02)00692-0

Lu, Z., Chen, T. C., Zhang, A., Persons, K. S., Kohn, N., Berkowitz, R., et al. (2007). An evaluation of the vitamin D_3 content in fish: is the vitamin D content adequate to satisfy the dietary requirement for vitamin D? *J. Steroid Biochem. Mol. Biol.* 103, 642–644. doi: 10.1016/j.jsbmb.2006.12.010

MacLaughlin, J., and Holick, M. F. (1985). Aging decreases the capacity of human skin to produce vitamin D_3. *J. Clin. Invest.* 76, 1536–1538. doi: 10.1172/JCI112134

Martineau, A. R., Timms, P. M., Bothamley, G. H., Hanifa, Y., Islam, K., Claxton, A. P., et al. (2011). High-dose vitamin D_3 during intensive-phase antimicrobial treatment of pulmonary tuberculosis: a double-blind randomised controlled trial. *Lancet* 377, 242–250. doi: 10.1016/S0140-6736(10)61889-2

Matsuoka, L. Y., Wortsman, J., Haddad, J. G., Kolm, P., and Hollis, B. W. (1991). Racial pigmentation and the cutaneous synthesis of vitamin D. *Arch. Dermatol.* 127, 536–538. doi: 10.1001/archderm.1991.04510010104011

Mattila, P., Konko, K., Eurola, M., Pihlava, J. M., Astola, J., Vahteristo, L., et al. (2001). Contents of vitamins, mineral elements, and some phenolic compounds in cultivated mushrooms. *J. Agric. Food Chem.* 49, 2343–2348. doi: 10.1021/jf001525d

Mattila, P., Lehikoinen, K., Kiiskinen, T., and Piironen, V. (1999a). Cholecalciferol and 25-hydroxycholecalciferol content of chicken egg yolk as affected by the cholecalciferol content of feed. *J. Agric. Food Chem.* 47, 4089–4092. doi: 10.1021/jf990183c

Mattila, P., Piironen, V., Bäckman, C., Asunmaa, A., Uusi-Rauva, E., and Koivistoinen, P. (1992). Determination of vitamin D_3 in egg yolk by high-performance liquid chromatography with diode array detection. *J. Food Compost. Anal.* 5, 281–290. doi: 10.1016/0889-1575(92)90062-O

Mattila, P., Piironen, V., Haapala, R., Hirvi, T., and Uusi-Rauva, E. (1997). Possible factors responsible for the high variation in the cholecalciferol contents of fish. *J. Agric. Food Chem.* 45, 3891–3896. doi: 10.1021/jf970243j

Mattila, P., Piironen, V., Uusi-Rauva, E., and Koivistoinen, P. (1995a). Cholecalciferol and 25-hydroxycholecalciferol contents in fish and fish products. *J. Food Compost. Anal.* 8, 232–243. doi: 10.1006/jfca.1995.1017

Mattila, P., Ronkainen, R., Lehikoinen, K., and Piironen, V. (1999b). Effect of Household Cooking on the Vitamin D content in Fish, Eggs, and Wild Mushrooms. *J. Food Compost. Anal.* 12, 153–160. doi: 10.1006/jfca.1999.0828

Mattila, P. H., Piironen, V. I., Uusi-Rauva, E. J., and Koivistoinen, P. E. (1994). Vitamin D contents in edible mushrooms. *J. Agric. Food Chem.* 42, 2449–2453. doi: 10.1021/jf00047a016

Mattila, P. H., Piironen, V. I., Uusi-Rauva, E. J., and Koivistoinen, P. E. (1995b). Contents of cholecalciferol, ergocalciferol, and their 25-hydroxylated metabolites in milk products and raw meat and liver as determined by HPLC. *J. Agric. Food Chem.* 43, 2394–2399. doi: 10.1021/jf00057a015

Mau, J. L., Chen, P. R., and Yang, J. H. (1998). Ultraviolet irradiation increased vitamin D_2 content in edible mushrooms. *J. Agric. Food Chem.* 46, 5269–5272. doi: 10.1021/jf980602q

Max Rubner-Institut. (2008). *Nationale Verzehrsstudie II. Ergebnisbericht Teil 2. Die bundesweite Befragung zur Ernaehrung von Jugendlichen und Erwachsenen (National Food Intake Study II. Results Part 2. Countrywide Assessment of Nutrition in Adolescents and Adults)*. Karlsruhe: Max Rubner-Institut Available online at: http://www.was-esse-ich.de/uploads/media/NVSII_Abschlussbericht_Teil_2.pdf

McCann, J. C., and Ames, B. N. (2009). Vitamin K, an example of triage theory: is micronutrient inadequacy linked to diseases of aging? *Am. J. Clin. Nutr.* 90, 889–907. doi: 10.3945/ajcn.2009.27930

Mellanby, E. (1919). An experimental investigation on rickets. *Lancet* 193, 407–412. doi: 10.1016/S0140-6736(01)25465-8

Mellanby, E. (1976). Nutrition classics. the lancet 1:407-12, 1919. An experimental investigation on rickets. *Nutr. Rev.* 34, 338–340. doi: 10.1111/j.1753-4887.1976.tb05815.x

Millen, A. E., Voland, R., Sondel, S. A., Parekh, N., Horst, R. L., Wallace, R. B., et al. (2011). Vitamin D status and early age-related macular degeneration in postmenopausal women. *Arch. Ophthalmol.* 129, 481–489. doi: 10.1001/archophthalmol.2011.48

Mitchell, K., Griffiths, C. J., and Martineau, A. R. (2011). Vitamin D and tuberculosis. *Curr. Respir. Med. Rev.* 7, 435–439. doi: 10.2174/157339811798072685

Mithal, A., Wahl, D. A., Bonjour, J. P., Burckhardt, P., Dawson-Hughes, B., Eisman, J. A., et al. (2009). Global vitamin D status and determinants of hypovitaminosis D. *Osteoporos Int.* 20, 1807–1820. doi: 10.1007/s00198-009-0954-6

Montgomery, J. L., Carr, M. A., Kerth, C. R., Hilton, G. G., Price, B. P., Galyean, M. L., et al. (2002). Effect of vitamin D_3 supplementation level on the postmortem tenderization of beef from steers. *J. Anim. Sci.* 80, 971–981.

Montgomery, J. L., King, M. B., Gentry, J. G., Barham, A. R., Barham, B. L., Hilton, G. G., et al. (2004). Supplemental vitamin D_3 concentration and biological type of steers. II. Tenderness, quality, and residues of beef. *J. Anim. Sci.* 82, 2092–2104.

Montgomery, J. L., Parrish, F. C., Beitz, D. C., Horst, R. L., Huff-Lonergan, E. J., and Trenkle, A. H. (2000). The use of vitamin D_3 to improve beef tenderness. *J. Anim. Sci.* 78, 2615–2621.

Nesby-O'dell, S., Scanlon, K. S., Cogswell, M. E., Gillespie, C., Hollis, B. W., Looker, A. C., et al. (2002). Hypovitaminosis D prevalence and determinants among African American and white women of reproductive age: third National Health and Nutrition Examination Survey, 1988–1994. *Am. J. Clin. Nutr.* 76, 187–192.

Ng, K., Meyerhardt, J. A., Wu, K., Feskanich, D., Hollis, B. W., Giovannucci, E. L., et al. (2008). Circulating 25-hydroxyvitamin d levels and survival in patients with colorectal cancer. *J. Clin. Oncol.* 26, 2984–2991. doi: 10.1200/JCO.2007.15.1027

Ng, K., Scott, J. B., Drake, B. F., Chan, A. T., Hollis, B. W., Chandler, P. D., et al. (2014). Dose response to vitamin D supplementation in African Americans: results of a 4-arm, randomized, placebo-controlled trial. *Am. J. Clin. Nutr.* 99, 587–598. doi: 10.3945/ajcn.113.067777

Nnoaham, K. E., and Clarke, A. (2008). Low serum vitamin D levels and tuberculosis: a systematic review and meta-analysis. *Int. J. Epidemiol.* 37, 113–119. doi: 10.1093/ije/dym247

Norman, A. W. (2012). The history of the discovery of vitamin D and its daughter steroid hormone. *Ann. Nutr. Metab.* 61, 199–206. doi: 10.1159/000343104

Norman, A. W., Ishizuka, S., and Okamura, W. H. (2001). Ligands for the vitamin D endocrine system: different shapes function as agonists and antagonists for genomic and rapid response receptors or as a ligand for the plasma vitamin D binding protein. *J. Steroid. Biochem. Mol. Biol.* 76, 49–59. doi: 10.1016/S0960-0760(00)00145-X

Norman, A. W., Okamura, W. H., Bishop, J. E., and Henry, H. L. (2002). Update on biological actions of 1alpha,25$(OH)_2$-vitamin D_3 (rapid effects) and 24R,25$(OH)_2$-vitamin D_3. *Mol. Cell. Endocrinol.* 197, 1–13. doi: 10.1016/S0303-7207(02)00273-3

Ostermeyer, U., and Schmidt, T. (2006). Vitamin D and provitamin D in fish: determination by HPLC with electrochemical detection. *Eur. Food Res. Technol.* 222, 403–413. doi: 10.1007/s00217-005-0086-y

Ovesen, L., Brot, C., and Jakobsen, J. (2003). Food contents and biological activity of 25-hydroxyvitamin D: a vitamin D metabolite to be reckoned with? *Ann. Nutr. Metab.* 47, 107–113. doi: 10.1159/000070031

Parekh, N., Chappell, R. J., Millen, A. E., Albert, D. M., and Mares, J. A. (2007). Association between Vitamin D and age-related macular degeneration in the third National Health and Nutrition Examination Survey, 1988 through 1994. *Arch. Ophthalmol.* 125, 661–669. doi: 10.1001/archopht.125.5.661

Parrish, D. B. (1979). Determination of vitamin D in foods: a review. *CRC Crit. Rev. Food Sci. Nutr.* 12, 29–57. doi: 10.1080/10408397909527272

Perrine, C. G., Sharma, A. J., Jefferds, M. E. D., Serdula, M. K., and Scanlon, K. S. (2010). Adherence to vitamin D recommendations among US infants. *Pediatrics* 125, 627–632. doi: 10.1542/peds.2009-2571

Pfeifer, M., Begerow, B., Minne, H. W., Abrams, C., Nachtigall, D., and Hansen, C. (2000). Effects of a short-term vitamin D and calcium supplementation on body sway and secondary hyperparathyroidism in elderly women. *J. Bone Miner. Res.* 15, 1113–1118. doi: 10.1359/jbmr.2000.15.6.1113

Pfeifer, M., Begerow, B., Minne, H. W., Suppan, K., Fahrleitner-Pammer, A., and Dobnig, H. (2009). Effects of a long-term vitamin D and calcium supplementation on falls and parameters of muscle function in community-dwelling older individuals. *Osteoporos. Int.* 20, 315–322. doi: 10.1007/s00198-008-0662-7

Phillips, K. M., Ruggio, D. M., Horst, R. L., Minor, B., Simon, R. R., Feeney, M. J., et al. (2011). Vitamin D and sterol composition of 10 types of mushrooms from retail suppliers in the United States. *J. Agric. Food Chem.* 59, 7841–7853. doi: 10.1021/jf104246z

Pike, J. W., Lee, S. M., and Meyer, M. B. (2014). Regulation of gene expression by 1,25-dihydroxyvitamin D in bone cells: exploiting new approaches and defining new mechanisms. *Bonekey Rep.* 3, 482. doi: 10.1038/bonekey.2013.216

Pilz, S., Gaksch, M., O'Hartaigh, B., Tomaschitz, A., and März, W. (2013a). The role of vitamin D deficiency in cardiovascular disease: where do we stand in 2013? *Arch. Toxicol.* 87, 2083–2103. doi: 10.1007/s00204-013-1152-z

Pilz, S., Kienreich, K., Rutters, F., De Jongh, R., Van Ballegooijen, A. J., Grubler, M., et al. (2013b). Role of vitamin D in the development of insulin resistance and type 2 diabetes. *Curr. Diab. Rep.* 13, 261–270. doi: 10.1007/s11892-012-0358-4

Pittas, A. G., Lau, J., Hu, F. B., and Dawson-Hughes, B. (2007). The role of vitamin D and calcium in type 2 diabetes. A systematic review and meta-analysis. *J. Clin. Endocrinol. Metab.* 92, 2017–2029. doi: 10.1210/jc.2007-0298

Platt, B. S. (1956). Sir Edward Mellanby, 8 April 1884-30 January 1955. *Br. J. Nutr.* 10, 177–181.

Prentice, A. (2008). Vitamin D deficiency: a global perspective. *Nutr. Rev.* 66, S153–S164. doi: 10.1111/j.1753-4887.2008.00100.x

Prince, R. L., Austin, N., Devine, A., Dick, I. M., Bruce, D., and Zhu, K. (2008). Effects of ergocalciferol added to calcium on the risk of falls in elderly high-risk women. *Arch. Int. Med.* 168, 103–108. doi: 10.1001/archinternmed.2007.31

Ramagopalan, S. V., Heger, A., Berlanga, A. J., Maugeri, N. J., Lincoln, M. R., Burrell, A., et al. (2010). A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution. *Genome Res.* 20, 1352–1360. doi: 10.1101/gr.107920.110

Ramos-Lopez, E., Jansen, T., Ivaskevicius, V., Kahles, H., Klepzig, C., Oldenburg, J., et al. (2006). Protection from type 1 diabetes by vitamin D receptor haplotypes. *Ann. N Y. Acad. Sci.* 1079, 327–334. doi: 10.1196/annals.1375.050

Redzic, M., Lewis, R. M., and Thomas, D. T. (2013). Relationship between 25-hydoxyvitamin D, muscle strength, and incidence of injury in healthy adults: a systematic review. *Nutr. Res.* 33, 251–258. doi: 10.1016/j.nutres.2013.02.007

Roberts, J. S., Teichert, A., and McHugh, T. H. (2008). Vitamin D2 formation from post-harvest UV-B treatment of mushrooms (Agaricus bisporus) and retention during storage. *J. Agricultur. Food Chem.* 56, 4541–4544. doi: 10.1021/jf0732511

Romagnoli, E., Mascia, M. L., Cipriani, C., Fassino, V., Mazzei, F., D'Erasmo, E., et al. (2008). Short and long-term variations in serum calciotropic hormones after a single very large dose of ergocalciferol (vitamin D2) or cholecalciferol (vitamin D_3) in the elderly. *J. Clin. Endocrinol. Metab.* 93, 3015–3020. doi: 10.1210/jc.2008-0350

Rosen, C. J., Adams, J. S., Bikle, D. D., Black, D. M., Demay, M. B., Manson, J. E., et al. (2012). The nonskeletal effects of vitamin D: an Endocrine Society scientific statement. *Endocr. Rev.* 33, 456–492. doi: 10.1210/er.2012-1000

Ross, A. C., Manson, J. E., Abrams, S. A., Aloia, J. F., Brannon, P. M., Clinton, S. K., et al. (2011). The 2011 report on dietary reference intakes for calcium and vitamin d from the institute of medicine: what clinicians need to know. *J. Clin. Endocrinol. Metab.* 96, 53–58. doi: 10.1210/jc.2010-2704

Salahuddin, N., Ali, F., Hasan, Z., Rao, N., Aqeel, M., and Mahmood, F. (2013). Vitamin D accelerates clinical recovery from tuberculosis: results of the SUCCINCT Study [Supplementary Cholecalciferol in recovery from tuberculosis]. A randomized, placebo-controlled, clinical trial of vitamin D supplementation in patients with pulmonary tuberculosis'. *BMC Infect. Dis.* 13:22. doi: 10.1186/1471-2334-13-22

Schmid, A., and Walther, B. (2013). Natural vitamin D content in animal products. *Adv. Nutr.* 4, 453–462. doi: 10.3945/an.113.003780

Schottker, B., Herder, C., Rothenbacher, D., Perna, L., Muller, H., and Brenner, H. (2013). Serum 25-hydroxyvitamin D levels and incident diabetes mellitus type 2: a competing risk analysis in a large population-based cohort of older adults. *Eur. J. Epidemiol.* 28, 267–275. doi: 10.1007/s10654-013-9769-z

Schroten, N. F., Ruifrok, W. P., Kleijn, L., Dokter, M. M., Sillje, H. H., Lambers Heerspink, H. J., et al. (2013). Short-term vitamin D_3 supplementation lowers plasma renin activity in patients with stable chronic heart failure: an open-label, blinded end point, randomized prospective trial (VitD-CHF trial). *Am. Heart J.* 166, 357–364.e352. doi: 10.1016/j.ahj.2013.05.009

Schwalfenberg, G. K. (2011). A review of the critical role of vitamin D in the functioning of the immune system and the clinical implications of vitamin D deficiency. *Mol. Nutr. Food Res.* 55, 96–108. doi: 10.1002/mnfr.201000174

Sohl, E., Van Schoor, N. M., De Jongh, R. T., Visser, M., Deeg, D. J., and Lips, P. (2013). Vitamin d status is associated with functional limitations and functional decline in older individuals. *J. Clin. Endocrinol. Metab.* 98, E1483–E1490. doi: 10.1210/jc.2013-1698

Solomon, A. J., and Whitham, R. H. (2010). Multiple sclerosis and vitamin D: a review and recommendations. *Curr. Neurol. Neurosci. Rep.* 10, 389–396. doi: 10.1007/s11910-010-0131-5

Strobel, N., Buddhadasa, S., Adorno, P., Stockham, K., and Greenfield, H. (2013). Vitamin D and 25-hydroxyvitamin D determination in meats by LC-IT-MS. *Food Chem.* 138, 1042–1047. doi: 10.1016/j.foodchem.2012.08.041

Sundar, I. K., and Rahman, I. (2011). Vitamin D and susceptibility of chronic lung diseases: role of epigenetics. *Front. Pharmacol.* 2:50. doi: 10.3389/fphar.2011.00050

Takeuchi, A., Okano, T., Ayame, M., Yoshikawa, H., Teraoka, S., Murakami, Y., et al. (1984). High-performance liquid chromatographic determination of vitamin D_3 in fish liver oils and eel body oils. *J. Nutr. Sci. Vitaminol.* 30, 421–430. doi: 10.3177/jnsv.30.421

Takeuchi, A., Okano, T., Sayamoto, M., Sawamura, S., Kobayashi, T., Motosugi, M., et al. (1986). Tissue distribution of 7-dehydrocholesterol, vitamin D_3 and 25-hydroxyvitamin D_3 in several species of fishes. *J. Nutr. Sci. Vitaminol.* 32, 13–22. doi: 10.3177/jnsv.32.13

Tamez, H., and Thadhani, R. I. (2012). Vitamin D and hypertension: an update and review. *Curr. Opin. Nephrol. Hypertens.* 21, 492–499. doi: 10.1097/MNH.0b013e3283557bf0

Tarroni, P., Villa, I., Mrak, E., Zolezzi, F., Mattioli, M., Gattuso, C., et al. (2012). Microarray analysis of $1,25(OH)_2D_3$ regulated gene expression in human primary osteoblasts. *J. Cell. Biochem.* 113, 640–649. doi: 10.1002/jcb.23392

Teichmann, A., Dutta, P. C., Staffas, A., and Jägerstad, M. (2007). Sterol and vitamin D_2 concentrations in cultivated and wild grown mushrooms: effects of UV irradiation. *LWT Food Sci. Technol.* 40, 815–822. doi: 10.1016/j.lwt.2006.04.003

Tieland, M., Brouwer-Brolsma, E. M., Nienaber-Rousseau, C., Van Loon, L. J., and De Groot, L. C. (2013). Low vitamin D status is associated with reduced muscle mass and impaired physical performance in frail elderly people. *Eur. J. Clin. Nutr.* 67, 1050–1055. doi: 10.1038/ejcn.2013.144

Tippetts, M., Martini, S., Brothersen, C., and McMahon, D. J. (2012). Fortification of cheese with vitamin D_3 using dairy protein emulsions as delivery systems. *J. Dairy Sci.* 95, 4768–4774. doi: 10.3168/jds.2011-5134

Trang, H. M., Cole, D. E., Rubin, L. A., Pierratos, A., Siu, S., and Vieth, R. (1998). Evidence that vitamin D_3 increases serum 25-hydroxyvitamin D more efficiently than does vitamin D_2. *Am. J. Clin. Nutr.* 68, 854–858.

Trenerry, V. C., Plozza, T., Caridi, D., and Murphy, S. (2011). The determination of vitamin D_3 in bovine milk by liquid chromatography mass spectrometry. *Food Chem.* 125, 1314–1319. doi: 10.1016/j.foodchem.2010.09.097

Troesch, B., Hoeft, B., McBurney, M., Eggersdorfer, M., and Weber, P. (2012). Dietary surveys indicate vitamin intakes below recommendations are common in representative Western countries. *Br. J. Nutr.* 108, 692–698. doi: 10.1017/S0007114512001808

Tsur, A., Feldman, B. S., Feldhammer, I., Hoshen, M. B., Leibowitz, G., and Balicer, R. D. (2013). Decreased serum concentrations of 25-hydroxycholecalciferol are associated with increased risk of progression to impaired fasting glucose and diabetes. *Diabetes Care* 36, 1361–1367. doi: 10.2337/dc12-1050

U.S. Department of Agriculture. (2013). *USDA National Nutrient Database for Standard Reference, Release 26. Nutrient Data Laboratory Home Page*. Available online at: http://www.ars.usda.gov/ba/ bhnrc/ndl (Accessed February 10, 2014).

Van Belle, T. L., Gysemans, C., and Mathieu, C. (2013). Vitamin D and diabetes: the odd couple. *Trends Endocrinol. Metab.* 24, 561–568. doi: 10.1016/j.tem.2013.07.002

van Rossum, C. T. M., Fransen, H. P., Verkaik-Kloosterman J., Buurma-Rethans, E. J. M., and Ocke, M. C. (2011). *Dutch National Food Consumption Survey 2007–2010: Diet of Children and Adults Aged 7 to 69 Years*. Bilthoven: RIVM.

Wacker, M., and Holick, M. (2013). Vitamin D—effects on skeletal and extraskeletal health and the need for supplementation. *Nutrients* 5, 111–148. doi: 10.3390/nu5010111

Wagner, D., Sidhom, G., Whiting, S. J., Rousseau, D., and Vieth, R. (2008). The bioavailability of vitamin D from fortified cheeses and supplements is equivalent in adults. *J. Nutr.* 138, 1365–1371.

Wahl, D. A., Cooper, C., Ebeling, P. R., Eggersdorfer, M., Hilger, J., Hoffmann, K., et al. (2012). A global representation of vitamin D status in healthy populations. *Arch. Osteoporos.* 7, 155–172. doi: 10.1007/s11657-012-0093-0

Webb, A. R., Kline, L., and Holick, M. F. (1988). Influence of season and latitude on the cutaneous synthesis of vitamin D_3: exposure to winter sunlight in boston and edmonton will not promote vitamin D_3 synthesis in human skin. *J. Clin. Endocrinol. Metab.* 67, 373–378. doi: 10.1210/jcem-67-2-373

Welsh, J. (2012). Cellular and molecular effects of vitamin D on carcinogenesis. *Arch. Biochem. Biophys.* 523, 107–114. doi: 10.1016/j.abb.2011.10.019

White, J. H. (2012). Vitamin D metabolism and signaling in the immune system. *Rev. Endocr. Metab. Disord.* 13, 21–29. doi: 10.1007/s11154-011-9195-z

Windaus, A., Schenck, F., and Von Werder, F. (1936). Über das antirachitisch wirksame Bestrahlungsprodukt aus 7-Dehydrocholesterin. *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* 241, 100–103. doi: 10.1515/bchm2.1936.241.1-3.100

Zadshir, A., Tareen, N., Pan, D., Norris, K., and Martins, D. (2005). The prevalence of hypovitaminosis D among US adults: data from the NHANES III. *Ethn. Dis.* 15, S5-97–S95-101.

Zhao, Y., Sun, Y., Ji, H. F., and Shen, L. (2013). Vitamin D levels in Alzheimer's and Parkinson's diseases: a meta-analysis. *Nutrition* 29, 828–832. doi: 10.1016/j.nut.2012.11.018

Conflict of Interest Statement: The authors are employees of DSM Nutritional Products Ltd. and declare to have no conflict of interest.

Received: 24 March 2014; accepted: 14 June 2014; published online: 11 July 2014.
*Citation: Bendik I, Friedel A, Roos FF, Weber P and Eggersdorfer M (2014) Vitamin D: a critical and essential micronutrient for human health. Front. Physiol. **5**:248. doi: 10.3389/fphys.2014.00248*
This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 21 April 2014
doi: 10.3389/fphys.2014.00151

Impact of vitamin D on immune function: lessons learned from genome-wide analysis

Rene F. Chun[1], Philip T. Liu[1], Robert L. Modlin[2], John S. Adams[1] and Martin Hewison[1]*

[1] *Department of Orthopaedic Surgery, Orthopedic Hospital Research Center, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, CA, USA*
[2] *Division of Dermatology, Department of Medicine, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, CA, USA*

Edited by:
Carsten Carlberg, University of Eastern Finland, Finland

Reviewed by:
Dieter Steinhilber, Goethe Universitat, Germany
Mieke Verstuyf, Katholieke Universiteit Leuven, Belgium

***Correspondence:**
Martin Hewison, Orthopedic Hospital Research Center, 615 Charles E. Young Drive South, Room 410D, Los Angeles, CA 90095-7358, USA
e-mail: mhewison@mednet.ucla.edu

Immunomodulatory responses to the active form of vitamin D (1,25-dihydroxyvitamin D, 1,25D) have been recognized for many years, but it is only in the last 5 years that the potential role of this in normal human immune function has been recognized. Genome-wide analyses have played a pivotal role in redefining our perspective on vitamin D and immunity. The description of increased vitamin D receptor (VDR) and 1α-hydroxylase (CYP27B1) expression in macrophages following a pathogen challenge, has underlined the importance of intracrine vitamin D as key mediator of innate immune function. It is now clear that both macrophages and dendritic cells (DCs) are able to respond to 25-hydroxyvitamin D (25D), the major circulating vitamin D metabolite, thereby providing a link between the function of these cells and the variations in vitamin D status common to many humans. The identification of hundreds of primary 1,25D target genes in immune cells has also provided new insight into the role of vitamin D in the adaptive immune system, such as the modulation of antigen-presentation and T cells proliferation and phenotype, with the over-arching effects being to suppress inflammation and promote immune tolerance. In macrophages 1,25D promotes antimicrobial responses through the induction of antibacterial proteins, and stimulation of autophagy and autophagosome activity. In this way variations in 25D levels have the potential to influence both innate and adaptive immune responses. More recent genome-wide analyses have highlighted how cytokine signaling pathways can influence the intracrine vitamin D system and either enhance or abrogate responses to 25D. The current review will discuss the impact of intracrine vitamin D metabolism on both innate and adaptive immunity, whilst introducing the concept of disease-specific corruption of vitamin D metabolism and how this may alter the requirements for vitamin D in maintaining a healthy immune system in humans.

Keywords: macrophage, dendritic cell, intracrine, antigen-presentation, antibacterial, CYP27B1, VDR

INTRODUCTION

Amongst the many reported extra-skeletal effects of vitamin D, its ability to regulate immunity through effects on both the innate and adaptive systems has received considerable attention. This stems in part from homage to studies carried out more than a century ago by a then relatively unknown scientist, Dr. Nils Finsen. In 1903 Dr. Finsen won the Nobel Prize for Medicine or Physiology for showing that he could cure the epidermal form of tuberculosis (TB), lupus vulgaris, using concentrated light irradiation (Moller et al., 2005). The subsequent discovery that exposure to ultra-violet light promotes epidermal synthesis of vitamin D led to further studies describing the successful use of oral vitamin D supplementation to treat lupus vulgaris, and other mycobacterial infections such as leprosy (Airey, 1946; Herrera, 1949). The advent of antibiotic therapies for infectious diseases appeared to have consigned these studies to the history books. However, in 2006 the work of Finsen returned to center stage as a consequence of a series of genome-wide analyses that revealed pathogen-induction of an intracrine vitamin D system in monocytes (Liu et al., 2006), and an associated mechanism for anti-mycobacterial actions of vitamin D (Wang et al., 2004), whilst also shedding light on how these responses may vary according to the vitamin D "status" of any given individual. With increasing awareness of vitamin D-deficiency across the globe (Holick, 2007), and ongoing discussions concerning the physiological and clinical relevance of this (Holick et al., 2011; Ross et al., 2011), these genome-wide analyses have played a pivotal role in defining our new perspective on non-classical vitamin D physiology. The current review will detail these developments and how they have helped to define a role for vitamin D in normal immune function.

ANTIBACTERIAL RESPONSES TO VITAMIN D

Despite its early use in the treatment of mycobacterial diseases such as TB and leprosy (Airey, 1946; Herrera, 1949), the immunomodulatory actions of vitamin D did not become clear until much later. Elucidation of this important non-classical action of vitamin D stemmed from two key observations. Firstly, most proliferating cells within the immune system express the nuclear receptor for active 1,25-dihydroxyvitamin D (1,25D)—the vitamin D receptor (VDR). Initial studies focused on 1,25D

binding capacity in cells from the adaptive immune system such as T and B lymphocytes (T and B cells) (Bhalla et al., 1983; Provvedini et al., 1983), with subsequent reports describing specific intracellular binding of 1,25D in cells from the innate immune system such as monocytes/macrophages (Kreutz et al., 1993), dendritic cells (DC) (Brennan et al., 1987), neutrophils (Takahashi et al., 2002), and monocytic cell lines (Mangelsdorf et al., 1984). The functional significance of these data was not immediately clear but, nevertheless, it was assumed that VDR-expressing immune cells were able to respond the circulating active 1,25D in a similar fashion to classical vitamin D target tissues such as the intestine, kidney, and bone. However, this assumption was challenged by the second major observation linking vitamin D and the immunity, namely the discovery of active vitamin D metabolism by cells from the immune system.

Elevated serum levels of 1,25D reported for some patients with the granulomatous disease sarcoidosis were shown to be due to conversion of pro-hormone 25D to 1,25D by tissue and systemic macrophages in these patients (Barbour et al., 1981; Adams et al., 1983). Similar observations for other inflammatory and granulomatous diseases (Kallas et al., 2010) suggested that immune activity of the enzyme that catalyzes metabolism of 25D to 1,25D, 25-hydroxyvitamin D-1α-hydroxylase (1α-hydroxylase) was a disease-related phenomenon. However, other studies, *in vitro*, highlighting the potential for macrophage 1α-hydroxylase activity in the absence of disease (Koeffler et al., 1985; Reichel et al., 1986) supported the exciting possibility that synthesis of 1,25D is part of normal immune function. Despite this, it was another 20 years before evidence to support this proposal was reported. Significantly, the major advances that provided this evidence involved genome-wide strategies that explored both the regulation and function of vitamin D by immune cells.

The first of the genome-wide studies to shed light on extra-skeletal actions of vitamin D was published by John White and colleagues at McGill University in Montreal and utilized a combination of DNA array and *in silico* strategies. In this report, DNA array analysis of 1,25D-regulated genes in squamous cell carcinoma cells *in vitro* (Akutsu et al., 2001; Lin et al., 2002) was combined with *in silico* analysis of genomic VDR binding sites to provide a comprehensive overview of potential 1,25D-VDR target genes (Wang et al., 2005). Genome-wide analysis of DNA sequences that are able to bind liganded VDR revealed consensus vitamin D response elements (VDRE) within the gene promoters for two antibacterial proteins, cathelicidin (*CAMP*) and β-defensin 2 (*DEFB4*) (Wang et al., 2005). Interestingly, although both of these genes exhibited classical proximal promoter direct-repeat 3 (DR3) consensus VDREs, only *CAMP* appeared to be transcriptionally induced by 1,25D in monocytes (Wang et al., 2005). The underlying mechanism for the differential regulation of monocyte *CAMP* and *DEFB4* by 1,25D was elucidated in subsequent studies, the first of which described increased expression of monocyte *DEFB4* following co-treatment with 1,25D and the inflammatory cytokine interleukin-1 (IL-1) (Liu et al., 2009). Based on these observations and promoter analysis for the *CAMP* and *DEFB4* genes, it was concluded that transcriptional induction of *DEFB4* requires cooperative occupancy of nuclear factor-κB (NF-κB) response elements as well as VDRE within the *DEFB4* gene promoter. By contrast, induction of *CAMP* appears to be primarily dependent on binding of VDR to promoter VDRE (Liu et al., 2009). The importance of NF-κB and VDR as co-inducers of *DEFB4* transcription was further emphasized by studies of the intracellular pathogen sensing protein NOD2 which is itself transcriptionally induced by 1,25D (Wang et al., 2010b). Cells co-treated with 1,25D and the ligand for NOD2, muramyl dipeptide (MDP), a cell wall product of Gram-positive and Gram-negative bacteria, showed potent NF-κB-dependent induction of *DEFB4* (Wang et al., 2010b). In these studies expression of *CAMP* was also enhanced by 1,25D-MDP co-treatment, suggesting that NF-κB may cooperate with VDR in a variety of immunomodulatory functions (**Figure 1**).

Subsequent functional studies confirmed that dose-dependent vitamin D induction of *CAMP* transcription involves direct interaction between liganded VDR and VDRE in the *CAMP* gene promoter (Wang et al., 2004; Gombart et al., 2005). Intriguingly, the VDRE initially identified within the *CAMP* promoter appear to be specific for subhuman and human primates, as there are no similar motifs within equivalent genes for lower mammals (Gombart et al., 2005). Acquisition of a VDRE by the *CAMP* gene appears to have occurred following the introduction of an

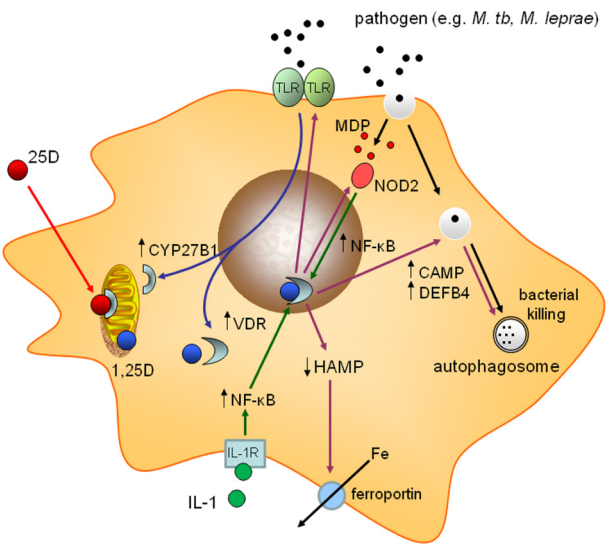

FIGURE 1 | Mechanisms for induction of vitamin D-mediated antibacterial responses in monocytes. Schematic representation of monocyte/macrophage responses to infection with a pathogen such as *Mycobacterium tuberculosis* (*M. tb*). Pattern recognition receptors (TLR2/1) sense *M. tb* and signal to induce expression of 1α-hydroxylase (CYP27B1) and the vitamin D receptor (VDR). The resulting intracrine system for vitamin D (blue arrows) converts 25-hydroxyvitamin D (25D) to 1,25-dihydroxyvitamin D (1,25D), which then binds to VDR and promotes transcriptional regulation. Prominent responses to intracrine activation of vitamin D (pink arrows) include: induction of antibacterial cathelicidin (CAMP) and β-defensin 2 (DEFB4); suppression of iron-regulatory hepcidin (HAMP); promotion of autophagy; induction of NOD2 expression; feedback regulation of toll-like receptor (TLR) expression; increased bacterial killing. For some responses (e.g., induction of DEFB4) accessory immune signals (MDP binding to NOD2, and IL-1 responsiveness) cooperate with intracrine vitamin D via nuclear factor-κB (NF-κB) (green arrows).

Alu short interspersed nuclear element (SINE) that placed *CAMP* under the control of 1,25D-VDR (Gombart et al., 2009b). This primate-specific adaptation has been conserved in humans and apes as well as Old World and New World primates, suggesting that a mechanism for transcriptional regulation of *CAMP* by vitamin D confers biological advantages. The assumption is that this mechanism will be potently activated by the relatively high circulating levels of 25D and 1,25D that are characteristic of non-human primates (Adams et al., 1985). A similar mechanism would also have been advantageous in early *Homo sapiens* whose existence is likely to have been defined by routine exposure to ultra-violet light and increased cutaneous vitamin D production, with associated high circulating levels of 25D. Conversely, in modern man where serum 25D status is more variable, this antibacterial mechanism may be less effective. Whilst this hypothetical mechanism broadly supports beneficial innate immune effects of vitamin D supplementation, the important question still remaining is how variations in serum levels of inactive 25D are able to influence immune responses driven by intracellular 1,25D and VDR. The answer to this question has been pivotal to our perspective on the non-classical actions of vitamin D and was, again, provided by genome-wide analyses.

For cells from both within and outside the classical immune system, recognition of and response to pathogens involves surveillance of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRR). Prominent amongst these PRRs is the extended family of Toll-like receptor (TLR) non-catalytic transmembrane receptors which interact with specific PAMPs (Moresco et al., 2011). To clarify the role of the TLR2/1 heterodimer in mediating innate immune responses to the TB pathogen, *Mycobacterium tuberculosis* (*M. tb*), Liu and colleagues carried out DNA array analysis of gene expression in human macrophages and DCs following treatment with one of the putative PRRs for *M. tb* (Liu et al., 2006). Amongst the many macrophage genes shown to be differentially regulated by 19 kDa lipoprotein (a TLR2-interacting PAMP), expression of both *CYP27B1* and *VDR* was increased (Liu et al., 2006) (see **Figure 1**). This genome-wide approach provided the first unbiased evidence of a role for vitamin D metabolism and signaling in innate immune responses to a pathogen. Crucially, the TLR2/1-stimulus induced expression of both *CYP27B1* and *VDR*, suggesting that macrophage responses to *M. tb* involve an endogenous, intracrine, vitamin D system. Further studies showed that macrophages co-treated with the TLR2/1-ligand, 19 kDa lipoprotein were responsive to both active 1,25D and inactive 25D, confirming the functional efficacy of the intracrine model. Moreover, inhibition of either 1α-hydroxylase activity, or VDR function blocked the actions of 25D, underlining the importance of cell-specific metabolism as a determinant of vitamin D immunoregulation.

The specific functional read-outs used in the TLR2/1-*M.tb* study were induction of mRNA for the vitamin D catabolic enzyme 24-hydroxylase (*CYP24A1)* and the antibacterial protein *CAMP* (Liu et al., 2006). Parallel analysis of the other antibacterial target gene for 1,25D, *DEFB4,* did not reveal significant induction of this gene in the presence of 19 kDa lipoprotein and 25D. However, as outlined above, subsequent experiments demonstrated that co-treatment with either IL-1 (Liu et al., 2009), or the NOD2 ligand MDP (Wang et al., 2010b) cooperates with the TLR2/1 ligand and 25D to stimulate expression of *DEFB4*. Antibacterial proteins such as CAMP and DEFB4 play a crucial role in vitamin D-mediated killing of intracellular bacteria. Monocytes treated with increasing concentrations of CAMP peptide show a dose-dependent decrease in the viability of internalized *M. tb* (Liu et al., 2006); a similar inhibition of macrophage *M. tb* viability occurs in the presence of 25D, with this effect being abrogated by a VDR antagonist. In other studies knockdown of either *CAMP* or *DEFB4* decreased killing of *M. tb* in macrophages, suggesting that both antibacterial proteins are important in mediating vitamin D-induced responses to mycobacterial infection (Liu et al., 2009). Both CAMP and DEFB4 are detectable in the circulation where they are able to support innate immune responses to extra-cellular pathogens including anti-viral responses (Barlow et al., 2011; Tripathi et al., 2013). However, pathogens such as mycobacteria are internalized by phagocytosis, and bacterial killing can then take place following fusion of the resulting phagosome with a lysosome to form a phagolysosome. To evade this antibacterial process and maintain intracellular viability *M. tb* can subvert the transition of phagosomes to phagolysosomes (Vergne et al., 2004). In this situation, the host cell can restore pathogen encapsulation by evoking alternative mechanisms such as autophagy, in which encapsulation of organelles, cell proteins or intracellular pathogens in a double-membrane autophagosome occurs prior to fusion with lysosomes (Gutierrez et al., 2004). Although a well-recognized feature of eukaryotic cells cytosolic homeostasis (Klionsky and Emr, 2000), autophagy also appears to play a pivotal role in cellular response to infection (Gutierrez et al., 2004; Deretic and Levine, 2009). The ability of 1,25D and its synthetic analogs to promote autophagy is well established (Hoyer-Hansen et al., 2005; Wang et al., 2008), but recent data suggest that induction of autophagy may be particularly important for vitamin D-induced antibacterial responses to *M. tb* infection (Yuk et al., 2009; Shin et al., 2011) (**Figure 1**). The precise mechanism for this is not clear and may involve inhibition of the mammalian target of rapamycin (mTOR) intracellular signaling system (O'Kelly et al., 2006; Lisse et al., 2011) with mTOR acting to suppress the induction of autophagy (Sanjuan et al., 2009). It has also been suggested that vitamin D-induced autophagy occurs via an indirect mechanism, in that RNA-interference (RNAi) knockdown of antibacterial *CAMP* was sufficient to abrogate 1,25D-induced autophagy in monocytes (Yuk et al., 2009). In common with effects on expression of antibacterial proteins, it was noted that monocyte autophagy following activation of TLR2/1 involves enhanced expression of VDR and CYP27B1 (Shin et al., 2011), further highlighting the importance of intracrine 25D metabolism and action in normal human innate immunity.

Intracrine synthesis of 1,25D has also been shown to regulate expression of another antibacterial protein, hepcidin antibacterial protein (HAMP) (Bacchetta et al., 2013b). However, in contrast to CAMP and DEFB4, the direct microbiocidal properties of HAMP appear to be relatively weak. Instead, the major function of HAMP appears to be suppression of the cell membrane protein ferroportin, the only known exporter of intracellular iron

(Ganz, 2011). This link between HAMP and ferroportin in cells such as enterocytes, hepatocytes and monocytes plays a key role in the so-called anemia of infection or chronic disease (Ganz, 2009). Because pathogens such as bacteria utilize iron to maintain growth, restriction of circulating iron concentrations provides an important host response to systemic infection (Drakesmith and Prentice, 2012). However, for pathogens such as *M. tb* that attempt to evade immune surveillance at the intracellular level, accumulation of iron within this environment may promote the growth of internalized pathogens such as *Salmonella typhimurium* (Nairz et al., 2007), *M. tb* (Schaible et al., 2002; Sow et al., 2007, 2009), and *Chlamydia psittaci* (Paradkar et al., 2008). Conversely, innate immune and viral stimuli are known to stimulate the expression of *HAMP* (Sow et al., 2009; Armitage et al., 2011). In this setting suppression, rather than induction, of HAMP by 25D and 1,25D may be beneficial by abrogating HAMP-induced suppression of ferroportin which, in turn, will favor iron export and lower intracellular concentrations of iron. In studies carried out by our group at UCLA, we have shown that 25D and 1,25D suppress transcription of *HAMP* in monocytes and hepatocytes, leading to increased membrane expression of ferroportin, and decreased expression of ferritin (a surrogate biomarker for intracellular iron concentrations) (Bacchetta et al., 2013b). Moreover, in contrast to CAMP and DEFB4, elevated serum 25D levels (but not 1,25D) following vitamin D supplementation of human subjects *in vivo* were associated with potent suppression of circulating concentrations of HAMP (Bacchetta et al., 2013b). It therefore appears that regulation of the HAMP-ferroportin axis is another key facet of vitamin D-mediated innate immune function, complimentary to its reported effects on antibacterial proteins (Liu et al., 2006; Adams and Hewison, 2008; Hewison, 2011), and autophagy (Yuk et al., 2009; Shin et al., 2011) (see **Figure 1**). However, it is important to recognize that the effect of vitamin D on serum levels of hepcidin may have additional consequences that are both positive (suppression of anemia) and negative (decreased hepcidin for systemic infection). This may be particularly important for patients with chronic kidney disease (CKD) who commonly present with impaired circulating levels of 25D and 1,25D, and who are at higher risk of infection. In CKD, low serum 25D has been shown to correlate inversely with anemia (Lac et al., 2010) and directly with blood hemoglobin levels (Kiss et al., 2011). These effects may be due to dysregulation of normal HAMP-ferroportin function under conditions of vitamin D-deficiency, further emphasizing the importance of vitamin D supplementation in these patients.

Vitamin D may also target other innate immunity mechanisms. For example, studies *in vitro* have shown that 1,25D promotes hyporesponsiveness to PAMPs by downregulating expression of TLR2 and TLR4 on monocytes (Sadeghi et al., 2006). In this way, vitamin D appears to promote feedback control pathways that limit antibacterial activity and other innate immune responses, thereby preventing potential inflammatory events that arise from an over-elaboration of immune responses, notably inflammatory T cell responses. Paradoxically, vitamin D can also promote responses that amplify innate immune function. Recent studies have described 1,25D-mediated induction of the triggering receptor on myeloid cells-1 (TREM-1) (Kim et al., 2013), a cell surface protein associated with cytokine and chemokine production (Bouchon et al., 2000) that can also act to amplify TLR signaling (Bouchon et al., 2001). The biological significance of this is still not clear and this mechanism may be more important for cells such as neutrophils which are the principal source of circulating CAMP. Neutrophils express VDR but, unlike monocytes/macrophages, they do not appear to express a functional 1α-hydroxylase and are therefore not subject to intracrine activation of innate immune function. In this setting, activation of proteins such as TREM-1 may help to promote neutrophil responses to circulating 1,25D rather than 25D through enhanced TLR-signaling. This, in turn, would stimulate expression of VDR and sensitivity to 1,25D.

A similar cooperative TLR response has also been described for epithelial keratinocytes, where basal expression of *CYP27B1* is insufficient to facilitate intracrine induction of antibacterial proteins by serum 25D. However, following skin wounding, locally generated transforming growth factor β (TGFβ) enhances expression of CYP27B1 (Schauber et al., 2007). The resulting TGFβ-driven *CYP27B1* expression is then able to stimulate intracrine generation of antibacterial proteins such as CAMP to combat potential infections associated with epidermal injury (Schauber et al., 2007). Interestingly, the TGFβ-induced 1α-hydroxylase activity was also associated with increased keratinocyte expression of TLR2 which further enhances surveillance of infectious bacteria, but also suggests that the effects of vitamin D on TLR expression are likely to be cell-specific. TGFβ and 1,25D may also cooperate to promote expression of other pathways linked to enhanced innate immune responses to infection such as induction of the enzyme 5-lipoxygenase (5-LO) that catalyzes synthesis of leukotrienes. Expression of 5-LO in human monocytes is induced by both 1,25D and TGFβ (Harle et al., 1998), with 1,25D enhancing expression of 5-LO through novel promoter-independent VDRE within exons 10 and 12 and intron M of the 5-LO gene (Stoffers et al., 2010). Although commonly associated with bronchial dilation and asthma, leukotrienes are also known to participate in leukocyte accumulation at sites of infection and phagocytosis of bacteria (Peters-Golden et al., 2005). Leukotrienes have also been shown to trigger the processing of antibacterial CAMP by neutophils (Wan et al., 2007).

Vitamin D-mediated innate immune responses may also be species-specific. VDR-mediated induction of *CAMP* and *DEFB4*, as well as suppression of *HAMP*, appears to be primate-specific; other mammals may therefore utilize alternative innate immunity targets for intracrine 1,25D. For example reactive oxygen species (ROS) can be bacteriocidal; previous studies have shown that macrophages infected with *M. tb* in the presence of 1,25D produce high levels of the superoxide anion ROS via the NADPH oxidase system (Sly et al., 2001). More recent studies have shown that another ROS, nitric oxide (NO), is produced by mouse macrophages as part of innate immune responses to infection, with bacteriocidal consequences (Kohchi et al., 2009). The NO pathway appears to play a pivotal role in mouse responses to *M. tb* infection (Chan et al., 1992), but its importance to human *M. tb* infection is less clear. Moreover, one study using 1,25D and mouse macrophages has reported decreased expression of the enzyme inducible nitric oxide synthase (iNOS) and its NO

product, suggesting that the link between vitamin D and NO in innate immune function is more complex than originally thought (Chang et al., 2004). Irrespective of the antibacterial mechanism that is utilized by animals such as mice, it is generally assumed that vitamin D-mediated induction of these responses will occur via the same intracrine monocyte mechanism that has been described for humans. Although expression of CYP27B1 and 1α-hydroxylase activity has been described for murine macrophages *in vitro* (Esteban et al., 2004; Stoffels et al., 2007), the relative importance of this *in vivo* is still unclear. Indeed recent studies using the CYP27B1 KO mouse have suggested that $CD8^+$ cytotoxic T cells are the predominant source of extra-renal 1,25D within the murine immune system (Ooi et al., 2014). Further studies are required to fully clarify the physiological importance of this observation.

Genome-wide analyses and associated *ex vivo* and *in vitro* experiments have clearly demonstrated the potential importance of vitamin D in maintaining optimal innate antibacterial responses in humans. However, these studies have also prompted three further crucially important questions: (1) how important is vitamin D for the adaptive immune system? (2) can vitamin D supplementation *in vivo* enhance these antibacterial responses? (3) what happens to the vitamin D system in human immune diseases? Each of these questions will be considered in the remaining sections of this review.

VITAMIN D AND ANTIGEN PRESENTATION

In the seminal DNA array analysis of monocyte TLR2/1 responses by Liu et al that highlighted induction of *CYP27B1* and *VDR* by *M. tb*, it was notable that DCs did not produce the same response when challenged with 19 kDa lipoprotein, despite expressing TLR2/1 (Liu et al., 2006). Monocytes/macrophages belong to the same hematopoietic lineage as DCs, and both types of cells are able to act as antigen-presenting cells (APCs) to promote T cell and B cells responses. Furthermore like, monocytes, DCs express *VDR* and *CYP27B1*, and exhibit an active intracrine vitamin D system (Brennan et al., 1987; Fritsche et al., 2003; Hewison et al., 2003). However, in contrast to monocytes/macrophages, the primary function on intracrine vitamin D in DCs appears to be as a regulator of cell maturation, and ability of DCs to present antigen to T cells (Hewison et al., 2003). Differentiation of DCs toward a mature APC is associated with increased expression of *CYP27B1* but, paradoxically, a reciprocal decrease in *VDR* (Hewison et al., 2003). It therefore seems likely that DCs will utilize a paracrine vitamin D system, with immature DCs expressing VDR and responding to 1,25D produced by mature DCs with lower VDR expression. Such a mechanism may be biologically advantageous in that it allows some DCs to mature and promote T cell activation as part of normal adaptive immune responses, whilst preventing an over-elaboration of this response that could lead to inflammatory complications. A similar pattern of differential regulation of CYP27B1 and VDR has also been described for monocytes differentiating toward macrophages (Kreutz et al., 1993). The importance of 1,25D as a modulator of DC function is endorsed by studies of *VDR* and *CYP27B1* knockout mice, which present with lymphatic abnormalities consistent with increased numbers of mature DCs (Griffin et al., 2001; Panda et al., 2001) and dysregulated DC trafficking (Enioutina et al., 2009).

In a similar fashion to macrophages, DCs can be divided into distinct sub-types, specifically myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). These cells exhibit different cytokine and chemokine profiles and exert complementary effects on T cells; mDCs are efficient promoters of naïve T cell function (Liu, 2005), whilst pDCs are more closely associated with attenuation of T cell function (Steinman et al., 2003). *In vitro*, 1,25D preferentially regulates mDCs, with associated suppression of naïve T cell activation (Penna et al., 2007). However mDC and pDC express similar levels of VDR, so tolerogenic pDC may also respond to 1,25D, possibly via local, intracrine mechanisms (Penna et al., 2007). Alternatively, 1,25D generated by pDCs may not act to regulate pDC maturation but may, instead, act in a paracrine fashion on VDR-expressing T-cells. The ability of vitamin D to influence the differentiation and function of DCs provides another layer of innate immune function that complements its antibacterial properties. However, this interaction between 1,25D and DC will also have downstream effects on cells that interact with APCs, namely cells from the adaptive immune system.

Consistent with the DNA array analyses that shed light on the antibacterial function of vitamin D in monocytes and macrophages (Liu et al., 2006), genome-wide analysis of DCs has revealed diverse responses to vitamin D in these cells. Proteomic analyses using matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF)/TOF strategies has defined the key proteins associated with tolerogenic responses to 1,25D (Ferreira et al., 2012). Intriguingly, the dominant effect of 1,25D treatment of monocyte-derived DCs described in this study was the alteration of proteins associated with the cytoskeleton and metabolic function. The induction of cytoskeletal proteins was shown to be consistent with DC responses to other tolerogenic steroid hormones, such as glucococorticoids (Ferreira et al., 2012). However, by contrast, the potent effects of 1,25D on metabolic pathways in DCs appear to be distinct from the effects of glucocorticoids. In particular, 1,25D induced significantly more proteins associated with carbohydrate metabolism, gluconeogenesis and the TCA cycle relative to the glucocorticoid dexamethasone, whilst 1,25D and dexamethasone shared induction of other groups of proteins such as those associated with glycolysis (Ferreira et al., 2012). This particular study also illustrates a key advantage of genome/proteome-wide analyses, which is the ability to group changes in gene/protein expression according to specific properties such as metabolism or cytoskeletal function. Moreover, more recent developments allow researchers to utilize tools such as Ingenuity Pathway Analysis (IPA) or DAVID to cluster altered genes and show protein or gene interaction networks (Hong et al., 2009). These "interactomes" provide a picture of the cooperativity of responses to a particular cell treatment. For example, recent proteomic analysis of responses to the synthetic 1,25D analog TX527 in immature and mature DCs showed that 65–75% of the proteins identified as TX527 responsive made up a statistically significant interactome, with some commonality between the two DC types (Ferreira et al., 2009). In this particular study the authors used multiple sets of data including the Biomolecular Interaction Network Database (BIND) (Bader et al.,

2003), and the Molecular Interaction Database (MINT) (Zanzoni et al., 2002) to maximize potential interactions. The increasing availability of gene/protein expression databases for different cell types means that this type of strategy is likely to become a more prominent feature of genome-wide expression analyses in the future.

VITAMIN D AND ADAPTIVE IMMUNITY

As outlined above, one of the initial observations linking vitamin D with the immune system was the presence of VDR in activated lymphocytes (Bhalla et al., 1983; Provvedini et al., 1983). The development of lymphocytes takes place in the thymus with VDR being expressed in medullary thymocytes but not in the less mature cortical thymocytes (Ravid et al., 1984). However, once cells leave the thymus and enter the circulation as T or B cells VDR expression is lost until these cells are activated to proliferate by mitogens (Bhalla et al., 1983; Provvedini et al., 1983). Indeed, 1,25D is a potent inhibitor of T-cell proliferation, blocking the transition from early G1 phase to late G1 phase (Bhalla et al., 1984; Nunn et al., 1986), but having no effect on transition from Go (resting) to early G1 or from late G1 to S phase (Rigby et al., 1985). Studies using T cells isolated from lymphatic tissue have shown that expression of VDR and responsiveness to 1,25D is proportional to the rate of cell proliferation (Karmali et al., 1991). Although these early studies have highlighted a role for 1,25D as a regulator of T and B cell proliferation, it has become increasingly clear that the predominant effects of vitamin D on adaptive immune function involve the modulation of T cell phenotype.

T cells consists of several sub-groups including cytotoxic $CD8^+$ T cells, natural killer cells, γδ T cells, memory cells, $CD4^+$ helper T cells (Th cells), and regulatory T cells (Treg). The best characterized vitamin D responses have been described for Th cells, with 1,25D regulating T cell proliferation and cytokine production (Lemire et al., 1985). Activation of naïve Th cells by antigen and APCs generates pluripotent Th_0 cells which can then differentiate into further Th sub-groups based on distinct cytokine profiles. Two of these sub-groups, Th_1 (IL-2, IFNγ, tumor necrosis factor alpha) and Th_2 (IL-3, IL-4, IL-5, IL-10) T cells, respectively support cell-mediated and humoral immunity (Abbas et al., 1996; Romagnani, 2006). *In vitro* 1,25D inhibits expression of Th_1 cytokines (Lemire et al., 1995), whilst promoting Th_2 cytokines (Boonstra et al., 2001). More recently, other Th cell sub-groups have been identified, including interleukin-17 (IL-17)-secreting T-cells (Th_{17} cells) and these cells are also targets for vitamin D. In the autoimmune disease-susceptible non-obese diabetic (NOD) mouse treatment with 1,25D decreased expression of IL-17 (Penna et al., 2006). In a similar fashion, 1,25D suppression of murine retinal autoimmunity involves inhibition of Th17 activity (Tang et al., 2009).

In addition to its effects on Th cells, vitamin D may also act on $CD8^+$ cytotoxic T cells which express relatively high levels of VDR (Rigby et al., 1987; Provvedini and Manolagas, 1989; Veldman et al., 2000). As outlined above, $CD8^+$ cells in mice have also been reported to express the vitamin D-activating enzyme 1α-hydroxylase (Ooi et al., 2014). $CD8^+$ T cells are known to be involved in autoimmune disease such as multiple sclerosis (MS) (Babbe et al., 2000), but do not mediate the effects of 1,25D in suppressing the murine form of MS, experimental autoimmune encepholmyelitis (EAE) (Meehan and DeLuca, 2002). More recent studies have reported a link between vitamin D and a variant of $CD8^+$ T cells, CD8αα cells. Unlike $CD8^+$ T cells, CD8αα cells are not cytotoxic and may play a role in suppressing gastrointestinal inflammation (Cheroutre and Lambolez, 2008). VDR knockout mice exhibit decreased numbers of CD8αα cells (Yu et al., 2008), due to decreased T cell expression of the chemokine receptor CCR9 preventing T cell homing to the gastrointestinal tract. T cell homing defects provides a potential explanation for the increased colonic inflammation observed in VDR knockout mice when crossed with colitis disease-susceptible mice (Froicu et al., 2003). Vitamin D metabolites may also influence T cell homing in other tissues. In the skin, 1,25D stimulates expression of the chemokine receptor 10 (CCR10) which recognizes the chemokine CCL27 secreted by keratinocytes (Sigmundsdottir et al., 2007).

As well as acting as a modulator of Th cell phenotype and function, vitamin D can also influence adaptive immunity by promoting suppressor T cells known as regulatory T cells (Treg) (Barrat et al., 2002). The precise mechanism by which vitamin D regulates Tregs is still somewhat controversial. Initial studies *in vitro* suggested that the ability of 1,25D to promote $CD4^+$ $CD25^+$ Treg was due to indirect effects on antigen-presenting DCs, specifically suppression of DC maturation and increased expression of DC cytokines such as CCL22 (Penna et al., 2007). However, subsequent studies have also described direct effects of 1,25D on T cells to generate CTLA4-positive Treg (Jeffery et al., 2009). Significantly, these studies were focused on the use of active 1,25D as the immunomodulator, and it is only in more recent studies that the role of pro-hormone 25D in Treg development has been investigated (Jeffery et al., 2012). Data from this study demonstrated the ability of 25D to promote the generation of Treg through intracrine/paracrine effects on CYP27B1/VDR-expressing DCs. Notably, this report also highlighted the impact of vitamin D binding protein (DBP) on DC responses to 25D, and concluded that non-DBP-bound (free) 25D is the form of 25D that is biologically active for generation of Tregs (Jeffery et al., 2012). The importance of Treg as a facet of vitamin D immunomodulation is illustrated by various studies *in vivo*. In patients with CKD, systemic administration of 1,25D has been shown to increase numbers of circulating Treg (Ardalan et al., 2007). Conversely, in patients with MS, serum concentrations of 25D correlate with Treg activity (Royal et al., 2009; Smolders et al., 2009), underlining the importance of intracrine pathways in mediating effects of vitamin D on adaptive, as well as innate immunity. In mice, topical application of 1,25D (Gorman et al., 2007) or its synthetic analog calcipotriol (Ghoreishi et al., 2009) have been shown to increase numbers of Treg.

The effects of vitamin D on adaptive immunity have to date been very much focused on its ability to modulate T cell proliferation and phenotype. Nevertheless, early studies reported that 1,25D could also suppress the development of immunoglobulin (Ig)-secreting B cells following mitogenic stimulation (Shiozawa et al., 1985; Iho et al., 1986). Initial experiments suggested that the most likely mechanism for this was an indirect effect through inhibition of Th cells (Lemire et al., 1985), but more recent work

has shown that 1,25D can suppress the differentiation of two types of B cell, plasma cells and class-switched memory cells, through apparent direct effects (Chen et al., 2007). Other reports have shown that 1,25D can regulate B cell IL-10 (Heine et al., 2008) and CCR10 (Shirakawa et al., 2008), suggesting that the effects of 1,25D on these cells is not restricted to their capacity to produce immunoglobulin.

Although genome-wide screening has played a pivotal role in identifying pivotal mechanisms for the interaction between vitamin D and innate immunity, the same cannot be said for vitamin D and adaptive immunity, where genome-wide analyses have complemented an already well-established field of research. Nevertheless, it is interesting to note reports where this strategy has been applied. In some cases these analyses have revealed a role for the vitamin D system, similar to the seminal studies of *M. tb* induction of CYP27B1 and VDR. For example, transcriptional profiling of γδT cells reported induction of VDR following activation of these cells with non-peptidic monoalkyl phosphate ligands (Chen et al., 2005). This small sub-set of T cells plays an important role in inflammatory diseases, and it was therefore speculated that 1,25D may act to suppress these cells as part of a more generalized anti-inflammatory response. Further array analyses have also identified VDR as one of a discrete number of genes involved in the formation of B cell germinal centers (Nakayama et al., 2006).

Array analyses have also been used to characterize the gene regulatory effects associated with immunomodulatory responses to 1,25D. These studies have focused primarily on the effects of 1,25D and its synthetic analogs on DCs, with results underlining the ability of 1,25D to promote decreased antigen presentation and a tolerogenic phenotype in these cells (Griffin et al., 2004; Shen and Zheng, 2004; Pedersen et al., 2009; Szeles et al., 2009). Notably, one of these array studies showed that the effects of 1,25D on DC gene expression were independent of DC differentiation status, suggesting a specific role for 1,25D as a regulator of DC function (Szeles et al., 2009). This particular study also reported that key changes in DC gene expression could be achieved using either 1,25D or 25D, further emphasizing the functional importance of the intracrine vitamin D system in these cells. DNA microarray analyses have also been used to assess 1,25D-mediated regulation of gene expression in $CD4^+$ Th cells following activation of these cells by phorbol myristate acetate and a calcium ionophore to induce VDR (Mahon et al., 2003). The diverse array of gene targets regulated by 1,25D in this particular array analysis suggests that 1,25D can influence Th cells both directly, as well as via effects on antigen-presenting DCs.

VITAMIN D STATUS AND IMMUNE FUNCTION

It is important to recognize that most of the genome-wide analyses that have explored the immunomodulatory effects of vitamin D *in vitro* have focused on treatments using active 1,25D or one of its synthetic analogs. However, as outlined above, pathogen-induction of an intracrine system in cells such as monocytes/macrophages strongly suggests that regulation of immunity *in vivo* is independent of endocrine, systemic 1,25D. Instead it is likely to be primarily driven by local activation of 25D, the major circulating form of vitamin D and determinant of vitamin D status in any given individual. Thus, it is not surprising that translational studies have focused on the relationship between serum 25D and human immune function, including effects on both innate and adaptive immunity.

Epidemiology has shown that vitamin D-insufficiency (serum 25D <30 ng/ml) is associated with increased risk of TB (Wilkinson et al., 2000; Ustianowski et al., 2005; Williams et al., 2008; Wejse et al., 2009). Several clinical trials of vitamin supplementation, as an adjunct to conventional antibiotic therapy, have also been reported with varying success (Nursyam et al., 2006; Martineau et al., 2007; Wejse et al., 2009). Supplementation using 4 × 100,000 IU vitamin D was successful in raising serum concentrations of 25D in TB patients, but this resulted in no overall improvement in sputum conversion time between vitamin D- and placebo-treated patients (Martineau et al., 2011). However, improved sputum conversion time was observed in a specific subset of TB patients with a *Taq1* single nucleotide polymorphism (SNP) within the VDR gene (Martineau et al., 2011), suggesting that genetic factors may influence immune responses to vitamin D supplementation. In a follow-up report to this TB vitamin D supplementation trial, it was shown that raised serum 25D was associated with improved resolution of TB disease (Coussens et al., 2012). Thus, in situations where infectious disease has already become established, it is possible that the role of 25D is primarily focused on anti-inflammatory adaptive immune responses. The link between vitamin D and infection is not restricted to TB. In patients with sepsis, circulating 25D levels have been shown to correlate directly with serum concentrations of CAMP, and inversely with critical illness in these patients (Jeng et al., 2009). Low serum 25D has also been linked to upper respiratory infections such as influenza (Cannell et al., 2006), and in patients with CKD low serum is associated with increased risk of infection and mortality (Gombart et al., 2009a).

To date, the application of genome-wide analyses to further elucidate the impact of serum vitamin D (25D) status on immune function has been limited. In a recent study by Holick and colleagues, array analysis of gene expression in peripheral blood mononuclear cells from vitamin D-sufficient (serum 25D >20 ng/ml, $n = 4$ subjects) and vitamin D-deficient (serum 25D <20 ng/ml, $n = 4$ subjects) revealed 66 differentially expressed genes (>1.5-fold change, $p < 0.01$) (Hossein-nezhad et al., 2013). However, after vitamin D supplementation (2000 IU vitamin D/day for 2 months), there was no significant change in the expression of these genes, even though serum 25D levels were increased in both sufficient and deficient subjects (Hossein-nezhad et al., 2013). Nevertheless, 291 additional genes were found to be differentially expressed in peripheral blood mononuclear cells following vitamin D supplementation (>1.5-fold, $p < 0.01$) (Hossein-nezhad et al., 2013). Similar array analyses carried out by our group using peripheral blood mononuclear cells from elderly vitamin D-deficient (18.8 ± 0.6 ng/ml serum 25D) and vitamin D–sufficient (58 ± 1.7 ng/ml serum 25D) patients, revealed 30 differentially regulated genes (**Figure 2**). These variations in gene expression occurred against a backdrop of no difference in serum 1,25D concentrations between vitamin D-sufficient and -deficient groups, underlining the importance of 25D, and the intracrine vitamin D system as regulators of immune cell

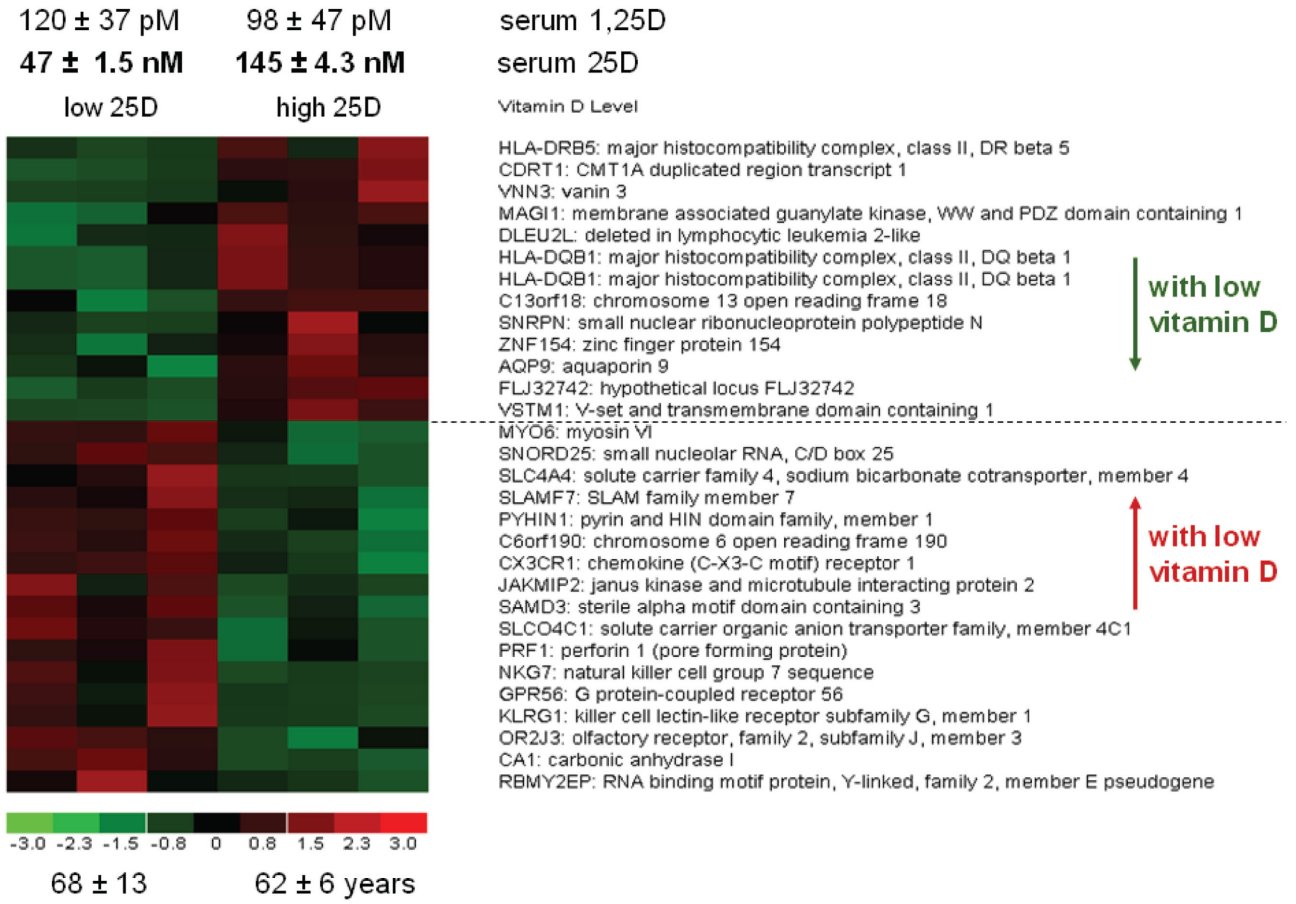

FIGURE 2 | Altered gene expression in leukocytes from vitamin D-deficient donors. Peripheral blood mononuclear cells (PBMC) were obtained from 6 healthy donors who had serum 25D levels that were either low (47 nM, 18.8 ng/ml) or high (145 nM, 58 ng/ml). After isolation of RNA and generation of cDNA, gene expression analyses in the PBMC samples was carried out by DNA array analyses (Affymetrix Exon Expr Chip.HuGene-1_0-st-v1). Data are shown as genes that were either increased (red) or decreased (green) in low 25D donors relative to high 25D donors (>1.5-fold change, $p < 0.05$, unpaired *t*-test).

function. In both this study and the Holick report, array analyses were carried out using mixed populations of systemic immune cells including both innate immunity APCs and lymphocytes of the adaptive immune system. The array analyses will therefore encapsulate both intracrine and paracrine activity of 25D, but will also reflect inherent donor to donor variations in immune cell composition.

Genome-wide analyses of immune responses to altered vitamin D status in mice are also very limited. Data from our group using colon tissue from vitamin D-deficient (serum 25D = 2.5 ± 0.1 ng/ml) vs. vitamin D-sufficient (serum 25D = 24.4 ± 1.8 ng/ml) identified 31 genes that were differentially expressed >2-fold ($p < 0.01$) (Lagishetty et al., 2010). Amongst these, vitamin D-deficient mice showed decreased expression of angiogenin-4 (Ang4), an antimicrobial protein which acts to minimize tissue invasion by enteric bacteria (Hooper et al., 2003). Further studies showed that decreased Ang4 in vitamin D-deficient mice was associated with increased levels of bacteria in the colon epithelium, consistent with compromised innate immune surveillance. Given that dysregulation of innate immune responses to enteric bacteria has been linked to the initiation of tissue inflammation associated with some types of inflammatory bowel disease (Packey and Sartor, 2009), it is possible that vitamin D plays a role in protecting against this disease via the induction of antibacterial Ang4.

Another genome-wide strategy with implications for vitamin D and the immune system, arose from studies aimed at determining the genetic component of vitamin D-deficiency. A recent Genome-Wide Association Study of almost 34,000 individuals showed that SNPs within the *DBP* gene are a key inherited determinant of low vitamin D status (serum 25D <75 nM or 30 ng/ml). Gene variations in *DBP* appear to act by influencing the serum concentrations of DBP protein (Lauridsen et al., 2001) which are known to be linked to serum levels of total 25D and 1,25D (Lauridsen et al., 2005; Wang et al., 2010a). Studies of other *DBP* SNPs suggest that genetic variants of *DBP* are linked to different binding affinities of 25D for DBP protein (Arnaud and Constans, 1993). Both the concentration and binding affinity of DBP protein are important for the serum transport of vitamin D metabolites (notably 25D which binds to DBP with a higher

affinity than 1,25D). However, DBP concentration and affinity also define the amount of 25D that is *not* bound to DBP. This "free" or "bioavailable" fraction of circulating 25D appears to be the form that accesses target cells such as monocytes (Chun et al., 2012), presumably via passive diffusion of lipid-soluble 25D through cell membranes—the so-called "free hormone hypothesis." Studies by our group have shown that antibacterial responses to 25D *in vitro* are more pronounced with low affinity forms of DBP that support higher levels of free 25D (Chun et al., 2010). Studies to date have been based on mathematical estimations of free 25D from total serum concentrations of 25D and DBP (Chun et al., 2012). However, future strategies using actual physical measurement of free 25D will greatly help to clarify the precise importance of total vs. free 25D in determining immune responses to vitamin D.

IMMUNE DISEASE AND THE DYSREGULATION OF VITAMIN D

Genome-wide strategies have played a pivotal role in elucidating the core mechanisms that trigger the intracrine vitamin D system and associated immune responses in cells such as monocytes/macrophages and DCs. Whether these studies have been carried out using freshly isolated preparations of immune cells, or cultured immune cells the resulting data have reflected the potential vitamin D-mediated responses that may occur following a pathogen challenge. What is less clear is how these responses function under conditions of actual human immune disease. An illuminating example of this strategy is provided by the disease leprosy which, like TB, involves a mycobacterial infection—in this case *Mycobacterium leprae* (*M. lep*) or *Mycobacterium lepromatis*. Similar to TB, vitamin D was at one time considered to be a putative therapy for leprosy (Herrera, 1949). However, unlike TB, leprosy can be divided into different disease sub-types, notably tuberculoid leprosy (T-lep) and lepromatous leprosy (L-lep). These two forms of leprosy have very different immune profiles and prognoses (Britton and Lockwood, 2004). DNA array analyses to define the gene expression profiles associated with the T-lep and L-lep forms of leprosy, highlighted elevated expression of CYP27B1, CYP24A1, and VDR in T-lep vs. L-lep lesions (Montoya et al., 2009). The over-arching conclusion from these studies is that the less aggressive form of leprosy, T-lep, is manifested by an intact vitamin D intracrine system that is able to support antibacterial responses to vitamin D. By contrast, L-lep, which is characterized by a high level of macrophage *M. lep* infection, and has a poor prognosis, exhibits an impaired vitamin D intracrine system. Thus, for patients with L-lep, successful elevation of serum 25D concentrations may be less effective in promoting intracrine-mediated regulation of antibacterial responses.

Several questions have arisen from the studies of vitamin D and leprosy. The first concerns the mechanism by which the vitamin D intracrine system is corrupted in L-lep patients. One possibility is that the T cell cytokine profiles that are characteristic of L-lep (e.g., increased IL-4, IL-10, and IFNα/β) exert a detrimental adjunct effect on the underlying TLR2/1-induced intracrine vitamin D system. At the same time, cytokine profiles associated with T-lep (e.g., increased IFNγ) may have more beneficial adjunct effects. Subsequent experiments *in vitro* support this hypothesis, with the Th1 cytokine IFNγ enhancing TLR2/1-induced vitamin D-activation and associated antibacterial activity (Edfeldt et al., 2010; Fabri et al., 2011). Conversely, the L-lep cytokines IL-4 (Edfeldt et al., 2010), IFNβ (Teles et al., 2013), and IL-10 (Teles et al., 2013) suppress antibacterial production (**Figure 3**). The effect of IFNβ appears to be mediated via IL-10 which acts to suppress expression of CYP27B1 (Teles et al., 2013), whereas IL-4 appears to act by stimulating activity of the vitamin D catabolic enzyme CYP24A1 (Edfeldt et al., 2010). The collective conclusion from these studies is that specific human diseases are characterized by T cell cytokines that act to either promote or corrupt the underlying pathogen-PRR-driven vitamin D intracrine system. Cytokine profiles such as this have also been described for active and inactive TB (Berry et al., 2010; Maertzdorf et al., 2012), providing an additional perspective on the varying success of vitamin D supplementation trials with this disease. Disease itself may therefore play a fundamental role in determining the efficacy of immunomodulatory vitamin D for any given patient; for example, it is possible that for diseases such as L-lep, higher levels of serum 25D will be required to achieve a specific antibacterial response. This is clearly an important topic for future research.

The differential regulation of the intracrine vitamin D pathway in T-lep and L-lep has also provided a platform for genome-wide analyses aimed at identifying factors other than T cell cytokines that may be involved in corrupting monocyte vitamin D responses. In a follow-up to the previous DNA array studies for

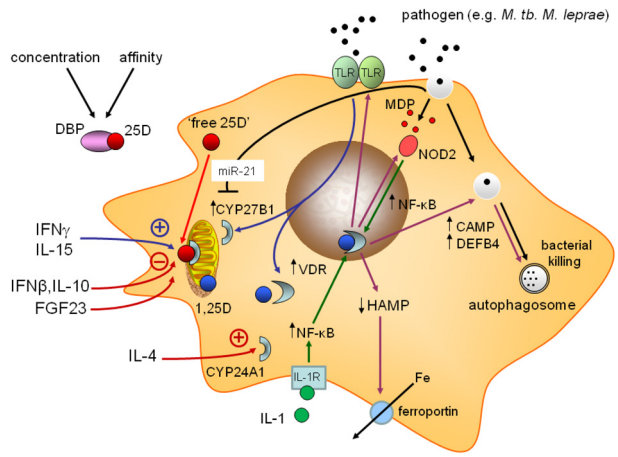

FIGURE 3 | Mechanisms for corruption of vitamin D-mediated antibacterial responses in monocytes. Schematic representation of monocyte/macrophage responses to infection with a pathogen such as *Mycobacterium tuberculosis* (*M. tb*), and associated adjunct signals. Positive effects on the vitamin D intracrine system include: induction of CYP27B1 expression by cytokines such as interferon γ (IFNγ) and interleukin-15 (IL-15). Negative effects include suppression of CYP27B1 by IL-10 and IFNβ, and induction of 24-hydroxylase activity by IL-4. Fibroblast growth factor 23 (FGF23) also suppresses CYP27B1 and microRNA-21 (miR-21) associated with some pathogen infections (e.g., *M. leprae*) can suppress expression of CYP27B1 by degradation of RNA and/or translation. The serum vitamin D binding protein (DBP) may also attenuate intracrine vitamin D by restricting monocyte bioavailability of free 25D.

leprosy, further array analysis of T-lep and L-lep tissues revealed distinct patterns of microRNA (miRNA) expression in these tissues (Liu et al., 2012). Recent studies have shown that miRNAs play a key role in fine-tuning gene expression by interacting with RNA to silence gene expression either by degrading transcripts, or by blocking their translation (Ketting, 2011). In L-lep, 16 miRNAs were found to be differentially induced relative to T-lep tissue, with miRNA-21 (miR-21) being the most prominent of these (Liu et al., 2012). In the context of innate immunity and leprosy, miR-21 may target several important mechanisms, including suppression of IL-1 expression which may, in turn, attenuate intracrine induction of antibacterial DEFB4 (see **Figure 3**). However, importantly, miR-21 is also predicted to interact with *CYP27B1* mRNA and suppress activity of 1α-hydroxylase and decrease localized synthesis of 1,25D. *In vitro*, siRNA knockdown of miR-21 in *M. lep* infected monocytes, restored CYP27B1 expression and 25D-mediated antibacterial responses (Liu et al., 2012). Despite these observations, relatively little is known about how miRNAs corrupt vitamin D signaling in disease situations. Based on genome-wide *in silico* analysis of miRNA target sequences, multiple miRNAs are predicted to influence the expression of proteins associated with vitamin D metabolism and signaling (reviewed in Lisse et al., 2013a). However, other than studies of miR-21, there have been few studies to validate the predicted effects of miRNAs on the vitamin D system. Analysis of ovarian granulosa and breast cancer cells has demonstrated increased expression of miR-125B in these tissue, and a concomitant dysregulation of two of its targets, *VDR* and *CYP24A1* mRNAs (Mohri et al., 2009). It seems likely that future studies will identify other miRNAs that modulate the vitamin D intracrine system in immune cells under disease conditions. Moreover, it is important to recognize that vitamin D itself is a potent regulator of miRNAs. To date, these studies have focused on cancer (Wang et al., 2009, 2011), and bone cells (Lisse et al., 2013b), but similar future studies of 1,25D-regulated non-coding RNAs in immune cells may provide an entirely new perspective on the immunomodulatory actions of vitamin D.

Some important questions about human disease and the immunomodulatory effects of vitamin D remain unanswered. For example, it is still not clear why there is aberrant synthesis of 1,25D by macrophages in granulomatous diseases (Kallas et al., 2010). It is also unclear what effect, if any, viral pathogens such as hepatitis C or HIV have on innate and adaptive immune actions of vitamin D, although HIV infection of some cells has been shown to suppress expression of *VDR* (Chandel et al., 2013). Future studies of vitamin D and the immune system may also explore non-traditional targets for immune regulation. For example, patients with end-stage kidney disease who routinely use dialysis are at high risk of infection and associated mortalities. These patients are also commonly vitamin D-deficient (Zehnder et al., 2007), and this may impair normal innate immune responses to infection. However, as with TB and leprosy, additional disease factors may act to further compromise the intracrine vitamin D system in these patients. Notably, circulating levels of fibroblast growth factor 23 (FGF23), which plays a key role in the endocrine regulation of phosphate homeostasis, are elevated very early in kidney disease (Danziger, 2008; Isakova et al., 2011). One of the important actions of FGF23 is to suppress renal production of 1,25D through the suppression of CYP27B1 expression (Shimada et al., 2004); in this way FGF23 acts as a counterpoint to parathyroid hormone which stimulates CYP27B1 and renal 1,25D production. Until recently, the effects of FGF23 were thought to be restricted to the mineral homeostasis endocrine system. However, work by our group has shown that FGF23 can also act on monocytes to suppress expression of CYP27B1 and the intracrine induction of antibacterial proteins (Bacchetta et al., 2013a). These data highlight a mechanism by which renal disease may compromise vitamin D-mediated immune function, similar to that observed for cytokines associated with infectious disease (see **Figure 3**). As well as providing an explanation for the increased risk of infection in kidney disease patients, these results also suggest a hitherto unrecognized link between the vitamin D endocrine system and its intracrine immune counterpart.

PERSPECTIVES

For many years, the link between vitamin D and the immune system was considered to be a non-classical response with only a pathophysiological relevance. The advent of genome-wide analyses has enabled a complete change in this perspective by providing an unbiased picture of how the vitamin D system is induced by pathogens, and how the resulting intracrine cellular machinery can promote both innate and adaptive immune responses to the pathogen. A key challenge going forward will be to relate these mechanisms to patient vitamin D status, and this is likely to herald a new wave of genome-wide analyses linked to placebo-controlled vitamin D supplementation trials. Interpretation of these studies is likely to be complex. Recent genome-wide analysis of patient tissues has shown that some immune diseases are characterized by corruption of the vitamin D system, so that conventional notions of vitamin D-sufficiency and vitamin D-deficiency may be very different for patients with specific diseases. A key objective for future studies will be to determine whether vitamin D-mediated-immune function is also applicable to mouse models, where genome-wide screening will help to identify immune targets that are related to, or distinct from, human data. Future studies will also need to better characterize disease corruption of vitamin D responses. Screening for disease-specific microRNAs will be particularly important to identify non-coding RNAs that can target components of the intracrine vitamin D. The current shift away from DNA array technology to RNAseq strategies will help to achieve these new objectives in a single genome-wide screen, at increasingly affordable prices.

ACKNOWLEDGMENTS

The authors acknowledge support from NIH grants DK0911672 and AR063910 (Martin Hewison), UL1TR000124 (Martin Hewison, Robert L. Modlin, John S. Adams), and AI073539 (Robert L. Modlin).

REFERENCES

Abbas, A. K., Murphy, K. M., and Sher, A. (1996). Functional diversity of helper T lymphocytes. *Nature* 383, 787–793. doi: 10.1038/383787a0

Adams, J., Gacad, M. A., Baker, A. J., Gonzales, B., and Rude, R. K. (1985). Serum concentrations of 1,25-dihydroxyvitamin D3 in platyrrhini and catarrhini:a phylogenetic appraisal. *Am. J. Primatol.* 28, 263–270.

Adams, J. S., and Hewison, M. (2008). Unexpected actions of vitamin D: new perspectives on the regulation of innate and adaptive immunity. *Nat. Clin. Pract. Endocrinol. Metab.* 4, 80–90. doi: 10.1038/ncpendmet0716

Adams, J. S., Sharma, O. P., Gacad, M. A., and Singer, F. R. (1983). Metabolism of 25-hydroxyvitamin D3 by cultured pulmonary alveolar macrophages in sarcoidosis. *J. Clin. Invest.* 72, 1856–1860. doi: 10.1172/JCI111147

Airey, F. S. (1946). Vitamin D as a remedy for lupus vulgaris. *Med. World* 64, 807–810.

Akutsu, N., Lin, R., Bastien, Y., Bestawros, A., Enepekides, D. J., Black, M. J., et al. (2001). Regulation of gene Expression by 1alpha,25-dihydroxyvitamin D3 and Its analog EB1089 under growth-inhibitory conditions in squamous carcinoma Cells. *Mol. Endocrinol.* 15, 1127–1139. doi: 10.1210/mend.15.7.0655

Ardalan, M. R., Maljaei, H., Shoja, M. M., Piri, A. R., Khosroshahi, H. T., Noshad, H., et al. (2007). Calcitriol started in the donor, expands the population of CD4+CD25+ T cells in renal transplant recipients. *Transplant. Proc.* 39, 951–953. doi: 10.1016/j.transproceed.2007.04.012

Armitage, A. E., Eddowes, L. A., Gileadi, U., Cole, S., Spottiswoode, N., Selvakumar, T. A., et al. (2011). Hepcidin regulation by innate immune and infectious stimuli. *Blood* 118, 4129–4139. doi: 10.1182/blood-2011-04-351957

Arnaud, J., and Constans, J. (1993). Affinity differences for vitamin D metabolites associated with the genetic isoforms of the human serum carrier protein (DBP). *Hum. Genet.* 92, 183–188. doi: 10.1007/BF00219689

Babbe, H., Roers, A., Waisman, A., Lassmann, H., Goebels, N., Hohlfeld, R., et al. (2000). Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. *J. Exp. Med.* 192, 393–404. doi: 10.1084/jem.192.3.393

Bacchetta, J., Sea, J. L., Chun, R. F., Lisse, T. S., Wesseling-Perry, K., Gales, B., et al. (2013a). Fibroblast growth factor 23 inhibits extrarenal synthesis of 1,25-dihydroxyvitamin D in human monocytes. *J. Bone Miner. Res.* 28, 46–55. doi: 10.1002/jbmr.1740

Bacchetta, J. Z. J., Sea, J. L., Chun, R. F., Lisse, T. S., Zavala, K., Nayak, A., et al. (2013b). Suppression of iron-regulatory hepcidin by vitamin D. *J. Am. Soc. Nephrol.* 25, 564–572. doi: 10.1681/ASN.2013040355

Bader, G. D., Betel, D., and Hogue, C. W. (2003). BIND: the biomolecular interaction network database. *Nucleic Acids Res.* 31, 248–250. doi: 10.1093/nar/gkg056

Barbour, G. L., Coburn, J. W., Slatopolsky, E., Norman, A. W., and Horst, R. L. (1981). Hypercalcemia in an anephric patient with sarcoidosis: evidence for extrarenal generation of 1,25-dihydroxyvitamin D. *N. Engl. J. Med.* 305, 440–443. doi: 10.1056/NEJM198108203050807

Barlow, P. G., Svoboda, P., Mackellar, A., Nash, A. A., York, I. A., Pohl, J., et al. (2011). Antiviral activity and increased host defense against influenza infection elicited by the human cathelicidin LL-37. *PLoS ONE* 6:e25333. doi: 10.1371/journal.pone.0025333

Barrat, F. J., Cua, D. J., Boonstra, A., Richards, D. F., Crain, C., Savelkoul, H. F., et al. (2002). *In vitro* generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J. Exp. Med.* 195, 603–616. doi: 10.1084/jem.20011629

Berry, M. P., Graham, C. M., McNab, F. W., Xu, Z., Bloch, S. A., Oni, T., et al. (2010). An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. *Nature* 466, 973–977. doi: 10.1038/nature09247

Bhalla, A. K., Amento, E. P., Clemens, T. L., Holick, M. F., and Krane, S. M. (1983). Specific high-affinity receptors for 1,25-dihydroxyvitamin D3 in human peripheral blood mononuclear cells: presence in monocytes and induction in T lymphocytes following activation. *J. Clin. Endocrinol. Metab.* 57, 1308–1310. doi: 10.1210/jcem-57-6-1308

Bhalla, A. K., Amento, E. P., Serog, B., and Glimcher, L. H. (1984). 1,25-Dihydroxyvitamin D3 inhibits antigen-induced T cell activation. *J. Immunol.* 133, 1748–1754.

Boonstra, A., Barrat, F. J., Crain, C., Heath, V. L., Savelkoul, H. F., and O'Garra, A. (2001). 1alpha,25-Dihydroxyvitamin d3 has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells. *J. Immunol.* 167, 4974–4980.

Bouchon, A., Dietrich, J., and Colonna, M. (2000). Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J. Immunol.* 164, 4991–4995.

Bouchon, A., Facchetti, F., Weigand, M. A., and Colonna, M. (2001). TREM-1 amplifies inflammation and is a crucial mediator of septic shock. *Nature* 410, 1103–1107. doi: 10.1038/35074114

Brennan, A., Katz, D. R., Nunn, J. D., Barker, S., Hewison, M., Fraher, L. J., et al. (1987). Dendritic cells from human tissues express receptors for the immunoregulatory vitamin D3 metabolite, dihydroxycholecalciferol. *Immunology* 61, 457–461.

Britton, W. J., and Lockwood, D. N. (2004). Leprosy. *Lancet* 363, 1209–1219. doi: 10.1016/S0140-6736(04)15952-7

Cannell, J. J., Vieth, R., Umhau, J. C., Holick, M. F., Grant, W. B., Madronich, S., et al. (2006). Epidemic influenza and vitamin D. *Epidemiol. Infect.* 134, 1129–1140. doi: 10.1017/S0950268806007175

Chan, J., Xing, Y., Magliozzo, R. S., and Bloom, B. R. (1992). Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages. *J. Exp. Med.* 175, 1111–1122. doi: 10.1084/jem.175.4.1111

Chandel, N., Sharma, B., Husain, M., Salhan, D., Singh, T., Rai, P., et al. (2013). HIV compromises integrity of the podocyte actin cytoskeleton through downregulation of the vitamin D receptor. *Am. J. Physiol. Renal Physiol.* 304, F1347–F1357. doi: 10.1152/ajprenal.00717.2012

Chang, J. M., Kuo, M. C., Kuo, H. T., Hwang, S. J., Tsai, J. C., Chen, H. C., et al. (2004). 1-alpha,25-Dihydroxyvitamin D3 regulates inducible nitric oxide synthase messenger RNA expression and nitric oxide release in macrophage-like RAW 264.7 cells. *J. Lab. Clin. Med.* 143, 14–22. doi: 10.1016/j.lab.2003.08.002

Chen, L., Cencioni, M. T., Angelini, D. F., Borsellino, G., Battistini, L., and Brosnan, C. F. (2005). Transcriptional profiling of gamma delta T cells identifies a role for vitamin D in the immunoregulation of the V gamma 9V delta 2 response to phosphate-containing ligands. *J. Immunol.* 174, 6144–6152.

Chen, S., Sims, G. P., Chen, X. X., Gu, Y. Y., Chen, S., and Lipsky, P. E. (2007). Modulatory effects of 1,25-dihydroxyvitamin d3 on human B cell differentiation. *J. Immunol.* 179, 1634–1647.

Cheroutre, H., and Lambolez, F. (2008). Doubting the TCR coreceptor function of CD8alphaalpha. *Immunity* 28, 149–159. doi: 10.1016/j.immuni.2008.01.005

Chun, R. F., Lauridsen, A. L., Suon, L., Zella, L. A., Pike, J. W., Modlin, R. L., et al. (2010). Vitamin D- binding protein directs monocyte responses to 25-hydroxy- and 1,25-dihydroxyvitamin D. *J. Clin. Endocrinol. Metab.* 95, 3368–3376. doi: 10.1210/jc.2010-0195

Chun, R. F., Peercy, B. E., Adams, J. S., and Hewison, M. (2012). Vitamin D binding protein and monocyte response to 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D: analysis by mathematical modeling. *PLoS ONE* 7:e30773. doi: 10.1371/journal.pone.0030773

Coussens, A. K., Wilkinson, R. J., Hanifa, Y., Nikolayevskyy, V., Elkington, P. T., Islam, K., et al. (2012). Vitamin D accelerates resolution of inflammatory responses during tuberculosis treatment. *Proc. Natl. Acad. Sci. U.S.A.* 109, 15449–15454. doi: 10.1073/pnas.1200072109

Danziger, J. (2008). The bone-renal axis in early chronic kidney disease: an emerging paradigm. *Nephrol. Dial. Transplant* 23, 2733–2737. doi: 10.1093/ndt/gfn260

Deretic, V., and Levine, B. (2009). Autophagy, immunity, and microbial adaptations. *Cell Host Microbe* 5, 527–549. doi: 10.1016/j.chom.2009.05.016

Drakesmith, H., and Prentice, A. M. (2012). Hepcidin and the iron-infection axis. *Science* 338, 768–772. doi: 10.1126/science.1224577

Edfeldt, K., Liu, P. T., Chun, R., Fabri, M., Schenk, M., Wheelwright, M., et al. (2010). T-cell cytokines differentially control human monocyte antimicrobial responses by regulating vitamin D metabolism. *Proc. Natl. Acad. Sci. U.S.A.* 107, 22593–22598. doi: 10.1073/pnas.1011624108

Enioutina, E. Y., Bareyan, D., and Daynes, R. A. (2009). TLR-induced local metabolism of vitamin D3 plays an important role in the diversification of adaptive immune responses. *J. Immunol.* 182, 4296–4305. doi: 10.4049/jimmunol.0804344

Esteban, L., Vidal, M., and Dusso, A. (2004). 1alpha-Hydroxylase transactivation by gamma-interferon in murine macrophages requires enhanced C/EBPbeta expression and activation. *J. Steroid Biochem. Mol. Biol.* 89–90, 131–137. doi: 10.1016/j.jsbmb.2004.03.092

Fabri, M., Stenger, S., Shin, D. M., Yuk, J. M., Liu, P. T., Realegeno, S., et al. (2011). Vitamin D is required for IFN-gamma-mediated antimicrobial activity of human macrophages. *Sci. Transl. Med.* 3, 104ra102. doi: 10.1126/scitranslmed.3003045

Ferreira, G. B., Kleijwegt, F. S., Waelkens, E., Lage, K., Nikolic, T., Hansen, D. A., et al. (2012). Differential protein pathways in 1,25-dihydroxyvitamin d(3) and dexamethasone modulated tolerogenic human dendritic cells. *J. Proteome Res.* 11, 941–971. doi: 10.1021/pr200724e

Ferreira, G. B., van Etten, E., Lage, K., Hansen, D. A., Moreau, Y., Workman, C. T., et al. (2009). Proteome analysis demonstrates profound alterations in human dendritic cell nature by TX527, an analogue of vitamin D. *Proteomics* 9, 3752–3764. doi: 10.1002/pmic.200800848

Fritsche, J., Mondal, K., Ehrnsperger, A., Andreesen, R., and Kreutz, M. (2003). Regulation of 25-hydroxyvitamin D3-1 alpha-hydroxylase and production of 1 alpha,25-dihydroxyvitamin D3 by human dendritic cells. *Blood* 102, 3314–3316. doi: 10.1182/blood-2002-11-3521

Froicu, M., Weaver, V., Wynn, T. A., McDowell, M. A., Welsh, J. E., and Cantorna, M. T. (2003). A crucial role for the vitamin D receptor in experimental inflammatory bowel diseases. *Mol. Endocrinol.* 17, 2386–2392. doi: 10.1210/me.2003-0281

Ganz, T. (2009). Iron in innate immunity: starve the invaders. *Curr. Opin. Immunol.* 21, 63–67. doi: 10.1016/j.coi.2009.01.011

Ganz, T. (2011). Hepcidin and iron regulation, 10 years later. *Blood* 117, 4425–4433. doi: 10.1182/blood-2011-01-258467

Ghoreishi, M., Bach, P., Obst, J., Komba, M., Fleet, J. C., and Dutz, J. P. (2009). Expansion of antigen-specific regulatory T cells with the topical vitamin d analog calcipotriol. *J. Immunol.* 182, 6071–6078. doi: 10.4049/jimmunol.0804064

Gombart, A. F., Bhan, I., Borregaard, N., Tamez, H., Camargo, C. A., Jr., Koeffler, H. P., et al. (2009a). Low plasma level of cathelicidin antimicrobial peptide (hCAP18) predicts increased infectious disease mortality in patients undergoing hemodialysis. *Clin. Infect. Dis.* 48, 418–424. doi: 10.1086/596314

Gombart, A. F., Borregaard, N., and Koeffler, H. P. (2005). Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3. *FASEB J.* 19, 1067–1077. doi: 10.1096/fj.04-3284com

Gombart, A. F., Saito, T., and Koeffler, H. P. (2009b). Exaptation of an ancient Alu short interspersed element provides a highly conserved vitamin D- mediated innate immune response in humans and primates. *BMC Genomics* 10:321. doi: 10.1186/1471-2164-10-321

Gorman, S., Kuritzky, L. A., Judge, M. A., Dixon, K. M., McGlade, J. P., Mason, R. S., et al. (2007). Topically applied 1,25-dihydroxyvitamin D3 enhances the suppressive activity of CD4+CD25+ cells in the draining lymph nodes. *J. Immunol.* 179, 6273–6283.

Griffin, M. D., Lutz, W., Phan, V. A., Bachman, L. A., McKean, D. J., and Kumar, R. (2001). Dendritic cell modulation by 1alpha,25 dihydroxyvitamin D3 and its analogs: a vitamin D receptor-dependent pathway that promotes a persistent state of immaturity *in vitro* and *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* 98, 6800–6805. doi: 10.1073/pnas.121172198

Griffin, M. D., Xing, N., and Kumar, R. (2004). Gene expression profiles in dendritic cells conditioned by 1alpha,25-dihydroxyvitamin D3 analog. *J. Steroid Biochem. Mol. Biol.* 89–90, 443–448. doi: 10.1016/j.jsbmb.2004.03.039

Gutierrez, M. G., Master, S. S., Singh, S. B., Taylor, G. A., Colombo, M. I., and Deretic, V. (2004). Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell* 119, 753–766. doi: 10.1016/j.cell.2004.11.038

Harle, D., Radmark, O., Samuelsson, B., and Steinhilber, D. (1998). Calcitriol and transforming growth factor-beta upregulate 5-lipoxygenase mRNA expression by increasing gene transcription and mRNA maturation. *Eur. J. Biochem.* 254, 275–281. doi: 10.1046/j.1432-1327.1998.2540275.x

Heine, G., Niesner, U., Chang, H. D., Steinmeyer, A., Zugel, U., Zuberbier, T., et al. (2008). 1,25-dihydroxyvitamin D(3) promotes IL-10 production in human B cells. *Eur. J. Immunol.* 38, 2210–2218. doi: 10.1002/eji.200838216

Herrera, G. (1949). Vitamin D in massive doses as an adjuvant to the sulfones in the treatment of tuberculoid leprosy. *Int. J. Lepr.* 17, 35–42.

Hewison, M. (2011). Antibacterial effects of vitamin D. *Nat. Rev. Endocrinol.* 7, 337–345. doi: 10.1038/nrendo.2010.226

Hewison, M., Freeman, L., Hughes, S. V., Evans, K. N., Bland, R., Eliopoulos, A. G., et al. (2003). Differential regulation of vitamin D receptor and its ligand in human monocyte-derived dendritic cells. *J. Immunol.* 170, 5382–5390.

Holick, M. F. (2007). Vitamin D deficiency. *N. Engl. J. Med.* 357, 266–281. doi: 10.1056/NEJMra070553

Holick, M. F., Binkley, N. C., Bischoff-Ferrari, H. A., Gordon, C. M., Hanley, D. A., Heaney, R. P., et al. (2011). Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *J. Clin. Endocrinol. Metab.* 96, 1911–1930. doi: 10.1210/jc.2011-0385

Hong, M. G., Pawitan, Y., Magnusson, P. K., and Prince, J. A. (2009). Strategies and issues in the detection of pathway enrichment in genome-wide association studies. *Hum. Genet.* 126, 289–301. doi: 10.1007/s00439-009-0676-z

Hooper, L. V., Stappenbeck, T. S., Hong, C. V., and Gordon, J. I. (2003). Angiogenins: a new class of microbicidal proteins involved in innate immunity. *Nat. Immunol.* 4, 269–273. doi: 10.1038/ni888

Hossein-nezhad, A., Spira, A., and Holick, M. F. (2013). Influence of vitamin D status and vitamin D3 supplementation on genome wide expression of white blood cells: a randomized double-blind clinical trial. *PLoS ONE* 8:e58725. doi: 10.1371/journal.pone.0058725

Hoyer-Hansen, M., Bastholm, L., Mathiasen, I. S., Elling, F., and Jaattela, M. (2005). Vitamin D analog EB1089 triggers dramatic lysosomal changes and Beclin 1-mediated autophagic cell death. *Cell Death Differ.* 12, 1297–1309. doi: 10.1038/sj.cdd.4401651

Iho, S., Takahashi, T., Kura, F., Sugiyama, H., and Hoshino, T. (1986). The effect of 1,25-dihydroxyvitamin D3 on *in vitro* immunoglobulin production in human B cells. *J. Immunol.* 136, 4427–4431.

Isakova, T., Wahl, P., Vargas, G. S., Gutierrez, O. M., Scialla, J., Xie, H., et al. (2011). Fibroblast growth factor 23 is elevated before parathyroid hormone and phosphate in chronic kidney disease. *Kidney Int.* 79, 1370–1378. doi: 10.1038/ki.2011.47

Jeffery, L. E., Burke, F., Mura, M., Zheng, Y., Qureshi, O. S., Hewison, M., et al. (2009). 1,25-Dihydroxyvitamin D(3) and IL-2 combine to inhibit T cell production of inflammatory cytokines and promote development of regulatory T cells expressing CTLA-4 and FoxP3. *J. Immunol.* 183, 5458–5467. doi: 10.4049/jimmunol.0803217

Jeffery, L. E., Wood, A. M., Qureshi, O. S., Hou, T. Z., Gardner, D., Briggs, Z., et al. (2012). Availability of 25-Hydroxyvitamin D3 to APCs controls the balance between regulatory and inflammatory T cell responses. *J. Immunol.* 189, 5155–5164. doi: 10.4049/jimmunol.1200786

Jeng, L., Yamshchikov, A. V., Judd, S. E., Blumberg, H. M., Martin, G. S., Ziegler, T. R., et al. (2009). Alterations in vitamin D status and anti-microbial peptide levels in patients in the intensive care unit with sepsis. *J. Transl. Med.* 7, 28. doi: 10.1186/1479-5876-7-28

Kallas, M., Green, F., Hewison, M., White, C., and Kline, G. (2010). Rare causes of calcitriol-mediated hypercalcemia: a case report and literature review. *J. Clin. Endocrinol. Metab.* 95, 3111–3117. doi: 10.1210/jc.2009-2673

Karmali, R., Hewison, M., Rayment, N., Farrow, S. M., Brennan, A., Katz, D. R., et al. (1991). 1,25(OH)2D3 regulates c-myc mRNA levels in tonsillar T lymphocytes. *Immunology* 74, 589–593.

Ketting, R. F. (2011). microRNA biogenesis and function : an overview. *Adv. Exp. Med. Biol.* 700, 1–14. doi: 10.1007/978-1-4419-7823-3_1

Kim, T. H., Lee, B., Kwon, E., Choi, S. J., Lee, Y. H., Song, G. G., et al. (2013). Regulation of TREM-1 expression by 1,25-dihydroxyvitamin D3 in human monocytes/macrophages. *Immunol. Lett.* 154, 80–85. doi: 10.1016/j.imlet.2013.08.012

Kiss, Z., Ambrus, C., Almasi, C., Berta, K., Deak, G., Horonyi, P., et al. (2011). Serum 25(OH)-cholecalciferol concentration is associated with hemoglobin level and erythropoietin resistance in patients on maintenance hemodialysis. *Nephron Clin. Pract.* 117, c373–c378. doi: 10.1159/000321521

Klionsky, D. J., and Emr, S. D. (2000). Autophagy as a regulated pathway of cellular degradation. *Science* 290, 1717–1721. doi: 10.1126/science.290.5497.1717

Koeffler, H. P., Reichel, H., Bishop, J. E., and Norman, A. W. (1985). gamma-Interferon stimulates production of 1,25-dihydroxyvitamin D3 by normal human macrophages. *Biochem. Biophys. Res. Commun.* 127, 596–603. doi: 10.1016/S0006-291X(85)80202-3

Kohchi, C., Inagawa, H., Nishizawa, T., and Soma, G. (2009). ROS and innate immunity. *Anticancer Res.* 29, 817–821.

Kreutz, M., Andreesen, R., Krause, S. W., Szabo, A., Ritz, E., and Reichel, H. (1993). 1,25-dihydroxyvitamin D3 production and vitamin D3 receptor expression are developmentally regulated during differentiation of human monocytes into macrophages. *Blood* 82, 1300–1307.

Lac, P. T., Choi, K., Liu, I. A., Meguerditchian, S., Rasgon, S. A., and Sim, J. J. (2010). The effects of changing vitamin D levels on anemia in chronic kidney disease patients: a retrospective cohort review. *Clin. Nephrol.* 74, 25–32. doi: 10.5414/CNP74025

Lagishetty, V., Misharin, A. V., Liu, N. Q., Lisse, T. S., Chun, R. F., Ouyang, Y., et al. (2010). Vitamin D deficiency in mice impairs colonic antibacterial activity and predisposes to colitis. *Endocrinology* 151, 2423–2432. doi: 10.1210/en.2010-0089

Lauridsen, A. L., Vestergaard, P., Hermann, A. P., Brot, C., Heickendorff, L., Mosekilde, L., et al. (2005). Plasma concentrations of 25-hydroxy-vitamin D and 1,25-dihydroxy-vitamin D are related to the phenotype of Gc (vitamin D-binding protein): a cross-sectional study on 595 early postmenopausal women. *Calcif. Tissue Int.* 77, 15–22. doi: 10.1007/s00223-004-0227-5

Lauridsen, A. L., Vestergaard, P., and Nexo, E. (2001). Mean serum concentration of vitamin D- binding protein (Gc globulin) is related to the Gc phenotype in women. *Clin. Chem.* 47, 753–756.

Lemire, J. M., Adams, J. S., Kermani-Arab, V., Bakke, A. C., Sakai, R., and Jordan, S. C. (1985). 1,25-Dihydroxyvitamin D3 suppresses human T helper/inducer lymphocyte activity *in vitro*. *J. Immunol.* 134, 3032–3035.

Lemire, J. M., Archer, D. C., Beck, L., and Spiegelberg, H. L. (1995). Immunosuppressive actions of 1,25-dihydroxyvitamin D3: preferential inhibition of Th1 functions. *J. Nutr.* 125, 1704S–1708S.

Lin, R., Nagai, Y., Sladek, R., Bastien, Y., Ho, J., Petrecca, K., et al. (2002). Expression profiling in squamous carcinoma cells reveals pleiotropic effects of vitamin D3 analog EB1089 signaling on cell proliferation, differentiation, and immune system regulation. *Mol. Endocrinol.* 16, 1243–1256. doi: 10.1210/mend.16.6.0874

Lisse, T. S., Adams, J. S., and Hewison, M. (2013a). Vitamin D and MicroRNAs in Bone. *Crit. Rev. Eukaryot. Gene Expr.* 23, 195–214. doi: 10.1615/CritRevEukaryotGeneExpr.2013007147

Lisse, T. S., Chun, R. F., Rieger, S., Adams, J. S., and Hewison, M. (2013b). Vitamin D activation of functionally distinct regulatory miRNAs in primary human osteoblasts. *J. Bone Miner. Res.* 28, 1478–1488. doi: 10.1002/jbmr.1882

Lisse, T. S., Liu, T., Irmler, M., Beckers, J., Chen, H., Adams, J. S., et al. (2011). Gene targeting by the vitamin D response element binding protein reveals a role for vitamin D in osteoblast mTOR signaling. *FASEB J.* 25, 937–947. doi: 10.1096/fj.10-172577

Liu, P. T., Schenk, M., Walker, V. P., Dempsey, P. W., Kanchanapoomi, M., Wheelwright, M., et al. (2009). Convergence of IL-1beta and VDR activation pathways in human TLR2/1-induced antimicrobial responses. *PLoS ONE* 4:e5810. doi: 10.1371/journal.pone.0005810

Liu, P. T., Stenger, S., Li, H., Wenzel, L., Tan, B. H., Krutzik, S. R., et al. (2006). Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* 311, 1770–1773. doi: 10.1126/science.1123933

Liu, P. T., Wheelwright, M., Teles, R., Komisopoulou, E., Edfeldt, K., Ferguson, B., et al. (2012). MicroRNA-21 targets the vitamin D-dependent antimicrobial pathway in leprosy. *Nat. Med.* 18, 267–273. doi: 10.1038/nm.2584

Liu, Y. J. (2005). IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu. Rev. Immunol.* 23, 275–306. doi: 10.1146/annurev.immunol.23.021704.115633

Maertzdorf, J., Weiner, J. 3rd., Mollenkopf, H. J., Network, T. B., Bauer, T., Prasse, A., et al. (2012). Common patterns and disease-related signatures in tuberculosis and sarcoidosis. *Proc. Natl. Acad. Sci. U.S.A.* 109, 7853–7858. doi: 10.1073/pnas.1121072109

Mahon, B. D., Wittke, A., Weaver, V., and Cantorna, M. T. (2003). The targets of vitamin D depend on the differentiation and activation status of CD4 positive T cells. *J. Cell. Biochem.* 89, 922–932. doi: 10.1002/jcb.10580

Mangelsdorf, D. J., Koeffler, H. P., Donaldson, C. A., Pike, J. W., and Haussler, M. R. (1984). 1,25-Dihydroxyvitamin D3-induced differentiation in a human promyelocytic leukemia cell line (HL-60): receptor-mediated maturation to macrophage-like cells. *J. Cell Biol.* 98, 391–398. doi: 10.1083/jcb.98.2.391

Martineau, A. R., Timms, P. M., Bothamley, G. H., Hanifa, Y., Islam, K., Claxton, A. P., et al. (2011). High-dose vitamin D(3) during intensive-phase antimicrobial treatment of pulmonary tuberculosis: a double-blind randomised controlled trial. *Lancet* 377, 242–250. doi: 10.1016/S0140-6736(10)61889-2

Martineau, A. R., Wilkinson, R. J., Wilkinson, K. A., Newton, S. M., Kampmann, B., Hall, B. M., et al. (2007). A single dose of vitamin d enhances immunity to mycobacteria. *Am. J. Respir. Crit. Care Med.* 176, 208–213. doi: 10.1164/rccm.200701-007OC

Meehan, T. F., and DeLuca, H. F. (2002). CD8(+) T cells are not necessary for 1 alpha,25-dihydroxyvitamin D(3) to suppress experimental autoimmune encephalomyelitis in mice. *Proc. Natl. Acad. Sci. U.S.A.* 99, 5557–5560. doi: 10.1073/pnas.082100699

Mohri, T., Nakajima, M., Takagi, S., Komagata, S., and Yokoi, T. (2009). MicroRNA regulates human vitamin D receptor. *Int. J. Cancer* 125, 1328–1333. doi: 10.1002/ijc.24459

Moller, K. I., Kongshoj, B., Philipsen, P. A., Thomsen, V. O., and Wulf, H. C. (2005). How Finsen's light cured lupus vulgaris. *Photodermatol. Photoimmunol. Photomed.* 21, 118–124. doi: 10.1111/j.1600-0781.2005.00159.x

Montoya, D., Cruz, D., Teles, R. M., Lee, D. J., Ochoa, M. T., Krutzik, S. R., et al. (2009). Divergence of macrophage phagocytic and antimicrobial programs in leprosy. *Cell Host Microbe* 6, 343–353. doi: 10.1016/j.chom.2009.09.002

Moresco, E. M., LaVine, D., and Beutler, B. (2011). Toll-like receptors. *Curr. Biol.* 21, R488–R493. doi: 10.1016/j.cub.2011.05.039

Nairz, M., Theurl, I., Ludwiczek, S., Theurl, M., Mair, S. M., Fritsche, G., et al. (2007). The co-ordinated regulation of iron homeostasis in murine macrophages limits the availability of iron for intracellular *Salmonella typhimurium*. *Cell. Microbiol.* 9, 2126–2140. doi: 10.1111/j.1462-5822.2007.00942.x

Nakayama, Y., Stabach, P., Maher, S. E., Mahajan, M. C., Masiar, P., Liao, C., et al. (2006). A limited number of genes are involved in the differentiation of germinal center B cells. *J. Cell. Biochem.* 99, 1308–1325. doi: 10.1002/jcb.20952

Nunn, J. D., Katz, D. R., Barker, S., Fraher, L. J., Hewison, M., Hendy, G. N., et al. (1986). Regulation of human tonsillar T-cell proliferation by the active metabolite of vitamin D3. *Immunology* 59, 479–484.

Nursyam, E. W., Amin, Z., and Rumende, C. M. (2006). The effect of vitamin D as supplementary treatment in patients with moderately advanced pulmonary tuberculous lesion. *Acta Med. Indones.* 38, 3–5.

O'Kelly, J., Uskokovic, M., Lemp, N., Vadgama, J., and Koeffler, H. P. (2006). Novel Gemini-vitamin D3 analog inhibits tumor cell growth and modulates the Akt/mTOR signaling pathway. *J. Steroid Biochem. Mol. Biol.* 100, 107–116. doi: 10.1016/j.jsbmb.2006.04.003

Ooi, J. H., McDaniel, K. L., Weaver, V., and Cantorna, M. T. (2014). Murine CD8+T cells but not macrophages express the vitamin D 1alpha-hydroxylase. *J. Nutr. Biochem.* 25, 58–65. doi: 10.1016/j.jnutbio.2013.09.003

Packey, C. D., and Sartor, R. B. (2009). Commensal bacteria, traditional and opportunistic pathogens, dysbiosis and bacterial killing in inflammatory bowel diseases. *Curr. Opin. Infect. Dis.* 22, 292–301. doi: 10.1097/QCO.0b013e32832a8a5d

Panda, D. K., Miao, D., Tremblay, M. L., Sirois, J., Farookhi, R., Hendy, G. N., et al. (2001). Targeted ablation of the 25-hydroxyvitamin D 1alpha -hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction. *Proc. Natl. Acad. Sci. U.S.A.* 98, 7498–7503. doi: 10.1073/pnas.131029498

Paradkar, P. N., De Domenico, I., Durchfort, N., Zohn, I., Kaplan, J., and Ward, D. M. (2008). Iron depletion limits intracellular bacterial growth in macrophages. *Blood* 112, 866–874. doi: 10.1182/blood-2007-12-126854

Pedersen, A. W., Holmstrom, K., Jensen, S. S., Fuchs, D., Rasmussen, S., Kvistborg, P., et al. (2009). Phenotypic and functional markers for 1alpha,25-dihydroxyvitamin D(3)-modified regulatory dendritic cells. *Clin. Exp. Immunol.* 157, 48–59. doi: 10.1111/j.1365-2249.2009.03961.x

Penna, G., Amuchastegui, S., Cossetti, C., Aquilano, F., Mariani, R., Sanvito, F., et al. (2006). Treatment of experimental autoimmune prostatitis in nonobese diabetic mice by the vitamin D receptor agonist elocalcitol. *J. Immunol.* 177, 8504–8511.

Penna, G., Amuchastegui, S., Giarratana, N., Daniel, K. C., Vulcano, M., Sozzani, S., et al. (2007). 1,25-dihydroxyvitamin d3 selectively modulates tolerogenic properties in myeloid but not plasmacytoid dendritic cells. *J. Immunol.* 178, 145–153.

Peters-Golden, M., Canetti, C., Mancuso, P., and Coffey, M. J. (2005). Leukotrienes: underappreciated mediators of innate immune responses. *J. Immunol.* 174, 589–594.

Provvedini, D. M., and Manolagas, S. C. (1989). 1 Alpha,25-dihydroxyvitamin D3 receptor distribution and effects in subpopulations of normal human T lymphocytes. *J. Clin. Endocrinol. Metab.* 68, 774–779. doi: 10.1210/jcem-68-4-774

Provvedini, D. M., Tsoukas, C. D., Deftos, L. J., and Manolagas, S. C. (1983). 1,25-dihydroxyvitamin D3 receptors in human leukocytes. *Science* 221, 1181–1183. doi: 10.1126/science.6310748

Ravid, A., Koren, R., Novogrodsky, A., and Liberman, U. A. (1984). 1,25-Dihydroxyvitamin D3 inhibits selectively the mitogenic stimulation of mouse medullary thymocytes. *Biochem. Biophys. Res. Commun.* 123, 163–169. doi: 10.1016/0006-291X(84)90394-2

Reichel, H., Koeffler, H. P., and Norman, A. W. (1986). Regulation of 25-hydroxyvitamin D3 metabolism in a human promyelocytic leukemia cell line (HL-60): 1,25-dihydroxyvitamin D3 stimulates the synthesis of 24,25-dihydroxyvitamin D3. *Arch. Biochem. Biophys.* 251, 222–231. doi: 10.1016/0003-9861(86)90069-X

Rigby, W. F., Noelle, R. J., Krause, K., and Fanger, M. W. (1985). The effects of 1,25-dihydroxyvitamin D3 on human T lymphocyte activation and proliferation: a cell cycle analysis. *J. Immunol.* 135, 2279–2286.

Rigby, W. F., Yirinec, B., Oldershaw, R. L., and Fanger, M. W. (1987). Comparison of the effects of 1,25-dihydroxyvitamin D3 on T lymphocyte subpopulations. *Eur. J. Immunol.* 17, 563–566. doi: 10.1002/eji.1830170420

Romagnani, S. (2006). Regulation of the T cell response. *Clin. Exp. Allergy* 36, 1357–1366. doi: 10.1111/j.1365-2222.2006.02606.x

Ross, A. C., Manson, J. E., Abrams, S. A., Aloia, J. F., Brannon, P. M., Clinton, S. K., et al. (2011). The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. *J. Clin. Endocrinol. Metab.* 96, 53–58. doi: 10.1210/jc.2010-2704

Royal, W. 3rd., Mia, Y., Li, H., and Naunton, K. (2009). Peripheral blood regulatory T cell measurements correlate with serum vitamin D levels in patients with multiple sclerosis. *J. Neuroimmunol.* 213, 135–141. doi: 10.1016/j.jneuroim.2009.05.012

Sadeghi, K., Wessner, B., Laggner, U., Ploder, M., Tamandl, D., Friedl, J., et al. (2006). Vitamin D3 down-regulates monocyte TLR expression and triggers hyporesponsiveness to pathogen-associated molecular patterns. *Eur. J. Immunol.* 36, 361–370. doi: 10.1002/eji.200425995

Sanjuan, M. A., Milasta, S., and Green, D. R. (2009). Toll-like receptor signaling in the lysosomal pathways. *Immunol. Rev.* 227, 203–220. doi: 10.1111/j.1600-065X.2008.00732.x

Schaible, U. E., Collins, H. L., Priem, F., and Kaufmann, S. H. (2002). Correction of the iron overload defect in beta-2-microglobulin knockout mice by lactoferrin abolishes their increased susceptibility to tuberculosis. *J. Exp. Med.* 196, 1507–1513. doi: 10.1084/jem.20020897

Schauber, J., Dorschner, R. A., Coda, A. B., Buchau, A. S., Liu, P. T., Kiken, D., et al. (2007). Injury enhances TLR2 function and antimicrobial peptide expression through a vitamin D-dependent mechanism. *J. Clin. Invest.* 117, 803–811. doi: 10.1172/JCI30142

Shen, Q. Y., and Zheng, S. S. (2004). Identification of genes differentially expressed in monocyte-derived dendritic cells with 1alpha,25-dihydroxyvitamin D3 using cDNA arrays. *J. Zhejiang Univ. Sci.* 5, 222–225. doi: 10.1631/jzus.2004.0222

Shimada, T., Hasegawa, H., Yamazaki, Y., Muto, T., Hino, R., Takeuchi, Y., et al. (2004). FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. *J. Bone Miner. Res.* 19, 429–435. doi: 10.1359/JBMR.0301264

Shin, D. M., Yuk, J. M., Lee, H. M., Lee, S. H., Son, J. W., Harding, C. V., et al. (2011). Mycobacterial lipoprotein activates autophagy via TLR2/1/CD14 and a functional vitamin D receptor signalling. *Cell. Microbiol.* 12, 1648–1665. doi: 10.1111/j.1462-5822.2010.01497.x

Shiozawa, K., Shiozawa, S., Shimizu, S., and Fujita, T. (1985). 1 alpha,25-dihydroxyvitamin D3 inhibits pokeweed mitogen-stimulated human B-cell activation: an analysis using serum-free culture conditions. *Immunology* 56, 161–167.

Shirakawa, A. K., Nagakubo, D., Hieshima, K., Nakayama, T., Jin, Z., and Yoshie, O. (2008). 1,25-dihydroxyvitamin D3 induces CCR10 expression in terminally differentiating human B cells. *J. Immunol.* 180, 2786–2795.

Sigmundsdottir, H., Pan, J., Debes, G. F., Alt, C., Habtezion, A., Soler, D., et al. (2007). DCs metabolize sunlight-induced vitamin D3 to "program" T cell attraction to the epidermal chemokine CCL27. *Nat. Immunol.* 8, 285–293. doi: 10.1038/ni1433

Sly, L. M., Lopez, M., Nauseef, W. M., and Reiner, N. E. (2001). 1alpha,25-Dihydroxyvitamin D3-induced monocyte antimycobacterial activity is regulated by phosphatidylinositol 3-kinase and mediated by the NADPH-dependent phagocyte oxidase. *J. Biol. Chem.* 276, 35482–35493. doi: 10.1074/jbc.M102876200

Smolders, J., Thewissen, M., Peelen, E., Menheere, P., Cohen Tervaert, J. W., Damoiseaux, J., et al. (2009). Vitamin D status is positively correlated with regulatory T cell function in patients with multiple sclerosis. *PLoS ONE* 4:e6635. doi: 10.1371/journal.pone.0006635

Sow, F. B., Alvarez, G. R., Gross, R. P., Satoskar, A. R., Schlesinger, L. S., Zwilling, B. S., et al. (2009). Role of STAT1, NF-kappaB, and C/EBPbeta in the macrophage transcriptional regulation of hepcidin by mycobacterial infection and IFN-gamma. *J. Leukoc. Biol.* 86, 1247–1258. doi: 10.1189/jlb.1208719

Sow, F. B., Florence, W. C., Satoskar, A. R., Schlesinger, L. S., Zwilling, B. S., and Lafuse, W. P. (2007). Expression and localization of hepcidin in macrophages: a role in host defense against tuberculosis. *J. Leukoc. Biol.* 82, 934–945. doi: 10.1189/jlb.0407216

Steinman, R. M., Hawiger, D., and Nussenzweig, M. C. (2003). Tolerogenic dendritic cells. *Annu. Rev. Immunol.* 21, 685–711. doi: 10.1146/annurev.immunol.21.120601.141040

Stoffels, K., Overbergh, L., Bouillon, R., and Mathieu, C. (2007). Immune regulation of 1alpha-hydroxylase in murine peritoneal macrophages: unravelling the IFNgamma pathway. *J. Steroid Biochem. Mol. Biol.* 103, 567–571. doi: 10.1016/j.jsbmb.2006.12.091

Stoffers, K. L., Sorg, B. L., Seuter, S., Rau, O., Radmark, O., and Steinhilber, D. (2010). Calcitriol upregulates open chromatin and elongation markers at functional vitamin D response elements in the distal part of the 5-lipoxygenase gene. *J. Mol. Biol.* 395, 884–896. doi: 10.1016/j.jmb.2009.10.022

Szeles, L., Keresztes, G., Torocsik, D., Balajthy, Z., Krenacs, L., Poliska, S., et al. (2009). 1,25-dihydroxyvitamin D3 is an autonomous regulator of the transcriptional changes leading to a tolerogenic dendritic cell phenotype. *J. Immunol.* 182, 2074–2083. doi: 10.4049/jimmunol.0803345

Takahashi, K., Nakayama, Y., Horiuchi, H., Ohta, T., Komoriya, K., Ohmori, H., et al. (2002). Human neutrophils express messenger RNA of vitamin D receptor and respond to 1alpha,25-dihydroxyvitamin D3. *Immunopharmacol. Immunotoxicol.* 24, 335–347. doi: 10.1081/IPH-120014721

Tang, J., Zhou, R., Luger, D., Zhu, W., Silver, P. B., Grajewski, R. S., et al. (2009). Calcitriol suppresses antiretinal autoimmunity through inhibitory effects on the Th17 effector response. *J. Immunol.* 182, 4624–4632. doi: 10.4049/jimmunol.0801543

Teles, R. M., Graeber, T. G., Krutzik, S. R., Montoya, D., Schenk, M., Lee, D. J., et al. (2013). Type I interferon suppresses type II interferon-triggered human anti-mycobacterial responses. *Science* 339, 1448–1453. doi: 10.1126/science.1233665

Tripathi, S., Tecle, T., Verma, A., Crouch, E., White, M., and Hartshorn, K. L. (2013). The human cathelicidin LL-37 inhibits influenza A viruses through a mechanism distinct from that of surfactant protein D or defensins. *J. Gen. Virol.* 94, 40–49. doi: 10.1099/vir.0.045013-0

Ustianowski, A., Shaffer, R., Collin, S., Wilkinson, R. J., and Davidson, R. N. (2005). Prevalence and associations of vitamin D deficiency in foreign-born persons with tuberculosis in London. *J. Infect.* 50, 432–437. doi: 10.1016/j.jinf.2004.07.006

Veldman, C. M., Cantorna, M. T., and DeLuca, H. F. (2000). Expression of 1,25-dihydroxyvitamin D(3) receptor in the immune system. *Arch. Biochem. Biophys.* 374, 334–338. doi: 10.1006/abbi.1999.1605

Vergne, I., Chua, J., Singh, S. B., and Deretic, V. (2004). Cell biology of mycobacterium tuberculosis phagosome. *Annu. Rev. Cell Dev. Biol.* 20, 367–394. doi: 10.1146/annurev.cellbio.20.010403.114015

Wan, M., Sabirsh, A., Wetterholm, A., Agerberth, B., and Haeggstrom, J. Z. (2007). Leukotriene B4 triggers release of the cathelicidin LL-37 from human neutrophils: novel lipid-peptide interactions in innate immune responses. *FASEB J.* 21, 2897–2905. doi: 10.1096/fj.06-7974com

Wang, J., Lian, H., Zhao, Y., Kauss, M. A., and Spindel, S. (2008). Vitamin D3 induces autophagy of human myeloid leukemia cells. *J. Biol. Chem.* 283, 25596–25605. doi: 10.1074/jbc.M801716200

Wang, T. J., Zhang, F., Richards, J. B., Kestenbaum, B., van Meurs, J. B., Berry, D., et al. (2010a). Common genetic determinants of vitamin D insufficiency: a genome-wide association study. *Lancet* 376, 180–188. doi: 10.1016/S0140-6736(10)60588-0

Wang, T. T., Dabbas, B., Laperriere, D., Bitton, A. J., Soualhine, H., Tavera-Mendoza, L. E., et al. (2010b). Direct and indirect induction by 1,25-dihydroxyvitamin D3 of the NOD2/CARD15-defensin beta2 innate immune pathway defective in Crohn disease. *J. Biol. Chem.* 285, 2227–2231. doi: 10.1074/jbc.C109.071225

Wang, T. T., Nestel, F. P., Bourdeau, V., Nagai, Y., Wang, Q., Liao, J., et al. (2004). Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. *J. Immunol.* 173, 2909–2912.

Wang, T. T., Tavera-Mendoza, L. E., Laperriere, D., Libby, E., MacLeod, N. B., Nagai, Y., et al. (2005). Large-scale *in silico* and microarray-based identification of direct 1,25-dihydroxyvitamin D3 target genes. *Mol. Endocrinol.* 19, 2685–2695. doi: 10.1210/me.2005-0106

Wang, W. L., Chatterjee, N., Chittur, S. V., Welsh, J., and Tenniswood, M. P. (2011). Effects of 1alpha,25 dihydroxyvitamin D3 and testosterone on miRNA and mRNA expression in LNCaP cells. *Mol. Cancer* 10:58. doi: 10.1186/1476-4598-10-58

Wang, X., Gocek, E., Liu, C. G., and Studzinski, G. P. (2009). MicroRNAs181 regulate the expression of p27Kip1 in human myeloid leukemia cells induced

to differentiate by 1,25-dihydroxyvitamin D3. *Cell Cycle* 8, 736–741. doi: 10.4161/cc.8.5.7870

Wejse, C., Gomes, V. F., Rabna, P., Gustafson, P., Aaby, P., Lisse, I. M., et al. (2009). Vitamin D as supplementary treatment for tuberculosis: a double-blind, randomized, placebo-controlled trial. *Am. J. Respir. Crit. Care Med.* 179, 843–850. doi: 10.1164/rccm.200804-567OC

Wilkinson, R. J., Llewelyn, M., Toossi, Z., Patel, P., Pasvol, G., Lalvani, A., et al. (2000). Influence of vitamin D deficiency and vitamin D receptor polymorphisms on tuberculosis among Gujarati Asians in west London: a case-control study. *Lancet* 355, 618–621. doi: 10.1016/S0140-6736(99)02301-6

Williams, B., Williams, A. J., and Anderson, S. T. (2008). Vitamin D deficiency and insufficiency in children with tuberculosis. *Pediatr. Infect. Dis. J.* 27, 941–942. doi: 10.1097/INF.0b013e31817525df

Yu, S., Bruce, D., Froicu, M., Weaver, V., and Cantorna, M. T. (2008). Failure of T cell homing, reduced CD4/CD8alphaalpha intraepithelial lymphocytes, and inflammation in the gut of vitamin D receptor KO mice. *Proc. Natl. Acad. Sci. U.S.A.* 105, 20834–20839. doi: 10.1073/pnas.0808700106

Yuk, J. M., Shin, D. M., Lee, H. M., Yang, C. S., Jin, H. S., Kim, K. K., et al. (2009). Vitamin D3 induces autophagy in human monocytes/macrophages via cathelicidin. *Cell Host Microbe* 6, 231–243. doi: 10.1016/j.chom.2009.08.004

Zanzoni, A., Montecchi-Palazzi, L., Quondam, M., Ausiello, G., Helmer-Citterich, M., and Cesareni, G. (2002). MINT: a Molecular INTeraction database. *FEBS Lett.* 513, 135–140. doi: 10.1016/S0014-5793(01)03293-8

Zehnder, D., Landray, M. J., Wheeler, D. C., Fraser, W., Blackwell, L., Nuttall, S., et al. (2007). Cross-sectional analysis of abnormalities of mineral homeostasis, vitamin D and parathyroid hormone in a cohort of pre-dialysis patients. The chronic renal impairment in Birmingham (CRIB) study. *Nephron Clin. Pract.* 107, c109–c116. doi: 10.1159/000108652

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 January 2014; accepted: 30 March 2014; published online: 21 April 2014.
Citation: Chun RF, Liu PT, Modlin RL, Adams JS and Hewison M (2014) Impact of vitamin D on immune function: lessons learned from genome-wide analysis. Front. Physiol. ***5****:151. doi: 10.3389/fphys.2014.00151*
This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 09 April 2014
doi: 10.3389/fphys.2014.00137

Vitamin D and gene networks in human osteoblasts

Jeroen van de Peppel and Johannes P. T. M. van Leeuwen*

Department of Internal Medicine, Bone and Calcium Metabolism, Erasmus MC, Rotterdam, Netherlands

Edited by:
Carsten Carlberg, University of Eastern Finland, Finland

Reviewed by:
Martin Hewison, University of California Los Angeles, USA
Lieve Verlinden, KU Leuven, Belgium

****Correspondence:***
Johannes P. T. M. van Leeuwen, Department of Internal Medicine, Bone and Calcium Metabolism, Erasmus MC, Room Ee585d, Dr. Molewaterplein 50, 3015 GE Rotterdam, Netherlands
e-mail: j.vanleeuwen@erasmusmc.nl

Bone formation is indirectly influenced by 1,25-dihydroxyvitamin D3 (1,25D3) through the stimulation of calcium uptake in the intestine and re-absorption in the kidneys. Direct effects on osteoblasts and bone formation have also been established. The vitamin D receptor (VDR) is expressed in osteoblasts and 1,25D3 modifies gene expression of various osteoblast differentiation and mineralization-related genes, such as alkaline phosphatase (ALPL), osteocalcin (BGLAP), and osteopontin (SPP1). 1,25D3 is known to stimulate mineralization of human osteoblasts *in vitro*, and recently it was shown that 1,25D3 induces mineralization via effects in the period preceding mineralization during the pre-mineralization period. For a full understanding of the action of 1,25D3 in osteoblasts it is important to get an integrated network view of the 1,25D3-regulated genes during osteoblast differentiation and mineralization. The current data will be presented and discussed alluding to future studies to fully delineate the 1,25D3 action in osteoblast. Describing and understanding the vitamin D regulatory networks and identifying the dominant players in these networks may help develop novel (personalized) vitamin D-based treatments. The following topics will be discussed in this overview: (1) Bone metabolism and osteoblasts, (2) Vitamin D, bone metabolism and osteoblast function, (3) Vitamin D induced transcriptional networks in the context of osteoblast differentiation and bone formation.

Keywords: vitamin D, osteoblast, differentiation, mineralization, autocrine/paracrine mechanisms, immune system

BONE METABOLISM AND OSTEOBLASTS

Bone is formed during fetal development by two processes: endochondral and intramembranous ossification (for review Bilezikian et al., 2002). Skull and flat bones are formed by intramembranous ossification where there is direct bone formation by condensation of the mesenchyme without a preformed cartilaginous scaffold. Long bones and most of the remaining bones are formed by endochondral ossification (Mackie et al., 2008, 2011; Nishimura et al., 2012). This type of bone formation is characterized by the transition of cartilage into mineralized bone tissue.

Two major processes occur in bone: bone modeling and bone remodeling. While bone modeling drives the growth of the skeleton, bone remodeling is responsible for the maintenance of healthy bone in the adulthood (Teti, 2011). Bone remodeling takes place throughout live and maintains the structural integrity and strength of the bone by removing old or damaged bone and replacing it by new, strong bone. Remodeling is a local process that can take place anywhere on the bone surface throughout the lifespan of a bone. Remodeling occurs in a temporary anatomic unit of osteoclasts and osteoblasts called a bone multicellular unit (BMU) (Martinello et al., 2012; Sims and Martin, 2014). The BMU is a sealed compartment in which bone resorption and subsequent formation are regulated. This coupled resorption and formation characterizes and differentiates bone remodeling from bone modeling, in which bone resorption and formation do not have to occur at the same time and site. For growth and for the maintenance of healthy bone, multiple cell types are of importance: mesenchymal stem cells (MSC), osteoblasts, osteocytes, and hematopoietic stem cells and osteoclasts. The osteoblasts play a pivotal role in bone metabolism by forming bone but also by controlling and regulating the formation and activity of the bone resorbing cell the osteoclast.

Osteoblasts originate from MSC. MSCs are located in the bone marrow but also in almost all other tissues undergoing continuous tissue homeostasis. MSCs can differentiate into osteoblasts, chondrocytes, fibroblasts, adipocytes or myocytes (Friedenstein et al., 1974; Minguell et al., 2001; Yin, 2006). During osteoblast differentiation several functional phases can be identified: proliferation, production and maturation of extracellular matrix (ECM) and ECM mineralization (Owen et al., 1991). Osteoblast differentiation can start by a trigger of certain growth factors (Wang, 1993) as well as hormones and other factors (Eijken et al., 2006). Mature osteoblasts produce and secrete ECM molecules (Owen et al., 1991). Osteoblasts synthesize the most abundant bone ECM protein collagen type I but also a broad range of non-collagenous ECM proteins. Mineralization of the ECM is likely induced by matrix vesicles which derive from osteoblasts (Anderson et al., 2005). When mature osteoblasts initiate mineralization of mature ECM, its fate may vary. Osteoblasts can further differentiate into osteocytes, become a bone lining cell or undergo apoptosis (Jilka et al., 1998; Weinstein et al., 1998). Osteoblasts become osteocytes by being entrapped in self-produced ECM, in which they may survive for decades. Osteocytes form a star-shaped network of cytoplasmic extensions. Osteocytes are thought to function as orchestrators of bone by

sensing and communicating mechanical stress (i.e., bone damage) via these extensions (Bonewald, 2011). It has become evident by genetic approaches that osteocytes play a role in regulation of bone turn-over (resorption and formation) (Nakashima et al., 2011; Atkins and Findlay, 2012). Bone lining cells are less well understood. They are covering the bone surface and prevent it from being in direct contact with the bone marrow. It has been reported that these cells "clean" resorption pits after osteoclasts retreated (Everts et al., 2002). Bone lining cells are considered as inactive osteoblasts. It has been suggested that these cells can be activated to become osteoblasts (Dobnig and Turner, 1995; Chow et al., 1998) but they also may represent the osteoblastic part of the stem cell niche and interact with the hematopoietic stem cells.

The osteoblasts/osteocytes guarantee the close coupling between bone formation and resorption in healthy bone remodeling. Osteoblasts and osteocytes produce the soluble osteoclast stimulating factors RANKL and M-CSF which upon binding to its receptors (RANK and c-Fms, respectively) induce differentiation of osteoclast progenitors and fusion of mononuclear cells into multinucleated tartrate-resistant acid phosphatase positive osteoclasts (Boyle et al., 2003). Besides RANKL, osteoblasts also produce a soluble decoy-receptor: osteoprotegerin (OPG). OPG binds RANKL with high affinity leading to inhibition of osteoclast stimulation and thus leading to less bone resorption (Lacey et al., 1998; Kostenuik and Shalhoub, 2001).

VITAMIN D, BONE METABOLISM, AND OSTEOBLAST

The biologically most active form of vitamin D, 1α,25-dihydroxyvitamin D3 [calcitriol or 1,25(OH)2D3 (1,25D3)] is formed by a stepwise process starting in the skin and involving the liver and kidney. Upon ultraviolet B exposure, 7-dehydrocholesterol (pro-vitamin D3) is transformed into (pre)vitamin D3 (cholecalciferol) in the skin. Subsequent hydroxylation at the C25 and 1α position in liver and kidney, respectively, produce 1,25D3 (Holick, 1995). In bone diseases, vitamin D is used as an anti-rickets agent (Kitanaka et al., 1998; McCollum et al., 2002; Tatsumi et al., 2007), which improves bone mineralization and is often prescribed in combination with other osteoporosis drugs to secure a positive calcium balance. However, a recent metaanalyes by Reid et al suggests that the supplementation of vitamin D for the prevention of osteoporosis is inappropriate without specific risk factors for vitamin D deficiency (Reid et al., 2014).

Up to today it is still in debate whether 1,25D3 effects on bone formation are indirect via intestinal and renal regulation of calcium levels or also via a direct effect on osteoblasts. It has been demonstrated that mice lacking the vitamin D receptor (VDR) gene display retarded growth, severe bone impairment, immune abnormalities, and premature death at only 15 weeks of age due to hypocalcemia (Li et al., 1997; Yoshizawa et al., 1997; Mathieu et al., 2001). A rescue diet restored all pathological effects suggesting that as long as calcium homeostasis is under control, bone itself does not seem to be affected by impaired VDR signaling. The importance of physiological 1,25D3 levels for bone is demonstrated by the mutation of the CYP27B1 gene. Subjects with a mutation in that gene develop vitamin-D-dependent rickets (Li et al., 1997). In a mouse model for rickets, greater extensibility and lower stiffness of fibrils resulted from a decreased grade of mineral deposition (Karunaratne et al., 2012). This further supports the importance of an optimal grade of mineralization for healthy bones (Kitanaka et al., 1998) and points to a role for 1,25D3 herein.

A direct positive effect an 1,25D3 analog on bone formation in ovariectomized rats with only slight changes in serum calcium points to the existence of a direct effect on bone formation (Shevde et al., 2002). This is supported by *in vitro* studies demonstrating direct effects on osteoblasts. The VDR is present in osteoblasts and its expression can be regulated by 1,25D3 itself and by other factors such as parathyroid hormone (PTH), glucocorticoids, transforming growth factor-β, and epidermal growth factor (Pols et al., 1988a,b; Reinhardt and Horst, 1990; van Leeuwen et al., 1991, 1992a,b; Godschalk et al., 1992). The expression of VDR allows 1,25D3 to directly affect osteoblast growth and differentiation. 1,25D3 has been shown to stimulate bone formation and mineralization in all studies using human osteoblasts and stimulate osteogenic differentiation from human mesenchymal stem/stromal cells (MSC) (Ueno et al., 1992; Prince et al., 2001; Jørgensen et al., 2004; Van Driel et al., 2006a,b; Zhou et al., 2006, 2012). 1,25D3 enhanced mineralization by effects on human osteoblasts prior to the onset of mineralization (Woeckel et al., 2010). Thus, 1,25D3 is not directly involved in the process of mineral deposition but more likely in a process preparing the environment/ECM for mineralization. 1,25D3 regulates the osteoblast differentiation marker ALPL and various bone ECM proteins such as COL1A1. Procollagen type I by human osteoblasts was stimulated (Franceschi et al., 1988; Hicok et al., 1998) as well as unaffected (Ingram et al., 1994; Hicok et al., 1998; Siggelkow et al., 1999) by vitamin D. However, gene expression profiling studies demonstrated that the 1,25D3 effect in the pre-mineralization phase is not likely primarily due to changes in expression of ECM proteins and thereby composition of the ECM (Woeckel et al., 2010). Production of alkaline phosphatase (ALPL) positive matrix vesicles was significantly induced by 1,25D3 in this period of osteoblast differentiation (Anderson, 1995) providing a means to enhance mineralization (Woeckel et al., 2010). In addition, previous studies have shown the importance of other factors like TGFβ, IGF-I, bone morphogenetic protein, interferon, PTH, hepatocyte growth factor, epidermal growth factor, and peroxisome proliferator-activated receptor ligands and Wnt signaling for the eventual effect of 1,25D3 on osteoblasts (Petkovich et al., 1987; Pols et al., 1988b; Scharla et al., 1991; Bonewald et al., 1992; Godschalk et al., 1992; van Leeuwen et al., 1992a,b; Ingram et al., 1994; Staal et al., 1994, 1996, 1998; Haussler et al., 1998; Yanagisawa et al., 1999; Sammons et al., 2004; Yarram et al., 2004; Fretz et al., 2007; Chen et al., 2012a, 2013; Woeckel et al., 2012; Yamaguchi and Weitzmann, 2012). These data stress the importance of studying and interpreting the effects of 1,25D3 on bone in a systems biological approach encompassing the different layers of regulation and interactions.

In contrast to human and rat studies, 1,25D3 inhibits differentiation and mineralization in cultures of murine osteoblasts (Shi et al., 2007; Chen et al., 2012a,b, 2013) and murine VDR deficient osteoblasts have increased osteogenic potential (Sooy et al., 2004). 1,25D3 increases in a VDR-dependent manner the expression of

progressive ankylosis (ANK) and ectonucleotide pyrophosphatase phosphodiesterase (ENPP1) in murine osteoblasts. This leads to an increase in the level of pyrophosphate (PPi) that inhibits mineralization (Lieben et al., 2012). 1,25D3 also increases osteopontin shown to inhibit mineralization (Staal et al., 1996). However, transgenic murine models with osteoblast-specific VDR overexpression show increased bone formation and mineralization (Gardiner et al., 2000; Misof et al., 2003; Xue et al., 2006). An 1,25D3 analog had a positive effect on bone nodule formation and mineralization in murine calvarial osteoblast cultures of wild type but not VDR null mice (Shevde et al., 2002) while one study showed increased mineralization in MC3T3 cell cultures (Matsumoto et al., 1991). In a recent study, Yamamoto et al. (2013) illustrated that mice lacking VDR in osteoblasts had an increased bone mass, due to decreased bone resorption.

Overall the present data show variation in effects of 1,25D3 on differentiation and mineralization with overall stimulatory effects in human and rat osteoblasts while overall an inhibitory effect in murine osteoblasts (Van Driel et al., 2006a). Following this, 1,25D3 has been shown to increase RUNX2 expression in human osteoblasts (Prince et al., 2001; Viereck et al., 2002; Maehata et al., 2006) while 1,25D3 suppresses RUNX2 promoter and reduces RUNX2 expression in murine osteoblasts (Prince et al., 2001; Drissi et al., 2002). Osteocalcin (BGLAP) is an interesting gene considering differences in 1,25D3 effects in human and murine osteoblasts (Thomas, 2000). 1,25D3 stimulates BGLAP expression in human and rat osteoblasts while it inhibits BGLAP expression in murine osteoblasts (Lian et al., 1997; Zhang et al., 1997), supporting differences between human/rat osteoblasts and murine with respect to 1,25D3 responsiveness and mineralization.

A full explanation for this apparent discrepancy between human and murine osteoblasts is absent. Both the extracellular milieu (i.e., presence/absence of growth factors, cytokines and other signaling molecules) and the intracellular milieu (e.g., the insulin-like growth factor binding protein-6 that can bind to the VDR and inhibit 1,25D3 induction of ALPL activity) of the cell is important for the eventual effect of 1,25D3 (Cui et al., 2011). Also the extracellular phosphate concentration may affect the 1,25D3 action (Ito et al., 2013). These characteristics may contribute to the differences in 1,25D3 effects observed in human and murine osteoblasts.

Besides stimulation of bone formation /mineralization by osteoblasts 1,25D3 has certain protective control mechanisms in place to avoid pathological over-mineralization. For example, 1,25D3 induces BGLAP and SPP1, established inhibitors of mineralization (Noda et al., 1990; MacDonald et al., 1993) and a stimulator of mineralization, bone sialoprotein (IBSP), is inhibited by 1,25D3 (Li and Sodek, 1993). As mentioned above also the presence or absence of other growth factors, cytokines or signaling molecules may limit the 1,25D3 effect. Examples of this in relation to mineralization are Activin A and follistatin. Activin A inhibits osteoblast differentiation and mineralization (Eijken et al., 2007). Activin A expression in human osteoblasts is stimulated by 1,25D3 (Woeckel et al., 2013), implicating that 1,25D3 as stimulator of human osteoblast differentiation and mineralization also stimulates the production of a mineralization inhibitor. A function in the prevention of over-mineralization is supported by the data that the activin A blocker follistatin enhances 1,25D3 stimulated mineralization (Woeckel et al., 2013). The above mentioned induction of carboxylated osteocalcin by 1,25D3 may fit this hypothesis on preventing over-mineralization. Accumulation of osteocalcin in the ECM of human osteoblast cultures stimulated by 1,25D3 is inhibited by warfarin (antagonist of vitamin K) while vitamin K2 (cofactor of γ-carboxylase) enhanced the 1,25 D3 effect (Koshihara and Hoshi, 1997). 1,25D3 stimulated mineralization was significantly augmented by warfarin (Woeckel et al., 2013). These data on activin A, follistatin, warfarin, and vitamin K put forward a 1,25D3 induced regulatory mechanism to guarantee optimal mineralization (Woeckel et al., 2013). Differences in these regulatory loops may also be part of the differences in 1,25D3 effects in human and murine osteoblast studies.

The most well-known mechanism to limit the biological activity of 1,25D3 is its degradation via 24-hydroxylation. 1,25D3 potently induces CYP24A1, which encodes for the enzyme 24-hydroxylase, in osteoblasts. 24-Hydroxylation is the first step in the degradation cascade of active 1,25D3 (Ohyama et al., 1994). However, hydroxylation at the C-24 position doesn't directly lead to an inactive vitamin D molecule. Henry and Norman demonstrated the significance of 24,25-dihydroxyvitamin D3 (24,25D3) for normal chicken egg hatchability and calcium and phosphorus homeostasis (Henry and Norman, 1978; Norman et al., 1980). Already in 1980 it was shown that 24,25D3 directly stimulates calcification of bone in interaction with PTH and that the number and size of resorption sites in bone is decreased by 24,25D3 (Endo et al., 1980; Galus et al., 1980). Several other studies supported a positive effect of 24,25D3 on bone metabolism (Matsumoto et al., 1985; Tam et al., 1986; Kato et al., 1998) while one study showed no effect of 24,25D3 on histomorphometric parameters in ovariectomized rats (Erben et al., 1992). Administration of 24,25D3 in combination with 1,25D3 improved fracture healing in chickens (Seo et al., 1997) and interestingly, 24,25D3 serum levels correlated to fracture healing (Seo and Norman, 1997). Studies with the CYP24A knockout mouse supported a role for 24,25D3 in fracture repair (St-Arnaud, 2010). Albeit in a human study no positive association with femoral fracture was observed (Weisman et al., 1978). However, a study in pre-dialysis renal insufficiency patients supported a direct, i.e., PTH-independent, functional role of 24,25D3 in bone (Birkenhäger-Frenkel et al., 1995). These data suggest a direct effect on osteoblasts. *In vitro* studies with human osteoblasts have shown that indeed 24,25D3 has direct effects similar to that of 1,25D3 (Van Driel et al., 2006b). A recent comparative gene expression profiling study of 1,25D3, 24,25D3, and 25D3 in primary human and mouse fibroblasts suggested induction of metabolite specific sets of genes and pathways (Tuohimaa et al., 2013). It is important to note that the fact whether biological active levels of 24,25D3 or 1,24,25-trihydroxyvitamin D3 (1,24,25D3) can be reached fully depends on the velocity of the subsequent steps in the degradation pathway after the initial 24-hydroxylation step.

We have shown that osteoblasts besides degradation of active 1,25D3, are able to convert 25-hydroxyvitamin D3 (25D3) into the biologically most active form 1,25D3, suggesting a direct relationship between 1,25D3 synthesis and bone (Van Driel et al.,

2006a). This study showed functionality of 1α-hydroxylation in human osteoblast differentiation. 25D3 induced expression of CYP24, osteocalcin and stimulated ALPL activity and mineralization, which was blocked by inhibition of 1α-hydroxylase by ketoconazole. Downregulation of CYP27B1 in human osteoblasts or perturbation of CYP27B1 supported the requirement of 1α-hydroxylase for the effect on human MSC proliferation and osteogenic differentiation (Atkins et al., 2007; Geng et al., 2011a). CYP27B1 expression is reduced in MSC of older subjects and resistance to 25D3 induced osteoblast formation points to an aging effect (Geng et al., 2011b). The 1α-hydroxylase-dependent 25D3 stimulation of ALPL activity in human MSC was blocked by histone deacytylase inhibition (Zhou et al., 2013). Of interest, 25D3 has been shown to regulate gene expression in a gene expression profiling study with CYP27B1 deficient fibroblasts (Tuohimaa et al., 2013). This suggests that 25D3 may act independent of 1α-hydroxylation.

Up to now the data on 1,25D3 production by osteoblasts are derived from *in vitro* studies. *In vivo* significance of CYP27B1 and 1,25D3 formation in osteoblasts needs yet to be proven, for example by knocking out CYP27B1 specifically in osteoblasts. However, the observed discrepancies in effects on human-murine osteoblasts may hamper this approach. Although yet *in vivo* proof is lacking, the principal of local synthesis of 1,25D3 in bone may explain the observed associations of 25D3 and not of 1,25D3 with bone as well as other parameters (Hewison et al., 2004; Anderson et al., 2013). Besides CYP27B1, osteoblasts also express the receptors megalin and cubulin that are involved in cellular uptake of 25D3 via endocytosis of the vitamin D binding protein (DBP) (Van Driel et al., 2006a; Atkins et al., 2007). Linking back to the above discussed interaction between locally produced growth factors and 1,25D3 is the regulation of CYP27B1 in osteoblasts. Albeit 1,25D3 itself inhibits CYP27B1 expression in MSC as well as in the kidney (Zhou et al., 2010), the regulation appears to be different and more complex than in the kidney involving local regulators. Several locally in bone produced factors affects CYP27B1 expression: TGFβ suppresses 5′-flanking region of CYP27B1 (Turner et al., 2007) and interferon-β reduces while interleukin-1 and IGF-I increase CYP27B1 expression in mature human osteoblasts (Van Driel et al., 2006a; Zhou et al., 2010; Woeckel et al., 2012). The effect of interleukin-1 points to the involvement of NF-κB in stimulation of CYP27B1 expression in human osteoblasts. This is supported by the interferon-β inhibition of NF-κB in synoviocytes (Van Holten et al., 2004) and CYP27B1 regulation in human dendritic cells (Hewison et al., 2003).

1,25D3 plays an important role in maintaining bone health either via controlling calcium and phosphate homeostasis or via direct effects on osteoblasts. This latter is supported by the direct effects of 1,25D3 on osteoblast differentiation, expression and activity of bone formation related proteins and enzymes, and mineralization. The complete vitamin D endocrine system, from receptor to enzymes involved in 1,25D3 synthesis and breakdown, is present in the osteoblast, pointing to an autocrine/paracrine 1,25D3 function in bone. This is the more so interesting as over the past decade it has become clear that osteoblasts are not only involved in bone metabolism but that they also form the hematopoietic stem cell (HSC) niche controlling renewal of HSCs and differentiation of the immune cells (Calvi et al., 2003). Moreover, these HSC niches are also the sites of bone metastasis (Shiozawa et al., 2011). Considering the 1,25D3 effect on the immune system and tumor cell growth it is tempting to speculate that autocrine/paracrine action of 1,25D3 is also beyond bone metabolism and important for other regulatory functions of osteoblasts. It is therefore of critical importance to understand the full picture of 1,25D3 effects on osteoblasts. One of the approaches to obtain information on the effects of 1,25D3 on osteoblasts and MSC in an unbiased way is by omics approaches in combination with bioinformatics. In the next paragraph the current available 1,25D3 gene expression profiling studies of osteoblasts will be discussed.

VITAMIN D AND GENE TRANSCRIPTION IN THE CONTEXT OF OSTEOBLAST DIFFERENTIATION AND BONE FORMATION

1,25D3 has been shown to regulate the expression of various genes related to osteoblast proliferation and differentiation. BMP-2 induced bone formation has been suggested to be enhanced by 1,25D3 induced c-MYC expression (Piek et al., 2010). Induction of Insulin-like growth factor-binding proteins (IGFBP)-2, -3, and -4 expression by 1,25D3 in human MSC may play a role in stimulation of osteogenic differentiation (Kveiborg et al., 2001). Recently, Li and coworkers (Li et al., 2013) demonstrated that IGFBP-3 interacts with the VDR and negatively regulates CYP24 and BGLAP expression. Overexpression of IGFBP-3 inhibited the 1,25D3 activation of ALP in MG-63 human osteosarcoma cells.

1,25D3 also regulated Forkhead Box O (FoxO) transcription factors in murine MC3T3 osteoblasts with FoxO3a being up-regulated while FoxO1 was down-regulated, and FoxO4 not affected. Knockdown of the FoxO's didn't change 1,25D3 inhibition of cell growth but led to increased accumulation of reactive oxygen species after 1,25D3 treatment (Eelen et al., 2013). This may be linked to cellular metabolism and the high energy demanding process of bone formation (Komarova et al., 2000; Chen et al., 2008; Bruedigam et al., 2010). Unfortunately, the effect of FoxO's knockdown on mineralization in these murine MC3T3 osteoblast cultures was not reported. 1,25D3 increased vascular endothelial growth factor (VEGF) expression in human and rat osteoblasts is interesting considering the relationship between bone formation and angiogenesis (Wang et al., 1996; Schlaeppi et al., 1997; Corrado et al., 2013). VEGF has been shown to be involved in the 1,25D3 bone anabolic effect (Wang et al., 1997).

Recent studies placed miRNAs in the 1,25D3 mechanism of action spectrum in osteoblasts. Five miRNAs were found to be differentially expressed in primary human osteoblast after 6 h of treatment with 1,25D3 (Lisse et al., 2013a,b). Interestingly, miR-637 and miR-1228 are two miRNAs located intergenic in DAPK3 and LRP1, respectively. miR-1228 was upregulated and coexpressed with its host gene LRP1 suggesting a conventional VDRE- mediated transactivation upon 1,25D3 treatment. Since LRP1 is known to mediate the canonical Wnt pathway in fibroblasts (Terrand et al., 2009), this suggests an indirect regulation of Wnt signaling by 1,25D3 adding to other data on 1,25D3 and Wnt signaling interaction (Fretz et al., 2007; Haussler et al., 2010).

The target of miR-1228, BMP2K, was previously identified to be increased in mouse osteoblasts upon treatment with BMP2 (Kearns et al., 2001). Stable expression of BMP2K in mouse osteoprogenitor cells decreased ALPL activity and osteocalcin mRNA levels. This suggests that 1,25D3 induced expression of miR-1228 may affect osteoblast differentiation via down-regulation of BMP2K.

On the contrary, 1,25D3 upregulated miR-637 while its host gene was downregulated suggesting a different way of regulation of the two transcripts. miR-637 stimulated the degradation of COL4A mRNA levels that is expressed in the basement membrane and is downregulated during early differentiation of mouse MC3T3-E1 osteoblasts (Hong et al., 2010). It is becoming evident that miRNAs play an important role in osteoblast differentiation and bone formation (Lian et al., 2012) and in the near future more data on their role in 1,25D3 action in osteoblasts will come forward (Lisse et al., 2013a).

IDENTIFICATION OF 1,25D3 TARGET GENES IN OSTEOBLASTS

In the past various studies have investigated the effects of 1,25D3 on target gene expression and VDR binding to DNA response elements. Only a few of these genome-wide studies have investigated the effects of 1,25D3 in the context of osteoblasts (**Table 1**). The studies that carried out are very heterogenic with regard to the differentiation stage of the cells (MSC vs. primary osteoblasts vs. Cell line), time points of treatment (2–6 h after treatment) and the 1,25D3 concentration that is used (1–100 nM). Together this makes it difficult to compare the different studies. Systematic analyses of both mRNA gene expression profiling and VDR binding experiments at early time points after induction with 1,25D3 will uncover direct target genes. Below we will address a few of these studies and the results obtained.

ChIP ANALYSES IN OSTEOBLASTS

Upon binding of 1,25D3 to the VDR, the VDR binds with its heterodimeric partner retinoid X receptor (RXR) on the vitamin D receptor response elements (VDRE). The VDRE consists of the hexameric sequence AGGTCAxxxAGGTCA (Ozono et al., 1990) but variants to the conserved sequence have been identified (Meyer, 2005). Due to the diversity of VDRE, bioinformatics approaches are limited in identifying whole genome VDR binding sites. To identify direct target genes of VDR, genome-wide approaches such as ChIP-chip or ChIP-seq approaches have to be performed. Systematic analyses of VDR binding upon activation by 1,25D3 combined with bioinformatics approaches identifies VDRE (VDR response elements) and subsequently direct targets of Vitamin D signaling. A few studies have started to identify 1,25D3 target genes is various cell types such as a human derived lymphoblastoid cell line (Ramagopalan et al., 2010) and monocytes (Heikkinen et al., 2011). Recently, the first VDR binding experiments in osteoblasts were published (Meyer et al., 2010). Meyer et al. analyzed the genomic locations that bind VDR, RXR, RNA polymerase II and acetylated H4 after 3 h treatment with 1,25D3 in mouse MCT3T-E1 osteoblasts. Interestingly, only 13% of the identified sites was located in classical promoter regions upstream vitamin D target genes. The majority of sites that were found to bind VDR, RXR and acetylated H4 were located distal (43%) and within intronic and exonic regions (44%). This demonstrates that distal transcriptional control contributes to the majority of vitamin D3-mediated transcription. Genome wide ChIP-seq analyses with human osteoblasts should illustrate whether binding of VDR at distal locations is conserved.

Pilot analysis of our gene expression profiles of osteogenic and adipogenic MSCs illustrated that many known 1,25D3 responsive genes (on basis of Ingenuity database; www.ingenuity.com) are dynamically expressed during adipogenic as well as osteogenic differentiation (data not published). This data does not directly show that these genes are regulated by 1,25D3 but it suggests that 1,25D3-responsive genes can have a role during the differentiation of mesenchymal precursors. Many of the two-fold regulated genes during osteogenic differentiation and those that were identified previously to be regulated by 1,25D3 are involved in Cell Cycle (41/162; GO:0007049), response to steroid hormone (21/162; GO:0048545), regulation of phosphate metabolic process (26/162, GO:0019220), regulation of apoptosis (31/162, GO:0042981), extracellular region part (36/162; GO:0044421). ChIP analyses using VDR and expression profiling of 1,25D3

Table 1 | Genome-wide studies of vitamin D and osteoblasts.

Publication	Experiment	Species	Cell type	Treatment
Lisse et al., 2013a,b	Expression profiling miRNA	Homo sapiens	Primary osteoblasts	1,25D3 10^{-8} M 6 h
Woeckel et al., 2012	Expression profiling mRNA	Homo sapiens	Pre-osteoblasts svHFO	1,25D3 10^{-8} M 2 and 24 h
Tarroni et al., 2012	Expression profiling mRNA	Homo sapiens	Primary osteoblasts	1,25D3 10^{-7} M 24 h
Grundberg et al., 2011	Expression profiling mRNA	Homo sapiens	Trabecular bone	1,25D3 10^{-7} M 2 and 24 h
Piek et al., 2010	Expression profiling mRNA	Homo sapiens	MSCs	1,25D3 10^{-8} M 0, 1, 3, 6, 12, 24, 48, 72, 120, 192, and 288 h
Meyer et al., 2010	VDR localization ChIP-chip	Mus musculus	Pre-osteoblasts MC3T3-E1	1,25D3 10^{-7} M 3 h
Woeckel et al., 2010	Expression profiling mRNA	Homo sapiens	Pre-osteoblasts svHFO	1,25D3 10^{-8} M 3, 7, 12, and 19 days
Eelen et al., 2004	Expression profiling mRNA	Mus musculus	Pre-osteoblasts MC3T3-E1	1,25D3 10^{-8} M 6 and 12 h
Farach-Carson and Xu, 2002	Expression profiling mRNA	Rattus norvegicus	Osteosarcoma ROS 17/2.8	1,25D3 10^{-9} M 0, 6 and 24 h

Database searches were performed using Bone[Title/Abstract] OR osteoblast[Title/Abstract] AND vitamin D AND microarray in Pubmed (http://www.ncbi.nlm.nih.gov/pubmed) and GEO (http://www.ncbi.nlm.nih.gov/geo/).

transcriptional activity against the backdrop of osteogenic MSC will be needed to demonstrate the importance of VDR—1,25D3 binding in osteoblast function either in bone formation, regulation of osteoclast formation and activity or in the stem cell niche.

GENE EXPRESSION PROFILING IN OSTEOBLASTS

Besides binding of the VDR to gene regulatory elements, important information on the effect of 1,25D3 on osteoblasts comes from expression profiling studies upon 1,25D3 treatment. Several gene expression profiling studies have been performed to examine the effect of 1,25D3 on RNA expression in osteoblasts. Gene expression profiling in murine MC3T3 cells showed down-regulation of DNA replication genes (Eelen et al., 2004) which fits the earlier observed inhibition of proliferation in these cells. Gene profiling of 1,25D3 treated human osteoblasts at multiple days during the differentiation phase before mineralization did not show regulation a specific set of DNA replication genes (Woeckel et al., 2010). Cell death, RNA splicing translation, and cell cycle genes were identified by Gene Ontology analyses as being most significantly overrepresented (Woeckel et al., 2010). Only 0.6 % (3 genes) of the genes changed in expression during the mineralizing period were also changed prior to mineralization (Woeckel et al., 2010). This study demonstrated that 1,25D3 has different effects on gene expression dependent on the differentiation stage of the cells and should be carefully addressed when investigating the effects of 1,25D3 on mesenchymal stem/stromal cells and differentiated osteoblasts.

Tarroni et al. found that upon 24 h treatment of human osteoblasts with 1,25D3 most genes were upregulated (136 up vs. 20 down) indicating the transcriptional activation of 1,25D3 (Tarroni et al., 2012). Pathway analyses identified various biological functions and/or diseases related to bone metabolism and cellular processes/molecular functions related to skeletal development. The link with skeletal development is supported by another study showing 1,25D3 induced expression in human and mouse osteoblasts of the odd-skipped related genes Osr1 and Osr2, known from expression in the developing limb (Verlinden et al., 2013).

Tarroni et al. also showed strong change in expression of genes linked to inflammation or immune and lymphatic system development (Tarroni et al., 2012). In line with this, is the observation of a gene profiling study showing interferon-related genes being overrepresented after 1,25D3 treatment of human osteoblasts. The interferon signaling related genes were down-regulated by 1,25D3 (Woeckel et al., 2012). The observations on processes related to the immune system are interesting from at least two points of view. Firstly, because of the link between the immune system and bone and the effect of immune cells-derived cytokines on bone metabolism, e.g., in conditions like rheumatoid arthritis. Secondly, considering the above mentioned role of the osteoblasts in the stem cell niche and control of hematopoietic stem cell renewal and differentiation. The expression profiling data and the identification of functions and processes related to the immune system may support a role of vitamin D in osteoblasts control the stem cell niche (Kawamori et al., 2010).

CONCLUSION

Vitamin D can regulate bone metabolism in an indirect way via controlling calcium and phosphate homeostasis but also via direct effects on osteoblasts. In fact, the complete vitamin D endocrine system is present in osteoblasts. This enables osteoblasts to respond not only to vitamin D via the VDR but also to synthesize the biological most active vitamin D metabolite 1,25D3 and to act in an autocrine/paracrine manner. Vitamin D directly regulates gene expression and stimulates mineralization in *ex vivo* cultures of human and rat osteoblasts. The effect on mineralization may depend on species and/or environmental context that can alter the eventual vitamin D effect. Besides effects on bone metabolism, vitamin D effects on osteoblasts may be related to additional functions of osteoblasts such as the hematopoietic stem cell niche. Interesting in this respect is that gene expression profiling studies on vitamin D-treated osteoblasts revealed genes and processes related to the immune system. Further studies are needed to delineate these non-bone metabolism related effects of vitamin D in osteoblasts in greater detail at cellular and molecular level. A future challenge will be to construct networks representing the effects of vitamin D, either in bone metabolism- or in non-bone metabolism-related processes, against the backdrop of osteoblast differentiation by systems biological approaches.

REFERENCES

Anderson, H. C. (1995). Molecular biology of matrix vesicles. *Clin. Orthop. Relat. Res.* 266–280. doi: 10.1097/00003086-199505000-00034

Anderson, H. C., Garimella, R., and Tague, S. E. (2005). The role of matrix vesicles in growth plate development and biomineralization. *Front. Biosci.* 10:1576. doi: 10.2741/1576

Anderson, P. H., Lam, N. N., Turner, A. G., Davey, R. A., Kogawa, M., Atkins, G. J., et al. (2013). The pleiotropic effects of vitamin D in bone. *J. Steroid Biochem. Mol. Biol.* 136, 190–194. doi: 10.1016/j.jsbmb.2012.08.008

Atkins, G. J., Anderson, P. H., Findlay, D. M., Welldon, K. J., Vincent, C., Zannettino, A. C. W., et al. (2007). Metabolism of vitamin D3 in human osteoblasts: evidence for autocrine and paracrine activities of 1α, 25-Dihydroxyvitamin D3. *Bone* 40, 1517–1528. doi: 10.1016/j.bone.2007.02.024

Atkins, G. J., and Findlay, D. M. (2012). Osteocyte regulation of bone mineral: a little give and take. *Osteoporos Int.* 23, 2067–2079. doi: 10.1007/s00198-012-1915-z

Bilezikian, J. P., Raisz, L. G., Rodan, G. A., and Karaplis, A. C. (2002). "Embryonic development of bone and the molecular regulation of intramembranous and endochondral bone formation," in *Principles of Bone Biology*, ed A. C. Karaplis (San Diego, CA: Academic Press), 53–84. doi: 10.1016/B978-0-12-373884-4.00025-2

Birkenhäger-Frenkel, D. H., Pols, H. A., Zeelenberg, J., Eijgelsheim, J. J., Schot, R., Nigg, A. L., et al. (1995). Effects of 24R,25-dihydroxyvitamin D3 in combination with 1 alpha-hydroxyvitamin D3 in predialysis renal insufficiency: biochemistry and histomorphometry of cancellous bone. *J. Bone Miner. Res.* 10, 197–204. doi: 10.1002/jbmr.5650100205

Bonewald, L. F. (2011). The amazing osteocyte. *J. Bone Miner. Res.* 26, 229–238. doi: 10.1002/jbmr.320

Bonewald, L. F., Kester, M. B., Schwartz, Z., Swain, L. D., Khare, A., Johnson, T. L., et al. (1992). Effects of combining transforming growth factor beta and 1,25-dihydroxyvitamin D3 on differentiation of a human osteosarcoma (MG-63). *J. Biol. Chem.* 267, 8943–8949.

Boyle, W. J., Simonet, W. S., and Lacey, D. L. (2003). Osteoclast differentiation and activation. *Nature* 423, 337–342. doi: 10.1038/nature01658

Bruedigam, C., Eijken, M., Koedam, M., van de Peppel, J., Drabek, K., Chiba, H., et al. (2010). A new concept underlying stem cell lineage skewing that explains the detrimental effects of thiazolidinediones on bone. *Stem Cells* 28, 916–927. doi: 10.1002/stem.405

Calvi, L. M., Adams, G. B., Weibrecht, K. W., Weber, J. M., Olson, D. P., Knight, M. C., et al. (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* 425, 841–846. doi: 10.1038/nature02040

Chen, C. T., Shih, Y. R., Kuo, T. K., Lee, O. K., and Wei, Y. H. (2008). Coordinated changes of mitochondrial biogenesis and antioxidant enzymes during osteogenic differentiation of human mesenchymal stem cells. *Stem Cells* 26, 960–968. doi: 10.1634/stemcells.2007-0509

Chen, J., Dosier, C. R., Park, J. H., De, S., Guldberg, R. E., Boyan, B. D., et al. (2013). Mineralization of three-dimensional osteoblast cultures is enhanced by the interaction of 1α,25-dihydroxyvitamin D3 and BMP2 via two specific vitamin D receptors. *J. Tissue Eng. Regen. Med.* doi: 10.1002/term.1770. [Epub ahead of print].

Chen, K., Aenlle, K. K., Curtis, K. M., Roos, B. A., and Howard, G. A. (2012a). Hepatocyte growth factor (HGF) and 1,25-dihydroxyvitamin D together stimulate human bone marrow-derived stem cells toward the osteogenic phenotype by HGF-induced up-regulation of VDR. *Bone* 51, 69–77. doi: 10.1016/j.bone.2012.04.002

Chen, Y.-C., Ninomiya, T., Hosoya, A., Hiraga, T., Miyazawa, H., and Nakamura, H. (2012b). 1α,25-dihydroxyvitamin D3 inhibits osteoblastic differentiation of mouse periodontal fibroblasts. *Arch. Oral Biol.* 57, 453–459. doi: 10.1016/j.archoralbio.2011.10.005

Chow, J. W., Wilson, A. J., Chambers, T. J., and Fox, S. W. (1998). Mechanical loading stimulates bone formation by reactivation of bone lining cells in 13-Week-Old Rats. *J. Bone Miner. Res.* 13, 1760–1767. doi: 10.1359/jbmr.1998.13.11.1760

Corrado, A., Neve, A., and Cantatore, F. P. (2013). Expression of vascular endothelial growth factor in normal, osteoarthritic and osteoporotic osteoblasts. *Clin. Exp. Med.* 13, 81–84. doi: 10.1007/s10238-011-0170-5

Cui, J., Ma, C., Qiu, J., Ma, X., Wang, X., Chen, H., et al. (2011). A novel interaction between insulin-like growth factor binding protein-6 and the vitamin D receptor inhibits the role of vitamin D3 in osteoblast differentiation. *Mol. Cell. Endocrinol.* 338, 84–92. doi: 10.1016/j.mce.2011.03.011

Dobnig, H., and Turner, R. T. (1995). Evidence that intermittent treatment with parathyroid hormone increases bone formation in adult rats by activation of bone lining cells. *Endocrinology* 136, 3632–3638. doi: 10.1210/en.136.8.3632

Drissi, H., Pouliot, A., Koolloos, C., Stein, J. L., Lian, J. B., Stein, G. S., et al. (2002). 1, 25-(OH)2-vitamin D3 suppresses the bone-related Runx2/Cbfa1 gene promoter. *Exp. Cell Res.* 274, 323–333. doi: 10.1006/excr.2002.5474

Eelen, G., Verlinden, L., Meyer, M. B., Gijsbers, R., Pike, J. W., Bouillon, R., et al. (2013). 1,25-dihydroxyvitamin D3 and the aging-related forkhead box O and sestrin proteins in osteoblasts. *J. Steroid Biochem. Mol. Biol.* 136, 112–119. doi: 10.1016/j.jsbmb.2012.09.011

Eelen, G., Verlinden, L., Van Camp, M., Mathieu, C., Carmeliet, G., Bouillon, R., et al. (2004). Microarray analysis of 1α,25-dihydroxyvitamin D3-treated MC3T3-E1 cells. *J. Steroid Biochem. Mol. Biol.* 89–90, 405–407. doi: 10.1016/j.jsbmb.2004.03.008

Eijken, M., Koedam, M., van Driel, M., Buurman, C. J., Pols, H. A. P., and van Leeuwen, J. P. T. M. (2006). The essential role of glucocorticoids for proper human osteoblast differentiation and matrix mineralization. *Mol. Cell. Endocrinol.* 248, 87–93. doi: 10.1016/j.mce.2005.11.034

Eijken, M., Swagemakers, S., Koedam, M., Steenbergen, C., Derkx, P., Uitterlinden, A. G., et al. (2007). The activin A-follistatin system: potent regulator of human extracellular matrix mineralization. *FASEB J.* 21, 2949–2960. doi: 10.1096/fj.07-8080com

Endo, H., Kiyoki, M., Kawashima, K., Naruchi, T., and Hashimoto, Y. (1980). Vitamin D3 metabolites and PTH synergistically stimulate bone formation of chick embryonic femur *in vitro*. *Nature* 286, 262–264. doi: 10.1038/286262a0

Erben, R. G., Weiser, H., Sinowatz, F., Rambeck, W. A., and Zucker, H. (1992). Vitamin D metabolites prevent vertebral osteopenia in ovariectomized rats. *Calcif. Tissue Int.* 50, 228–236. doi: 10.1007/BF00296287

Everts, V., Delaisse, J. M., Korper, W., Jansen, D. C., Tigchelaar-Gutter, W., Saftig, P., et al. (2002). The bone lining cell: its role in cleaning lacunae and initiating bone formation. *J. Bone Miner. Res.* 17, 77–90. doi: 10.1359/jbmr.2002.17.1.77

Farach-Carson, M. C., and Xu, Y. (2002). Microarray detection of gene expression changes induced by 1,25(OH)(2)D(3) and a Ca(2+) influx-activating analog in osteoblastic ROS 17/2.8 cells. *Steroids* 67, 467–470. doi: 10.1016/S0039-128X(01)00168-4

Franceschi, R. T., Romano, P. R., and Park, K. Y. (1988). Regulation of type I collagen synthesis by 1,25-dihydroxyvitamin D3 in human osteosarcoma cells. *J. Biol. Chem.* 263, 18938–18945.

Fretz, J. A., Zella, L. A., Kim, S., Shevde, N. K., and Pike, J. W. (2007). 1,25-dihydroxyvitamin D3 induces expression of the Wnt signaling co-regulator LRP5 via regulatory elements located significantly downstream of the genes transcriptional start site. *J. Steroid Biochem. Mol. Biol.* 103, 440–445. doi: 10.1016/j.jsbmb.2006.11.018

Friedenstein, A. J., Deriglasova, U. F., Kulagina, N. N., Panasuk, A. F., Rudakowa, S. F., Luriá, E. A., et al. (1974). Precursors for fibroblasts in different populations of hematopoietic cells as detected by the *in vitro* colony assay method. *Exp. Hematol.* 2, 83–92.

Galus, K., Szymendera, J., Zaleski, A., and Schreyer, K. (1980). Effects of 1α-hydroxyvitamin D3 and 24R, 25-dihydroxyvitamin D3 on bone remodeling. *Calcif. Tissue Int.* 31, 209–213. doi: 10.1007/BF02407183

Gardiner, E. M., Baldock, P. A., Thomas, G. P., Sims, N. A., Henderson, N. K., Hollis, B., et al. (2000). Increased formation and decreased resorption of bone in mice with elevated vitamin D receptor in mature cells of the osteoblastic lineage. *FASEB J.* 14, 1908–1916. doi: 10.1096/fj.99-1075com

Geng, S., Zhou, S., and Glowacki, J. (2011a). Effects of 25-hydroxyvitamin D(3) on proliferation and osteoblast differentiation of human marrow stromal cells require CYP27B1/1α-hydroxylase. *J. Bone Miner. Res.* 26, 1145–1153. doi: 10.1002/jbmr.298

Geng, S., Zhou, S., and Glowacki, J. (2011b). Age-related decline in osteoblastogenesis and 1α-hydroxylase/CYP27B1 in human mesenchymal stem cells: stimulation by parathyroid hormone. *Aging Cell* 10, 962–971. doi: 10.1111/j.1474-9726.2011.00735.x

Godschalk, M., Levy, J. R., and Downs, R. W. (1992). Glucocorticoids decrease vitamin D receptor number and gene expression in human osteosarcoma cells. *J. Bone Miner. Res.* 7, 21–27. doi: 10.1002/jbmr.5650070105

Grundberg, E., Adoue, V., Kwan, T., Ge, B., Duan, Q. L., Lam, K. C. L., et al. (2011). Global analysis of the impact of environmental perturbation on cis-regulation of gene expression. *PLoS Genet.* 7:e1001279. doi: 10.1371/journal.pgen.1001279

Haussler, M. R., Haussler, C. A., Whitfield, G. K., Hsieh, J.-C., Thompson, P. D., Barthel, T. K., et al. (2010). The nuclear vitamin D receptor controls the expression of genes encoding factors which feed the "Fountain of Youth" to mediate healthful aging. *J. Steroid Biochem. Mol. Biol.* 121, 88–97. doi: 10.1016/j.jsbmb.2010.03.019

Haussler, M. R., Whitfield, G. K., Haussler, C. A., Hsieh, J. C., Thompson, P. D., Selznick, S. H., et al. (1998). The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. *J. Bone Miner. Res.* 13, 325–349. doi: 10.1359/jbmr.1998.13.3.325

Heikkinen, S., Väisänen, S., Pehkonen, P., Seuter, S., Benes, V., and Carlberg, C. (2011). Nuclear hormone 1,25-Dihydroxyvitamin D3 elicits a genome-wide shift in the locations of VDR chromatin occupancy. *Nucleic Acids Res.* 39, 9181–9193. doi: 10.1093/nar/gkr654

Henry, H., and Norman, A. (1978). Vitamin D: two dihydroxylated metabolites are required for normal chicken egg hatchability. *Science* 201, 835–837. doi: 10.1126/science.684411

Hewison, M., Freeman, L., Hughes, S. V., Evans, K. N., Bland, R., Eliopoulos, A. G., et al. (2003). Differential regulation of vitamin D receptor and its ligand in human monocyte-derived dendritic cells. *J. Immunol.* 170, 5382–5390.

Hewison, M., Zehnder, D., Chakraverty, R., and Adams, J. S. (2004). Vitamin D and barrier function: a novel role for extra-renal 1α-hydroxylase. *Mol. Cell. Endocrinol.* 215, 31–38. doi: 10.1016/j.mce.2003.11.017

Hicok, K. C., Thomas, T., Gori, F., Rickard, D. J., Spelsberg, T. C., and Riggs, B. L. (1998). Development and characterization of conditionally immortalized osteoblast precursor cell lines from human bone marrow stroma. *J. Bone Miner. Res.* 13, 205–217. doi: 10.1359/jbmr.1998.13.2.205

Holick, M. F. (1995). Defects in the synthesis and metabolism of vitamin D. *Exp. Clin. Endocrinol. Diabetes* 103, 219–227. doi: 10.1055/s-0029-1211354

Hong, D., Chen, H.-X., Yu, H.-Q., Liang, Y., Wang, C., Lian, Q.-Q., et al. (2010). Morphological and proteomic analysis of early stage of osteoblast differentiation in osteoblastic progenitor cells. *Exp. Cell Res.* 316, 2291–2300. doi: 10.1016/j.yexcr.2010.05.011

Ingram, R. T., Bonde, S. K., Riggs, B. L., and Fitzpatrick, L. A. (1994). Effects of transforming growth factor beta (TGFβ) and 1,25 dihydroxyvitamin D3 on the function, cytochemistry and morphology of normal human osteoblast-like cells. *Differentiation* 55, 153–163. doi: 10.1046/j.1432-0436.1994.5520153.x

Ito, N., Findlay, D. M., Anderson, P. H., Bonewald, L. F., and Atkins, G. J. (2013). Extracellular phosphate modulates the effect of 1α,25-dihydroxy vitamin D3

(1,25D) on osteocyte like cells. *J. Steroid Biochem. Mol. Biol.* 136, 1–4. doi: 10.1016/j.jsbmb.2012.09.029

Jilka, R. L., Weinstein, R. S., Bellido, T., Parfitt, A. M., and Manolagas, S. C. (1998). Osteoblast programmed cell death (apoptosis): modulation by growth factors and cytokines. *J. Bone Miner. Res.* 13, 793–802. doi: 10.1359/jbmr.1998.13.5.793

Jørgensen, N. R., Henriksen, Z., Sørensen, O. H., and Civitelli, R. (2004). Dexamethasone, BMP-2, and 1,25-dihydroxyvitamin D enhance a more differentiated osteoblast phenotype: validation of an *in vitro* model for human bone marrow-derived primary osteoblasts. *Steroids* 69, 219–226. doi: 10.1016/j.steroids.2003.12.005

Karunaratne, A., Esapa, C. T., Hiller, J., Boyde, A., Head, R., Bassett, J. H. D., et al. (2012). Significant deterioration in nanomechanical quality occurs through incomplete extrafibrillar mineralization in rachitic bone: evidence from *in-situ* synchrotron X-ray scattering and backscattered electron imaging. *J. Bone Min. Res.* 27, 876–890. doi: 10.1002/jbmr.1495

Kato, A., Seo, E. G., Einhorn, T. A., Bishop, J. E., and Norman, A. W. (1998). Studies on 24R,25-dihydroxyvitamin D3: evidence for a nonnuclear membrane receptor in the chick tibial fracture-healing callus. *Bone* 23, 141–146. doi: 10.1016/S8756-3282(98)00085-4

Kawamori, Y., Katayama, Y., Asada, N., Minagawa, K., Sato, M., Okamura, A., et al. (2010). Role for vitamin D receptor in the neuronal control of the hematopoietic stem cell niche. *Blood* 116, 5528–5535. doi: 10.1182/blood-2010-04-279216

Kearns, A. E., Donohue, M. M., Sanyal, B., and Demay, M. B. (2001). Cloning and characterization of a novel protein kinase that impairs osteoblast differentiation *in vitro*. *J. Biol. Chem.* 276, 42213–42218. doi: 10.1074/jbc.M106163200

Kitanaka, S., Takeyama, K., Murayama, A., Sato, T., Okumura, K., Nogami, M., et al. (1998). Inactivating mutations in the 25-hydroxyvitamin D3 1alpha-hydroxylase gene in patients with pseudovitamin D-deficiency rickets. *N. Engl. J. Med.* 338, 653–661. doi: 10.1056/NEJM199803053381004

Komarova, S. V., Ataullakhanov, F. I., and Globus, R. K. (2000). Bioenergetics and mitochondrial transmembrane potential during differentiation of cultured osteoblasts. *Am. J. Physiol. Cell Physiol.* 279, C1220–C1229.

Koshihara, Y., and Hoshi, K. (1997). Vitamin K2 enhances osteocalcin accumulation in the extracellular matrix of human osteoblasts *in vitro*. *J. Bone Miner. Res.* 12, 431–438. doi: 10.1359/jbmr.1997.12.3.431

Kostenuik, P. J., and Shalhoub, V. (2001). Osteoprotegerin: a physiological and pharmacological inhibitor of bone resorption. *Curr. Pharm. Des.* 7, 613–635. doi: 10.2174/1381612013397807

Kveiborg, M., Flyvbjerg, A., Eriksen, E. F., and Kassem, M. (2001). 1,25-dihydroxyvitamin D3 stimulates the production of insulin-like growth factor-binding proteins-2, -3 and -4 in human bone marrow stromal cells. *Eur. J. Endocrinol.* 144, 549–557. doi: 10.1530/eje.0.1440549

Lacey, D. L., Timms, E., Tan, H. L., Kelley, M. J., Dunstan, C. R., Burgess, T., et al. (1998). Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 93, 165–176. doi: 10.1016/S0092-8674(00)81569-X

Li, J., Jin, D., Fu, S., Mei, G., Zhou, J., Lei, L., et al. (2013). Insulin-like growth factor binding protein-3 modulates osteoblast differentiation via interaction with vitamin D receptor. *Biochem. Biophys. Res. Commun.* 436, 632–637. doi: 10.1016/j.bbrc.2013.04.111

Li, J. J., and Sodek, J. (1993). Cloning and characterization of the rat bone sialoprotein gene promoter. *Biochem. J.* 289(Pt 3), 625–629.

Li, Y. C., Pirro, A. E., Amling, M., Delling, G., Baron, R., Bronson, R., et al. (1997). Targeted ablation of the vitamin D receptor: an animal model of vitamin D-dependent rickets type II with alopecia. *Proc. Natl. Acad. Sci. U.S.A.* 94, 9831–9835. doi: 10.1073/pnas.94.18.9831

Lian, J. B., Shalhoub, V., Aslam, F., Frenkel, B., Green, J., Hamrah, M., et al. (1997). Species-specific glucocorticoid and 1,25-dihydroxyvitamin D responsiveness in mouse MC3T3-E1 osteoblasts: dexamethasone inhibits osteoblast differentiation and vitamin D down-regulates osteocalcin gene expression. *Endocrinology* 138, 2117–2127.

Lian, J. B., Stein, G. S., van Wijnen, A. J., Stein, J. L., Hassan, M. Q., Gaur, T., et al. (2012). MicroRNA control of bone formation and homeostasis. *Nat. Revi. Endocrinol.* 8, 212–227. doi: 10.1038/nrendo.2011.234

Lieben, L., Masuyama, R., Torrekens, S., Van Looveren, R., Schrooten, J., Baatsen, P., et al. (2012). Normocalcemia is maintained in mice under conditions of calcium malabsorption by vitamin D–induced Inhibition of Bone Mineralization. *J. Clin. Invest.* 122, 1803–1815. doi: 10.1172/JCI45890

Lisse, T. S., Adams, J. S., and Hewison, M. (2013a). Vitamin D and microRNAs in bone. *Crit. Rev. Eukaryot. Gene Expr.* 23, 195–214. doi: 10.1615/CritRevEukaryotGeneExpr.2013007147

Lisse, T. S., Chun, R. F., Rieger, S., Adams, J. S., and Hewison, M. (2013b). Vitamin D activation of functionally distinct regulatory miRNAs in primary human osteoblasts. *J. Bone Miner. Res.* 28, 1478–1488. doi: 10.1002/jbmr.1882

MacDonald, P. N., Dowd, D. R., Nakajima, S., Galligan, M. A., Reeder, M. C., Haussler, C. A., et al. (1993). Retinoid X receptors stimulate and 9-cis retinoic acid inhibits 1,25-dihydroxyvitamin D3-activated expression of the rat osteocalcin gene. *Mol. Cell. Biol.* 13, 5907–5917.

Mackie, E. J., Ahmed, Y. A., Tatarczuch, L., Chen, K.-S., and Mirams, M. (2008). Endochondral ossification: how cartilage is converted into bone in the developing skeleton. *Int. J. Biochem. Cell Biol.* 40, 46–62. doi: 10.1016/j.biocel.2007.06.009

Mackie, E. J., Tatarczuch, L., and Mirams, M. (2011). The skeleton: a multi-functional complex organ. The growth plate chondrocyte and endochondral ossification. *J. Endocrinol.* 211, 109–121. doi: 10.1530/JOE-11-0048

Maehata, Y., Takamizawa, S., Ozawa, S., Kato, Y., Sato, S., Kubota, E., et al. (2006). Both direct and collagen-mediated signals are required for active vitamin D3-elicited differentiation of human osteoblastic cells: roles of osterix, an osteoblast-related transcription factor. *Matrix Biol.* 25, 47–58. doi: 10.1016/j.matbio.2005.09.001

Martinello, T., Bronzini, I., Volpin, A., Vindigni, V., Maccatrozzo, L., Caporale, G., et al. (2012). Successful recellularization of human tendon scaffolds using adipose-derived mesenchymal stem cells and collagen gel. *J. Tissue Eng. Regen. Med.* doi: 10.1002/term.1557. [Epub ahead of print].

Mathieu, C., Van Etten, E., Gysemans, C., Decallonne, B., Kato, S., Laureys, J., et al. (2001). *In vitro* and *in vivo* analysis of the immune system of vitamin D receptor knockout mice. *J. Bone Miner. Res.* 16, 2057–2065. doi: 10.1359/jbmr.2001.16.11.2057

Matsumoto, T., Ezawa, I., Morita, K., Kawanobe, Y., and Ogata, E. (1985). Effect of vitamin D metabolites on bone metabolism in a rat model of postmenopausal osteoporosis. *J. Nutr. Sci. Vitaminol.* 31, S61–S65. doi: 10.3177/jnsv.31.Supplement_S61

Matsumoto, T., Igarashi, C., Takeuchi, Y., Harada, S., Kikuchi, T., Yamato, H., et al. (1991). Stimulation by 1, 25-dihydroxyvitamin D3 of *in vitro* mineralization induced by osteoblast-like MC3T3-E1 cells. *Bone* 12, 27–32. doi: 10.1016/8756-3282(91)90051-J

McCollum, E. V., Pitz, W., Simmonds, N., Becker, J. E., Shipley, P. G., and Bunting, R. W. (2002). The effect of additions of fluorine to the diet of the rat on the quality of the teeth. 1925. Studies on experimental rickets. XXI. An experimental demonstration of the existence of a vitamin which promotes calcium deposition. 1922. The effect of addition. *J. Biol. Chem.* 277:E8.

Meyer, M. B. (2005). The human transient receptor potential vanilloid type 6 distal promoter contains multiple vitamin D receptor binding sites that mediate activation by 1,25-dihydroxyvitamin D3 in intestinal cells. *Mol. Endocrinol.* 20, 1447–1461. doi: 10.1210/me.2006-0031

Meyer, M. B., Goetsch, P. D., and Pike, J. W. (2010). Genome-wide analysis of the VDR/RXR cistrome in osteoblast cells provides new mechanistic insight into the actions of the vitamin D hormone. *J. Steroid Biochem. Mol. Biol.* 121, 136–141. doi: 10.1016/j.jsbmb.2010.02.011

Minguell, J. J., Erices, A., and Conget, P. (2001). Mesenchymal stem cells. *Exp. Biol. Med. (Maywood).* 226, 507–520.

Misof, B. M., Roschger, P., Tesch, W., Baldock, P. A., Valenta, A., Messmer, P., et al. (2003). Targeted overexpression of vitamin D receptor in osteoblasts increases calcium concentration without affecting structural properties of bone mineral crystals. *Calcif. Tissue Int.* 73, 251–257. doi: 10.1007/s00223-002-2139-6

Nakashima, T., Hayashi, M., Fukunaga, T., Kurata, K., Oh-hora, M., Feng, J. Q., et al. (2011). Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nat. Med.* 17, 1231–1234. doi: 10.1038/nm.2452

Nishimura, R., Hata, K., Ono, K., Amano, K., Takigawa, Y., Wakabayashi, M., et al. (2012). Regulation of endochondral ossification by transcription factors. *Front. Biosci.* 17:4076. doi: 10.2741/4076

Noda, M., Vogel, R. L., Craig, A. M., Prahl, J., DeLuca, H. F., and Denhardt, D. T. (1990). Identification of a DNA sequence responsible for binding of the 1,25-dihydroxyvitamin D3 receptor and 1,25-dihydroxyvitamin D3 enhancement of mouse secreted phosphoprotein 1 (SPP-1 or Osteopontin) gene expression. *Proc. Natl. Acad. Sci. U.S.A.* 87, 9995–9999. doi: 10.1073/pnas.87.24.9995