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Vitamin D Receptor and RXR in the Post-Genomic Era

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Abstract

Following the elucidation of the human genome and components of the epigenome, it is timely to revisit what is known of vitamin D receptor (VDR) function. Early transcriptomic studies using microarray approaches focused on the protein coding mRNA that were regulated by the VDR, usually following treatment with ligand. These studies quickly established the approximate size and surprising diversity of the VDR transcriptome, revealing it to be highly heterogenous and cell type and time dependent. Investigators also considered VDR regulation of non-protein coding RNA and again, cell and time dependency was observed. Attempts to integrate mRNA and miRNA regulation patterns are beginning to reveal patterns of co-regulation and interaction that allow for greater control of mRNA expression, and the capacity to govern more complex cellular events. Alternative splicing in the transcriptome has emerged as a critical process in transcriptional control and there is evidence of the VDR interacting with components of the spliceosome. ChIP-Seq approaches have proved to be pivotal to reveal the diversity of the VDR binding choices across cell types and following treatment, and have revealed that the majority of these are non-canonical in nature. The underlying causes driving the diversity of VDR binding choices remain enigmatic. Finally, genetic variation has emerged as important to impact the transcription factor affinity towards genomic binding sites, and recently the impact of this on VDR function has begun to be considered.

Transcription across the human genome

Understanding of the human genome and its regulation has undergone a rapid expansion in recent years, in large part thanks to the integrative genomic efforts of the ENCODE consortium (Birney, 2012; Maher, 2012; Stamatoyannopoulos, 2012; Rosenbloom et al., 2013) and FANTOM (Katayama et al., 2004; Sanli et al., 2013). These consortia, and other functional genomics investigators (Heikkinen et al., 2011a; Mendoza-Parra et al., 2011; Ram et al., 2011; Zhu et al., 2013; Daniel et al., 2014), have used next generation sequencing approaches to examine the global function of genes and proteins in the context of the whole human genome. This has resulted in an explosion in the volume of publically available data that relates to all aspects of gene characterization, expression, and function. These studies have demonstrated a hitherto unsuspected complexity in terms of the variation and diversity in many key steps in the control of transcription including; the distribution of transcription

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factor binding across the human genome; the functional differences in the spatial relationships between proximal and distal binding; the interplay between transcription factors and different co-regulating partners; the extent of the genome that is transcribed; the number and functionally different RNA-based molecules that are transcribed; and the impact of mechanisms that process and edit RNA molecules. Although the true meaning of these analyses is not without debate (Graur et al., 2013; Kellis et al., 2014), these efforts have already catalyzed new investigations and a reappraisal of transcription factor function.

The VDR Complex Regulates Transcription

The nuclear receptors (NRs) form one of the largest and best understood families of transcription factors, are expressed in many cell types, and are highly integrated at the functional level (Abedin et al., 2009; Battaglia et al., 2010; Long et al., 2014; Thorne and Campbell, 2014). In human diseases including cancer, these receptors represent some of the most successful examples of targeted therapies (Campbell et al., in press; Mongan and Gudas, 2007; Thorne and Campbell, 2008; Carlberg and Campbell, 2013), representing the targets for up to 15% of all drugs.

Within this family the vitamin D receptor (VDR/NR1I1) (Pike et al., 1980; Baker et al., 1988; Carlberg and Campbell, 2013) plays a prominent role in multiple aspects of human health and disease. Indeed, around the world, the VDR is currently the target of large scale prospective trials to examine its roles in a broad spectrum of human health outcomes including cancer, cardiovascular health, and other common disease phenotypes (Chlebowski et al., 2008; Manson et al., 2012; Litonjua et al., 2014). Therefore it is timely to review VDR genomic functions in human health and disease, and to consider how VDR actions are integrated with other members of the NR superfamily, and generally with other transcription factors. In turn, and in light of the renewed understanding of the distribution of transcriptional binding patterns and the diversity of RNA species, it is also timely to consider how these relationships illuminate VDR function.

Members of the nuclear receptors have been extensively investigated and are relatively well-understood transcription factors. Their actions have been dissected and modeled, and have generated the concept of cyclical gene regulation in which transcription factors oscillate between on and off states (Metivier et al., 2003; Reid et al., 2003; Kim et al., 2005; Vaisanen et al., 2005b; Carroll et al., 2006; Meyer et al., 2006; Yang et al., 2006; Zella et al., 2006; Saramaki et al., 2006a; Malinen et al., 2007; Seo et al., 2007).

Classical steroidal NRs exist as complexes in the cytoplasm with Heat Shock Factor proteins and therefore their transcriptional actions are largely mediated by ligand-induced release from this complex and shuttling into the nucleus. The VDR differs from these NRs by being located in the nucleus even in the absence of ligand and controls gene expression by switching between repressing and activating states, dependent upon the availability of ligand. Thus, the distribution of genomic binding sites, or so-called cistrome, of the VDR is biologically significant both in the absence and presence of even low levels of ligand.

The VDR–RXR α heterodimer complex (Quack and Carlberg, 2000) (hereafter termed VDR complex) is contained within larger complexes that are dependent on the binding of 1 α ,

25(OH)₂D₃ ligand to determine function. In the absence of ligand, the VDR complex associates with corepressors at enhancer and promoter regions to silence gene expression. The binding of ligand induces formation of the coactivator complex leading to target gene transactivation by direct and indirect mechanisms. The VDR–RXR α coactivator complex can bind directly at either the same or different sites on the same gene, or it can redistribute to genomic sites on previously unregulated genes. Indirectly, the loss of VDR–RXR α corepressor complex binding at a locus can be permissive for positive gene regulation by allowing binding of other transcription factors.

A number of proteins with transcriptional activator function have been identified in complex with VDR. For example, CBP/ p300 is a transcriptional co-integrator with histone acetylase activity (Wang et al., 2011a; Wang et al., 2013) and is associated with the VDR. Similarly, SNW1/NCOA62 has function as a transcriptional co-activator (Baudino et al., in press), as well as other proteins that have more recently been characterized to have coactivator function (e.g., CCND3) have been associated with VDR function (Cenciarelli et al., 1999; Lazaro et al., 2002; Despouy et al., 2003; Sarruf et al., 2005).

In the absence of ligand, basal levels of receptor remain in the nucleus associated with co-repressor complexes which leads to silencing of transcription (Malinen et al., 2008; Saramaki et al., 2009; Thorne et al., 2011; Doig et al., 2013). The VDR functionally interacts with a range of co-repressors such as NCOR1 (Saramaki et al., 2009; Doig et al., 2013), NCOR2/ SMRT (Khanim et al., 2004; Gynther et al., 2011), TRIP15/ALIEN (Polly et al., 2000; Cui et al., 2009) and DREAM (Scsucova et al., 2005). Similarly the corepressor HR is also identified by such protein-protein interactions (Hsieh et al., 2003).

A consequence of these intricate protein-protein interactions with the genome is the finely tuned control of epigenetic states at VDR binding regions, and more broadly across target gene loci. These epigenetic events play a central role in the transcriptional cycle to initiate, sustain and finally terminate transcription (Dobrzynski and Bruggeman, 2009). These events are varied in space across the genomic loci, and in time through the course of the transcriptional cycle. For example, different histone modifications can control the rate and magnitude of transcription (reviewed in [Goldberg et al., 2007]) and more recently evidence has emerged that these events are intertwined with DNA CpG methylation (Kangaspeska et al., 2008; Metivier et al., 2008; Le May et al., 2010). Specifically, the VDR containing complex can regulate histone modifications and DNA methylation processes (ref). For example, histone H3 lysine 4 tri-methylation (H3K4me₃) is found in the promoter regions of actively transcribed genes. This mark is mutually exclusive with H3K9me, which instead is associated with transcriptionally silent promoter regions. At distal regions from the TSS, histone marks can also mark poised, active or closed enhancer sites H3K4me₁ is associated with enhancer regions. H3K27 can be either acetylated or methylated, with acetylation associated with active gene transcription, and H3K27ac is enriched at active enhancers, whereas H3K27me₃ is associated with gene repression.

Thus the VDR complex is multifactorial and, in part through enzymatic control of histone modifications, modulates regional chromatin status in order to regulate transcription. In the relaxed chromatin state, the VDR complex recruits linking factors (Mediator/DRIP/TRAP

complex) and subsequently the basal transcriptional machinery. However this is not an indefinite signal as ligand is rapidly metabolized. Also the VDR itself is limited in function by proteasome-mediated receptor degradation (Peleg and Nguyen, 2010). The choreography of these actions gives rise to the periodicity of transcriptional activation and pulsatile mRNA and protein accumulation, reflecting intrinsic control mechanisms required to tightly regulate the precise expression of important signaling molecules.

Whilst these scenarios are relatively well characterized for the positive regulation of gene expression, it is probably not a complete understanding as the distribution of VDR binding in the genome (Ramagopalan et al., 2010; Heikkinen et al., 2011a; Meyer et al., 2012a; Ding et al., 2013) and the patterns of associated gene regulation suggest that the VDR is actually associated broadly with both gene activation and repression. The mechanisms that drive gene repression appear more diverse than gene activation and reflect differences in VDR complex formation and the precision of its binding.

The VDR Regulated Transcriptome

Anti-proliferative effects of $1\alpha,25(\text{OH})_2\text{D}_3$ have been demonstrated in a wide variety of cancer cell lines, including those from prostate, breast, and colon (Campbell et al., 1997; Colston et al., 1982; Colston et al., 1989; Koike et al., 1997; Elstner et al., 1999; Palmer et al., 2003). Therefore many of these models have been exploited to undertake VDR transcriptional studies. Initially, before transcriptomic methods were widely available, biologists undertook candidate gene based studies combined with classical biochemistry-based approaches to confirm VDR binding and function. These studies identified processes by which the VDR mediated its cellular effects, for example through the regulation of genes encoding the $1\alpha,25(\text{OH})_2\text{D}_3$ metabolizing enzyme *CYP24A1* (Dwivedi et al., 2000; Anderson et al., 2003) and the cell cycle regulator *CDKN1A* (encodes p21^(waf1/cip1)) (Schwaller et al., 1995).

Subsequently, with the emergence of comprehensive transcriptomic approaches including microarray technologies, an understanding was developed concerning the VDR transcriptome in different cell types and treatment conditions. For example, differential expression approaches in the context of $1\alpha,25(\text{OH})_2\text{D}_3$ were used to identify a number of cyclin dependent kinase inhibitors including *CDKN1A* as targets required to trigger myeloid differentiation (Liu et al., 1996). Similarly, studies in squamous models, following $1\alpha,25(\text{OH})_2\text{D}_3$ exposure, identified various targets included *GADD45A* (Akutsu et al., 2001b) which was confirmed by others in different cell types (Akutsu et al., 2001a; Jiang et al., 2003; Khamis et al., 2004). These early studies suggested central functions at the footprint of the VDR dependent transcriptome (reviewed in [Rid et al., 2013]) including cell cycle regulation (Akutsu et al., 2001a; Palmer et al., 2003; Eelen et al., 2004; Wang et al., 2005b). In many ways, these studies also highlighted the heterogeneity of VDR actions that was to be identified subsequently by ChIP-Seq studies. This heterogeneity may in part reflect experimental conditions (e.g., growth conditions, ligand exposures) with very different cell lines, but also genuine tissue-specific differences of co-factor expression that alter the amplitude and periodicity of VDR transcriptional actions.

In the transition to genome wide understanding, workers undertook MIAME compliant array experiments (Brazma et al., 2001; Do and Choi, 2006; Zhang et al., 2009), which were deposited in public archives such as GEO (Barrett et al., 2005; Barrett et al., 2013) and ArrayExpress at the EMBL (Parkinson et al., 2007) and Stanford microarray databases (Marinelli et al., 2008). These approaches identified networks of genes that trigger the response to wounding, protease inhibition, secondary metabolite biosynthesis, cellular migration, amine biosynthetic processes (Lin et al., 2002; Wang et al., 2005a) and a critical role for TGF β signaling was revealed to associate with VDR antiproliferative sensitivity towards 1 α ,25(OH) $_2$ D $_3$ in breast cancer cell models (Towsend et al., 2006).

Exploiting leukemia cell models with differential responsive-ness towards 1 α ,25(OH) $_2$ D $_3$ triggered-differentiation (Tagliafico et al., 2006) identified that certain VDR transcriptional targets could distinguish the aggressiveness of leukemia, again, focused around cell cycle regulators included *MS4A3* which can modulate the phosphorylation of CDK2 and therefore exert control over the cell cycle. The concept of VDR sensitive versus resistant models was also exploited in prostate cancer to identify the critical VDR transcriptional targets that mediate antiproliferative sensitivity which similarly identified cell cycle and signal transduction components including *GADD45A* and *MAPKAPK2* that were required to mediate the sensitivity of cells to 1 α ,25(OH) $_2$ D $_3$. Furthermore, these studies examined the epigenetic basis for the transcriptionally inert state of these targets in resistant models (Rashid et al., 2001; Khanim et al., 2004).

A final area to emerge from these agnostic studies of the VDR transcriptome is the impact that 1 α ,25(OH) $_2$ D $_3$ exposure exerts on a range of chromatin remodeling components. Interestingly, NCOR2/SMRT appears to be a target of VDR signaling (Dunlop et al., 2004), and adds to the concept that VDR signaling is cyclical and based on the functions of various negative feedback loops. Similarly, KDM6B/JMJD3 is a histone H3 lysine demethylase and expression is induced by the activated VDR. In this manner, VDR action can induce a feed-forward activation of its own transcriptional program by promoting H3K9 acetylation and gene action (Pereira et al., 2011). Indeed VDR can induce demethylation of the PDLIM2 promoter to upregulate it in breast cancer cells (Vanoirbeek et al., 2014).

VDR Regulation of Non-Coding RNA Species

The human genome project (ref) in many ways focused on the protein coding genes within the human genome as a key step in the initial alignment process was to leverage cDNA and EST libraries to the genomic sequences contained in the Bacterial artificial chromosomes libraries. The subsequent interpretation of the human genome, and other large scale approaches to investigating chromosomal function (Consortium et al., 2007; Tress et al., 2007), led to a growing awareness of the extent of non-coding RNA and at least suggested that there was an unknown. This uncertainty has been reflected in the debate within the biological community over the extent and roles of so-called Junk DNA (Kapranov and St. Laurent, 2012; Doolittle, 2013).

As a result, researchers have considered roles for non-coding RNA in the regulation of cell function and have begun to examine the interplay between the at least 20 different types of

different non-coding RNA (reviewed in [Ling et al., 2013]). Many of these RNA species are gene regulatory RNA and include microRNA miRNA and long noncoding RNA (lncRNA), whereas others are involved in the post-transcriptional modification of RNA, for example small nucleolar RNA (snoRNA).

Naturally, miRNA regulation by the VDR has now been investigated and sheds light on earlier findings. For example Studzinski and co-workers revisited the mechanistically enigmatic VDR-mediated control of p27^(kip1). A common anti-proliferative VDR function is associated with arrest at G₀/G₁ of the cell cycle, coupled with up-regulation of a number of cell cycle inhibitors. Previous studies had suggested that p27^(kip1) regulation reflected translational regulation and enhanced mRNA translation, and attenuating degrading mechanisms (Hengst and Reed, 1996; Wang et al., 1996; Huang et al., 2004; Li et al., 2004). MiRNA based studies subsequently demonstrated that VDR down-regulated miR181a, which when left unchecked degrades p27^(kip1) (Wang et al., 2009). Thus, indirectly, VDR activation elevates expression of p27^(kip1), initiates cell cycle arrest and commits cells towards differentiation.

Similar integration of miRNA and mRNA was revealed to control the regulation of *CDKN1A*. Dynamic patterns of *CDKN1A* mRNA accumulation have been observed in various cell systems (Thorne et al., 2011). This is in part explained by the epigenetic states of different VDR binding sites on the *CDKN1A* promoter. However VDR-dependent co-regulation of miR-106b also appears to modulate the precise timing of *CDKN1A* accumulation and expression of p21^(waf1/cip1) in a feed-forward loop and determine the final extent of the cell cycle arrest. 1 α ,25(OH)₂D₃ regulates the DNA helicase *MCM7* (Khanim et al., 2004) that encodes the miR-106b, in intron 13 of the *MCM7* gene, and together these co-regulation processes control p21^(waf1/cip1) through the balance of *MCM7* and *CDKN1A* (Saramaki et al., 2006b; Ivanovska et al., 2008).

The concept of miRNA-mediated negative regulatory aspects to normal gene regulation, for example as part of feed-forward loop motifs, appears common (Mangan and Alon, 2003; Mangan et al., 2006; Song and Wang, 2008; Gatfield et al., 2009; Sun et al., 2009; Wang et al., 2009), but is technically, and statistically, challenging to establish. This arises in part due to the many-to-many nature of miRNA; a given miRNA target many mRNA, and a given mRNA may have many miRNA targeting it. Thus, the computational challenges to resolve these relationships are not insignificant. These challenges notwithstanding, other co-regulated mRNA/miRNA relationships have emerged. For example, established miRNA targets of the VDR include miR-627 (Padi et al., 2013) that in turn targets *JMJD1A* (another histone H3 lysine demethylase), miR-98 (Ting et al., 2013) and let-7a-2 (Guan et al., 2013). However one of the more explored relationships between VDR and miRNA is the relationship between VDR and miR-125b. MiR-125b inhibits VDR (Mohri et al., 2009; Zhang et al., 2011) and in turn VDR can down-regulate miR-125b (Iosue et al., 2013; Zhou et al., 2014). Other targets of miR-125b include NCOR2/SMRT (Yang et al., 2012) (itself a VDR target gene) suggesting multiple levels of co-regulation and interdependent relationship between the VDR, the mRNA and miRNA transcriptomes, and the epigenome. Finally, it is interesting to note that altered levels of miRNA are associated with cancer

states and progression risks and indeed miR-125b is associated with aggressive prostate cancer (Shi et al., 2011; Amir et al., 2013; Singh et al., 2014).

Microarray analyses based on miRNA have also have identified various novel 1α , $25(\text{OH})_2\text{D}_3$ regulated networks, including the control of lipid metabolism (Wang et al., 2011b). Similarly, regulation by VDR of other types of non-coding RNA species is emerging and VDR regulation of lncRNA has been undertaken in keratinocytes, identifying a number of target lncRNA and in doing so has raised the curtain on new avenues of exploration (Jiang and Bikle, 2014)

Given the number of microarrays available, it is now timely to consider meta-analyses across identified transcriptomes to reveal common themes; this forward compatibility is one the key benefits of MIAME compliance. Meta-integration of array data has been shown to be surprisingly revealing in a range of studies. For example at the larger scale various workers have integrated multiple microarray data to reveal underlying patterns in the context of disease classification (Shah et al., 2009; Engreitz et al., 2011) but can also be applied to consider that specific phenotypes (Martinez-Climent et al., 2010; Rantala et al., 2010; Lai et al., 2014). It is therefore timely for these data to mined, and integrated with related nuclear receptor actions or other transcription factors that appear to co-operate with the VDR, for example SMADs. It is likely that the increased application and integration of microarray and next generation sequencing approaches will identify the key networks downstream of the VDR transcriptome, pertaining to both protein coding and non-coding RNA.

The Repertoire of VDR-Protein Interactions and Genomic Binding Sites

Appreciation is growing of the diversity of protein interactions that involve the VDR. Aside from these traditional roles for the VDR to modulate transcriptional actions, there is evidence to support a wider range of actions in the control of mRNA. The VDR-associated coactivator SNW1 also plays a role as a splicing factor. This latter function is also shared by another VDR interacting protein, namely SRPK1, which is a protein kinase that regulates the activity of various splicing factors (Hayes et al., 2006; Aubol et al., 2013). Other interactions allude to the cross-talk between the VDR and different signal transduction processes. For example, the VDR interacts with negative regulators of WNT signaling (NKD2) (Katoh and Katoh, 2007), substrates for PKC signaling (PRKCSH) (Gkika et al., 2004), p53 (Kommagani et al., 2006; Lambert et al., 2006; Maruyama et al., 2006; Saramaki et al., 2006b; Ellison et al., 2008) and SMAD3 (Ding et al., 2013; Ito et al., 2013; Zerr et al., 2014). Although some of these interactions were identified in candidate approaches, the increasing use of protein-protein screens is identifying unpredictable interactions containing the VDR. For example, proteomic screens in liver cells (Wang et al., 2011a) and muscle cells (Blandin et al., in press) combined with network approaches have identified more global roles for the VDR in signaling networks that were previously unsuspected for example associated with the YTHDC1; ZBTB16 in a pathways of complement activation (GO:0006957), and with GBA3; RPS6; in GO:0007243 protein kinase cascade.

Arguably, these catalogs of protein interactions only make sense in the context of the signaling networks that lead to altered genome binding and function. To date, much of the

complex body of knowledge concerning VDR genome interactions has been gathered by studies of the VDR associating with candidate genes, often by undertaking time-resolved studies, and considering how candidate histone modification or protein co-factors are involved (Vaisanen et al., 2005a; Saramaki et al., 2006b; Saramaki et al., 2009).

To identify VDR binding sites in an agnostic manner through the genome several groups have now applied ChIP-Seq approaches in different human cell types including immortalized lymphoblastoids (Ramagopalan et al., 2010), hepatic stellate cells (Ding et al., 2013) and cancer cell lines representing monocytic leukemia (Heikkinen et al., 2011b) and colon cancer (Meyer et al., 2012b). These studies differed in the time of cell exposure to $1\alpha, 25(\text{OH})_2\text{D}_3$, ranging from 40 min to 36 h. Depending on time of treatment with $1\alpha, 25(\text{OH})_2\text{D}_3$ and cell background, these studies varied to the extent of identified binding sites, on the order of hundreds to several thousands of loci. Interestingly, longer treatment tends to be associated with more binding sites. Despite the widespread enthusiasm to undertake next generation sequencing approaches, challenges remain with significant statistical complexity in terms of sequence alignment, peak calling and data interpretation. In essence, this technology remains far from a widespread and “plug and play” application. Reflecting this, the four existing VDR ChIP-Seq experiments have been analyzed differently by the four groups that drove the research, and this may in part give rise to some of the variation in findings of the number and significance of the VDR enriched peaks identified.

These differences in treatment and analytical approaches aside, the data sets reveal that fewer than 20% of the VDR binding sites are in common between the different cell types. This finding perhaps offers strong support for the concept that VDR transcription is extremely tailored in different cell types, presumably through interactions with either equal or more dominant co-factors that combine to determine its binding. Another important finding from these studies is that the canonical binding site for the VDR, termed the direct repeat (DR) spaced by 3 nucleotides (DR-3), which was identified by traditional biochemical approaches in candidate gene studies, appears to be the minority genomic element that directly binds the receptor. Fewer than 30% of genomic VDR binding sites contain a DR-3, although this number is increased following ligand treatment when there is increased enrichment for VDR binding to DR-3 elements (reviewed in [Carlberg and Campbell, 2013]). Also, consensus peaks (peaks that are found across multiple cell types) tend to have high DR3%. This lends credence to the concept that other dominant TFs drive most of the cell to cell differences.

Nonetheless, a range of other genomic elements were enriched in VDR binding peaks suggesting that the VDR cooperates closely with other factors to associate with the genome, both in the absence and presence of ligand. Indeed, the study of Evans and co-workers in the hepatic stellate cells (Ding et al., 2013) and the work of Pike and co-workers in colon cancer cells (Meyer et al., 2012b) both address this significant cross-talk of the VDR. In the case of the hepatic cells this is considered in the context of $\text{TGF}\beta$ and in the case of colon cancer cells this is with TCF4, downstream of β -catenin. Both of these studies therefore reflect the finding of VDR interactions with SMAD3 specifically, and more generally with regulators of WNT signaling.

Given the interaction of the VDR with RXR α , the group of Pike also analyzed the co-incident association of these two nuclear receptors in the colon cancer cells. Following treatment with 1 α ,25(OH) $_2$ D $_3$ there were more VDR binding sites in general, and approximately 75% of the 2209 VDR binding sites were enriched for RXR. However, the total number of binding sites for the RXR was over 4000, suggesting that following ligand treatment the VDR and cistrome shares approximately 50% of the RXR α cistrome.

Interestingly other workers have examined the RXR cistrome in different cell types. For example, in macrophages approximately 5,200 RXR α binding sites were identified (Daniel et al., 2014). Other studies exploited F9 cells that are acutely responsive to ATRA and identified approximately 13,000 RXR α binding sites with 4200 RAR γ sites (Mendoza-Parra et al., 2011). In both of these studies there is significant movement of binding sites towards genes in a ligand and treatment time dependent manner and the RXR-cistrome appeared to co-ordinate complex differentiation responses. It is tempting to speculate that in systems that are acutely responsive, there are over 10,000 RXR binding sites and obligate heterodimers such as the VDR or RARs have binding sites in the order of several 1000, and support the role of RXR as a central and integrative heterodimer partner.

One of the most complete approaches to date is the analyses from the White group who have undertaken cistromic analyses of multiple NRs and interacting transcription factors to construct a network level understanding of gene expression programs in breast cancer (Hua et al., 2009; Kittler et al., 2013). These approaches identified high complexity enhancer sites that integrated the actions of multiple NRs and other transcription factors. In particular, approximately 12000 RAR γ and 8000 VDR binding sites were identified in MCF-7 cells; RAR γ was amongst the most commonly found NR binding site. These sites were significantly enriched with other NRs (e.g. RAR α , PPAR γ , VDR, HNF4 α , ER α), transcription factors (FOXA1, SP1, STAT3) and co-regulators (CTCF). Key aspects of these associations, focused around the RAR γ and RAR α were related to clinical outcome in breast cancer patients and supported the role of larger networks of the VDR and other NRs to control cell fates. Specifically, the data support the concept that cross-talk occurs between RARs and VDR, which exert mitotic restraint, and other NRs, such as ER α , that drive proliferation and survival (Hua et al., 2009; Kittler et al., 2013).

Together these ChIP-Seq studies suggest that the VDR combines with other proteins in a network of interactions, quite likely in a cell type specific manner, to participate in diverse gene regulatory networks. It remains to be established how targeted or stochastic this is. The variation observed in both the type and position of binding sites for the VDR, depending on cell phenotype and disease state, suggests it is directed, and at least will establish a paradigm for hypothesis testing concerning what directs the VDR to bind and participate in gene transcription. The specificity of VDR signaling may arise due to integration with other perhaps more dominant transcription factors.

Again, for other nuclear receptors (e.g., AR and ER) the concept has emerged that receptor binding is guided by the actions of more dominant so-called pioneer factors including the Forkhead (FKH) family members (Lupien et al., 2008; Serandour et al., 2011; Sahu et al., 2013). However, efforts to define the major pioneer factors for the VDR have proven to be

less consistent between the different VDR ChIP-Seq studies and may reflect the biology of the VDR which, given that it exists in the nucleus both in the presence and absence of ligand, such that a single dominant pioneer factor is not so deterministic.

Network Behavior Centered on the VDR

Another approach to identify the interacting partners of the VDR has been to examine the gene networks it regulates and to cluster genes by known regulating transcription factors. Novershtern et al. (Novershtern et al., 2011) measured the transcriptome profiles of a large number of hematopoietic stem cells, multiple progenitor states and terminally differentiated cell types. They found distinct regulatory circuits in both stem cells and differentiated cells, which implicated dozens of new regulators in hematopoiesis. They identified 80 distinct modules of tightly co-expressed genes in the hematopoietic system. One of these modules is expressed in granulocytes and monocytes and includes genes encoding enzymes and cytokine receptors that are essential for inflammatory responses. Major players in this module are VDR together with the factors CEBP α and SPI1/PU.1. This indicates that VDR works together with this small set of transcription factors, in order to regulate granulocyte and monocyte differentiation. It is reasonable to anticipate that such modules exist in multiple cell types but are guided by the tissue specific expression of such factors.

Genetic Variation in VDR Binding

Many studies have addressed the impact of genetic variation in the VDR and other regulatory proteins (Uitterlinden et al., 2004; Gandini et al., 2014). We have recently pursued the role of disease and phenotype associated genetic variation throughout the genome in non-coding regions (van den Berg et al., 2014). The findings of widespread genetic variation and distal binding of transcription factors raises the possibility that phenotype and disease associated SNPs at distal regions are impacting transcription factor activity (Al Olama et al., 2014). Specifically, we investigated how the trait and disease associated SNPs those in linkage disequilibrium associated with VDR binding sites. The combination of these data allows the use of phenotype-driven data to identify what VDR binding sites, and interactions, are important.

A major hurdle to addressing this question is very large potential for Type 1 error owing to the large-scale data sets that need to be integrated, namely; all SNPs and those in linkage disequilibrium that are significantly associated with disease in replicated studies and all binding sites for a given transcription factor against the backdrop of the number of SNPs for a given trait, the platform used for identification and the total number of SNPs in the human genome. Several clear findings emerged from these analyses, including that the majority of SNPs identified to be significantly associated with VDR binding were related to immune phenotypes and that there was a very pronounced co-enrichment for binding sites for NF- κ B at these regions, again supporting a significant overlapping biology between these two transcription factors (ref). A finding that supports the anti-inflammatory actions of vitamin D identified by others (Sokoloski and Sartorelli, 1998; Wang et al., 2014)

Conclusions and Future Perspectives

Clearly, the VDR regulates transcriptional programs that control cell growth and differentiation and exerts these actions by binding through the genome as distal and proximal regions and in doing so, its actions are interegrated with other transcription factors including SMADs, TCF and NF- κ B.

To build on this comprehensive understanding it is now necessary to build predicative understanding of the epigenetic niche that characterizes the VDR binding. These analyses will require agnostic integration of multiple genomic data sets, for example histone modifications, transcription factor binding, chromatin conformation and transcriptomic data and application of machine learning approaches to reveal the significance of the underlying patterns, VDR binding and transcriptional activity. Whilst the VDR was not included in the ENCODE project, judicious choice of a cell line model for these studies, most likely a Tier 1 cell line from ENCODE, will enable leverage of a considerable volume of cistromic and epigenomic data to be combined with de novo VDR ChIP-Seq data. For example undertaking VDR ChIP-Seq and a limited number of RNA-Seq approaches in K562 cells has the potential to leverage a remarkable volume of data.

Another clear challenge will be to translate this global VDR understanding into a precision medicine context. Many aspects of VDR function can be interpreted by serum borne measurements, which are highly attractive owing to their ease of measurement. Serum levels of the pro-hormone, 25(OH) vitamin D₃, are strongly correlated with the generation of the active hormone 1 α ,25(OH)₂D₃ and VDR function. For example, reduced serum levels of 25(OH) vitamin D₃ levels are associated with increased risk of either cancer initiation and/or progression (Drake et al., 2010; Shanafelt et al., 2011). Therefore the serum level of 25(OH) vitamin D₃ can yield the “potential” of the VDR system to signal (Brader et al., 2014). This potential is impacted by the various cellular mechanisms outlined above. Of these, genetic variation that impacts VDR binding can obviously be measured in any cell in the body. The total transcriptome can be challenging to measure but perhaps small non-coding RNA represent a highly attractive marker of activity. Remarkably, miRNA are readily secreted into serum where they remain stable (El-Hefnawy et al., 2004; Goyal et al., 2006; Taylor and Gercel-Taylor, 2008) and can be reliably extracted and measured (Chen et al., 2008; Liu et al., 2008; Mitchell et al., 2008; Rabinowits et al., 2009). Using serum-borne molecules as prognostic markers is highly attractive for several reasons. First, they can overcome the limitations of inaccurate sampling for example in the case of the presence of cancer within a tumor biopsy. Second, they can encapsulate the effects of heterotypic cell interactions, again, for example within the tumor microenvironment. Third, they form a non-invasive test procedure. From a biostatistical perspective, given there are fewer miRNA than protein coding mRNA, genome-wide coverage is more readily achieved and avoids the statistical penalties typically associated mRNA genome wide testing (Lussier et al., 2012).

This raises the very exciting possibility that generating integrated models of VDR binding, the impact of genetic variation, the tissue specific differences in the VDR transcriptome and their relation to non-coding RNA species such as miRNA offers the opportunity to not only provide global understanding of the VDR in health and disease but to exploit this knowledge

for use in precision medicine. Similarly, serum expression levels of 25(OH) vitamin D3, genetic variation and miRNA expression will be able to predict accurately the capacity of VDR function and provide a platform for precise analyses in human in health and disease.

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