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Bioinformatic approaches to interrogating vitamin D receptor signaling

Moray J. Campbell

Division of Pharmaceutics and Pharmaceutical Chemistry, College of Pharmacy, 536 Parks Hall, The Ohio State University, Columbus, OH 43210, USA

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ABSTRACT

Bioinformatics applies unbiased approaches to develop statistically-robust insight into health and disease. At the global, or "20,000 foot" view bioinformatic analyses of vitamin D receptor (NR1I1/VDR) signaling can measure where the VDR gene or protein exerts a genome-wide significant impact on biology; VDR is significantly implicated in bone biology and immune systems, but not in cancer. With a more VDR-centric, or "2000 foot" view, bioinformatic approaches can interrogate events downstream of VDR activity. Integrative approaches can combine VDR ChIP-Seq in cell systems where significant volumes of publically available data are available. For example, VDR ChIP-Seq studies can be combined with genome-wide association studies to reveal significant associations to immune phenotypes. Similarly, VDR ChIP-Seq can be combined with data from Cancer Genome Atlas (TCGA) to infer the impact of VDR target genes in cancer progression. Therefore, bioinformatic approaches can reveal what aspects of VDR downstream networks are significantly related to disease or phenotype.

1. What is bioinformatics and how is it applied?

The future is the widespread application of technologies that currently exist but are restricted. For example, the first email was sent in 1971 between two physically adjacent computers. However, this technology only has an impact when there are large numbers of people with email addresses, who care to read email, and with ready access to hardware and software to run email programs. In short, the world of 1971 in Europe and North America was vastly different to today. One year earlier, in 1970, Paulien Hogeweg and Ben Hesper developed the term bioinformatics to refer to the study in biological systems of how information was encoded and determined cell development (reviewed in (Hogeweg, 2011)). In this manner Hogeweg and Hesper considered bioinformatics as the study of information in biology, thereby akin to biochemistry, or the study of chemistry in biology.

The roots of bioinformatics encapsulate theory and approaches that in many cases were themselves already well-established. For example, the desire to analyze and model morphogenesis in living systems had long been a central focus of biology. The theoretical analyzes of nucleic acids as the central conduit for information flow had been a focus of theoretical research in the first half of the twentieth century and crystalized in 1944 by the physicist, Erwin Schrödinger, in the book "*What Is Life? The Physical Aspect of the Living Cell*".

In much the same way that it took nearly half a century for the digital age to prevail, the wider application of bioinformatic approaches required progress in other disciplines accompanied by development of companion practices and technologies. For example, the further development of computational theory and computational hardware, as well as the development of statistical concepts were all absolutely necessary for the wide-spread application of bioinformatics. In short, the central components of bioinformatics are the statistical and computational sciences, which each have their own considerable histories. Bioinformatics is therefore a truly interdisciplinary approach with high transformative potential.

Currently, it isn't clear how the biological community stands in terms of the widespread application of bioinformatics. In terms of the email analogy, there are significant communities of researchers who are using emails, undertaking e-commerce and telecommuting and, for them, these technologies and approaches are truly transformative. Powerful examples are illustrated by the achievements of various genomic research consortia including the Human Genome Project (Roberts et al., 2001), ENCODE (Birney, 2012, Consortium et al., 2007), RoadMap Epigenome (Roadmap Epigenomics, Kundaje, Meuleman et al., 2015), FANTOM(Sanli

E-mail address: Campbell.1933@osu.edu.

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et al., 2013), IHEC (Bujold et al., 2016, Chen et al., 2016) and TCGA (Cancer Genome Atlas Research, Weinstein, Collisson et al., 2013).

However, the adoption of bioinformatics across the biological sciences is extremely uneven. In many parts of the biological community the steps are much more tentative and uncertain. Therefore, it is probably reasonable to argue that bioinformatics is far from being democratized, and not having its full transformative impact on the understanding of health and disease in humans and other organisms.

This raises the question as to how nimble the biological community can be in the adoption of new practices. An interesting parallel emerges from considering the development and application of molecular biology. Warren Weaver, the Director of the Rockefeller Foundation, invented the term molecular biology in 1938(Weaver, 1970) and for several decades the approaches were essentially restricted to a small number of university departments and institutes. However, a number of transformative technologies led to molecular biology democratization. These included the application of restriction enzymes (Danna and Nathans, 1971), the capacity to amplify targeted regions of DNA with PCR(Saiki et al., 1988) and rapid large-scale nucleic acid sequencing (Hunkapiller et al., 1991). In short, these were vital levers to ensure molecular biology transformed within 50 years to become a common and democratized approach, such that any laboratory, anywhere in the world, could undertake fairly complex molecular biology analyses.

We now stand almost 50 years after the conception of bioinformatics and it is far from clear that this science is becoming democratized, and will achieve its potential to transform biology. In fact, it is extremely challenging to make the case that bioinformatics will be democratized within 50 years of its conception, a time point that is only a few years away from the time of writing in 2017. Instead it remains a fairly restrictive practice. Arguably, this is deleterious for the biological and biomedical communities and needs to be addressed strategically, and urgently.

2. Bioinformatics approaches can identify genome-wide significant relationships for the VDR; the 20,000-foot view

The goal of bioinformatics is to reveal novel biological understanding. The premise for such analyses is that the comprehensive analyses of biological data, free from a bias as to what are the major biological drivers of a given phenotype, will reveal organizational insight that is neither obvious nor intuitive. In this respect, analyses that has no assumptions what are the biological drivers of a given phenotype can be achieved by applying algorithmic approaches that depend on discrete mathematics and information theory, combined with graph theory, data mining, computer science generally, with a central role for the statistical sciences. In this manner, bioinformatic approaches offer the promise to reveal underlying mechanisms of biology in health and disease. Importantly, such approaches hold the promise of revealing insight that is unlikely to be revealed by reductionist experimental approaches.

However, here a note of caution should also be considered. No analyses is free from bias. That is, many of the algorithms and statistical approaches applied, for example to next-generation sequencing approaches, themselves are built on certain assumptions on the distribution of the data and how in broad terms significance can be determined. In this regard, it is possible that an analytical workflow may contain certain biases as to how the biological material is captured, analyzed and interpreted. For example, a trivial example would emerge over considering how degradation rates of nucleic acid material could impact library preparation and how signal is considered significant (van Dijk et al., 2014). At the other end of the spectrum, statistical assumptions used in the models applied to measure changes in gene expression or DNA methylation can determine the numbers and magnitude of significance (Ren et al., 2017, Poirion et al., 2016, Choi et al., 2017, Love et al., 2014). These assumptions can be compounded when omic approaches are integrated (Kumar et al., 2016).

With awareness of these biases and statistical considerations in mind, investigators would apply bioinformatics approaches across data sets in a biological condition of choice, for example during cell development or cell transformation leading to cancer. These analyses would apply myriad omic technologies to reveal genomic structural variants and nucleotide polymorphisms, gene and protein expression patterns, protein post-translational modifications and metabolites. Bioinformatics analyses will then include all steps from data processing (e.g. filtering and normalization) to establishing reproducible changes, for example between cell state A and B, and more complex integrative and statistical approaches would then combine to identify the network changes between states and finally identify nodes that exert control. Such nodes would then form attractive targets for interventionist wet-lab based experiments.

Several points are worth stressing from this theoretical workflow. Firstly, study design in terms of replicates and time points applied to the appropriate cell phenotype description are both critical. Secondly, all analyses include a denominator (e.g. the genome, the detectable transcriptome etc) such that any change is considered against the backdrop of all events occurring in the cell. Thirdly, all data processing includes normalization across samples, including replicates and states, and subsequent filtering to remove the large component of the signal that is unchanging, and therefore limit the penalties of false discovery. Finally, the integrative steps have very high potential for creativity and novelty. That is, as the volume of publically available data grow, the statistical approaches and types of data integration that can occur are varied and represent where many of the key biological questions of the future will be framed. In contrast, in part because molecular biology has become so widely democratized, the wet lab component in this workflow has less potential for creativity and novelty.

The mechanics of VDR signaling is well-studied in both humans and genetic mouse models, and therefore extensively reviewed (Campbell, 2014, Carlberg and Campbell, 2013,Carlberg, 2016, Narvaez et al., 2014, White, 2012,Bikle, 2016, Jeffery et al., 2016, Suda et al., 2015, Demay, 2013). Therefore, the current review will focus on the application of bioinformatics approaches applied to place VDR signaling in an integrated context.

There are several different ways in which bioinformatics approaches could be applied to VDR. In the first instance, a strict interpretation can be applied whereby the question addressed is to consider where VDR biology been identified as significant following bioinformatics analyses that considers the genome as the denominator for analyses. The null hypothesis in this approach is that the VDR is not biologically significant and empiric bias doesn't impact either study design or analytical workflow. Instead these papers can reveal in what biological or disease settings does a significant role for the VDR emerge when considering all genetic variation, or expression of all genes and proteins?

Viewed from this perspective, relatively few studies have identified a significant role for the VDR (Table 1). For example, to address this question, literature in PubMed and the catalog of genome-wide association studies (GWAS) were mined in January 2017. Ten phenotypes were considered, which were the principal topics from the Vitamin D Workshop (Boston, 2016), for example including immunity, cancer and diabetes. In each case all RNA and protein omic studies in a phenotype were identified, and then the search was repeated to include the term VDR in the title or abstract. In this manner, 51,649 publications have applied omic approaches to the 10 principal phenotypes, 71 of which also included the term

Table 1
Genome-wide significant relationships between the VDR and the indicated phenotypes. The indicated search terms were undertaken in PubMed or the NHGRI GWAS catalog and the numbers indicate the number of publications. The specific papers are indicated (Pub)
1. (Mvubu et al., 2016)
2. (Sims et al., 2013)
3. (Bi et al., 2013)
4. (Li et al., 2012)
5. (Valta et al., 2009)
6. (Huang et al., 2014)
7. (Chen and Xia, 2014)
8. (Li et al., 2013)
9. (Nakou et al., 2010)
10. (Fowlkes et al., 2008)
11. (Huttunen et al., 2007)
12. (Mochizuki et al., 2009)
13. (Zhou et al., 2014)
14. (Zolotarenko et al., 2016)
15. (Bao et al., 2016)
16. (Jeong et al., 2012)
17. (Wang et al., 2016)
18. (Dorjgochoo et al., 2012)
19. (Jostins et al., 2012)
20. (Perry et al., 2014)
21. (Lai et al., 2012).

Phenotype	PubMed Search term						GWAS catalog
	(RNA-Seq [Title/Abstract]) OR microarray [Title/Abstract]) OR proteomic [Title/Abstract]) AND			(genome wide association study [Title/Abstract]) AND			VDR
	+Phenotype	+VDR	Pub	+Phenotype	+VDR	Pub	Pub
Immunity	1972	4	1,2	91	0	–	19
Infection	6210	4	–	216	0	–	–
Cancer	22,271	28	3–5	760	0	–	–
TCGA	344	0	–	7	0	–	–
Bone	2810	16	6–11	153	0	–	–
Muscle	3103	5	–	114	0	–	–
Intestine	597	1	12	11	0	–	–
Reproduction	747	1	13	25	0	–	20
Metabolism	11,679	10	14–16	300	2	17,18	–
Nutrition	387	0	–	31	0	–	–
Diabetes	1529	2	–	434	0	–	21

VDR. Inspection of these publications revealed that only 16 were designed to test the null hypothesis that could be used to determine where a significant role for VDR expression or function emerges in one of these 10 phenotypes. The other 55 publications were biased in the sense that the system examined was either treated with vitamin D ligands, of examined the impact of changing VDR expression, for example in a knockout mouse model. In this manner these publications all took the *a priori* assumption that the VDR was biologically significant.

Publications that reported genetic variation and could reveal a genome-wide significant role for the VDR was also searched. In the same manner, 2142 publications have aimed to identify roles for significant genetic variation associated with the ten phenotypes examined. Of these, 2 publications directly identified a genome-wide significant role for genetic variation associated with the VDR with one of the 10 phenotypes, specifically altered metabolism. In parallel, the GWAS catalog was mined with the term VDR and a further 3 publications were identified that annotated significant genetic variation to the VDR gene that associated with immunity, reproduction or diabetes.

In total, therefore, 21 papers, from 53,791 (0.039%) phenotype-associated studies that applied bioinformatics analyses identified a genome-wide significant relationship between VDR function and one of the phenotypes.

There are a number of caveats to these literature mining. The scientific literature is itself biased. There are approximately 5000 papers that consider the VDR and ten times as many that examine the androgen receptor (AR), and 1/100 as many that consider NR6A1. It is not possible to know if this is biologically based. Is the AR ten times more important in some biological sense than the VDR; is a man suffering from advanced prostate cancer more important than a child suffering with rickets? Similarly, in the arena of genetic variation, as data sets increase in size, and with more defined phenotypes, rare variants may emerge associated with the VDR. However, with over 5000 papers the VDR is fairly well studied, and it is possible to begin to infer where its actions are biologically-significant at the genome-wide level.

Within these 21 papers, approximately 25% establish an unbiased genome-wide significant relationship between bone phenotypes and VDR. Of course, the actions of the VDR has been examined extensively in the context of rickets and more specifically in the process of calcium signaling (Peacock et al., 1974). Other significant relationships are observed with phenotypes related to Immunity, Cancer, Intestine, Reproduction and Metabolism.

The phenotype of cancer has been very extensively investigated. There are approximately 22,000 omic papers in the cancer field. Furthermore the cancer research field now benefits from the genomic analyses of TCGA (Cerami et al., 2012, Gao et al., 2013). These data are derived from over 33 different cancer types that were collected from approximately 11,000 patients. Large consortia have analyzed these data with comprehensive statistical approaches and to date 344 papers have been published. None of these papers indicate a significant role for disruption or association of the VDR with tumor phenotypes. By comparison over 100 TCGA papers report a significant relationship with TP53. Together these findings suggest that the VDR alone does not act as a cancer-driver.

Often biological signals are extremely contextual and there is evidence to support a subset of VDR-gene relationships that are biologically impactful. For example, analyses of myeloid (Novershtern et al., 2011) and megakaryocyte (Fuhrken et al., 2008) cells illustrate there is a significant role for the VDR to act in specific transcriptional units that control aspects of cell differentiation at specific points. These findings reveal the intricate mechanistic basis to some of the earliest cancer studies on VDR signaling in leukemia (Yetgin and Ozsoylu, 1982,Koeffler, 1983), which revealed that exogenous vitamin D compounds can trigger cell differentiation.

In part, VDR signaling in leukemia was examined by workers because of the pharmacological targeting of RARs by all-*trans* retinoic acid (ATRA) in acute promyelocytic leukemia (APL). ATRA therapy in APL is a dramatic example of targeted cancer-therapies and also gave rise to the concept of differentiation therapy (Chen et al., 1991, Douer and Koeffler, 1982,Breitman et al., 1980, Castaigne et al., 1990, Huang et al., 1989, Grignani et al., 1998, Lin et al., 1998, Spira and Carducci, 2003, Ferrari and Waxman, 1994). ATRA-based therapies remains clinically-exploited (Lo-Coco et al., 2016, Uray et al., 2016) and were a major catalyst for studying RARs across cancers (Ferrari and Waxman, 1994, Bhutani and Koo, 2011,Campbell et al., 1998). In the case of VDR signaling, the lack of evidence from large-scale leukemia studies probably starts to suggest that this receptor is neither distorted to act as cancer-driver, nor can it be meaningfully exploited therapeutically.

However, reflecting the role VDR appears to be playing in myeloid systems, it is worth stressing the apparent importance of VDR in immune phenotypes. That is, two papers support a genome-wide significant role for VDR expression in immune phenotypes (Mvubu et al., 2016) (Sims et al., 2013), and GWAS studies has identified significant genetic variation annotated to the VDR also in immune phenotypes (Jostins et al., 2012).

Findings from other cancers, suggest that VDR does not function as a cancer-driver in the other major epithelial cancers if the standards are i. Identifying a genome-wide significant role for the receptor in the biology of the tissue and ii. Establishing that the receptor is disrupted in a significant manner and may act as a cancer-driver. One of the tissues where these aspects have been tested is in mammary gland function. Perhaps reflecting the reproduction-related functions of the VDR in murine systems (Zhou et al., 2014), the VDR −/− mice display a mammary gland phenotype, and this genotype can modulate cancer incidence in murine cancer models (Zinser and Welsh, 2004, Zinser and Welsh, 2004, Zinser et al., 2002). However, transcriptional and epigenomic control of breast epithelial systems in human cells does not reveal a genome-wide significant role for the VDR (Pellacani et al., 2016) and the major breast cancer papers from TCGA have not identified a genome-wide significant role for the VDR to act as a cancer-driver (Suo et al., 2015, Ciriello et al., 2015, Keenan et al., 2015, Robinson et al., 2013). A caveat however is that as statistical approaches are developed it is possible that cancer-drivers will be identified with more nuanced activities and may yet yield a more significant role for the VDR in the TCGA data (Sikdar and Datta, 2017).

Combining these findings together from leukemia, and common cancers suggests that the VDR itself does not act as a direct cancer driver either through loss or gain of function and is most likely not therapeutically relevant in the cancer context. These findings are reflected by the lack of clinical success in targeting the VDR by using vitamin D compounds as chemotherapies (Evans et al., 2002, Dalhoff et al., 2003, Flaig et al., 2006, Jarrard et al., 2016, Chadha et al., 2010, Osborn et al., 1995, Amir et al., 2010, Jacot et al., 2016). In all cases the regimens were well tolerated but clinical responses were scant.

By contrast in the immunomodulatory phenotypes it seems more clear the VDR plays a genomicaly significant biological role and therapeutic intervention remains an attractive possibility. It could be argued that the ongoing analyses of the literature in this manner could be used to direct where research efforts can be most readily justified. Given the ever increasing costs of biomedical research, it is perhaps reasonable that such literature mining that identifies genome-wide significant association of the VDR with a given phenotype may lead to more impactful research findings.

3. Bioinformatic approaches to VDR signaling events; the 2000-foot view

Alternative approaches to apply bioinformatic insight to dissecting VDR function are to adjust the denominator. For example, to consider VDR status compared only to a smaller sub-set of related genes or proteins, and to consider only the down-stream consequences of VDR, and then to apply unbiased approaches. This approach then is a mixture of biased and unbiased approaches. It starts with the premise that the VDR is significant in a given phenotype, and then tests the null hypothesis that VDR does not regulate genes significantly that are associated with a given phenotype. In this manner it's possible to dissect VDR function without consideration to what pathways are important.

Adjusting the denominator has the effect to reduce the number of comparisons made and therefore of course generates a more restricted answer. It is reasonable to assume that the VDR is significant as its actions are implicated in a range of biological settings as it is clear that the VDR ligand, $1\alpha,25(OH)_2D_3$, can regulate calcium signaling and control the proliferation of, for example, a wide variety on myeloid, prostate, breast, and colon non-malignant and malignant cell lines (Palmer et al., 2003, Koike et al., 1997, Campbell et al., 1997, Elstner et al., 1999, Peehl et al., 1994, Welsh et al., 2002, Colston et al., 1989, Colston et al., 1982).

Similarly, there is a significant history of VDR-centric transcriptomic studies that support the cell phenotypes observed (Savli et al., 2002, Akutsu et al., 2001) (Palmer et al., 2003, Eelen et al., 2004,Akutsu et al., 2001, Wang et al., 2005, Wang et al., 2005, Lin et al., 2002).

More recently, this has been complemented by VDR ChIP-Seq studies in which the VDR genomic binding patterns have been captured. For example, VDR ChIP-seq studies have been performed in several human cell types (Ding et al., 2013, Heikkinen et al., 2011, Meyer et al., 2012, Ramagopalan et al., 2010, Tuoresmaki et al., 2014), in the presence and/or absence of ligand, and revealed the impact of ligand binding on VDR genomic targeting.

Another approach to a VDR-centric analyses is to consider a number of large data sets can also be interrogated with no specialist computational skills. For example, the Genotype-Tissue Expression (GTEx) project (Mele et al., 2015) has undertaken expression profiling by RNA-seq in approximately 50 different tissues from large numbers of normal tissue donors. These data are readily available to interrogate through a graphical user interface (GUI) and therefore allows unparalleled and easy investigation of where the VDR is most and least abundant; it is most abundant in tissues of the colon and small intestine and least abundant in basal ganglia and brain tissues.

To complement the analyses of normal tissue, the TCGA data are available both for analyses in a GUI format or through API-type interrogation. Therefore, it is possible to ask more focused questions centered around the VDR and related genes, and thereby limit the penalties of false discovery. It is possible to examine in which cancer the VDR is most altered. It is deleted in approximately 40% of Adenoid Cystic Breast cancer, for example. However this gives no level of significance to the finding. It is a number but it is unclear what it means; are all genes deleted in 40% of Adenoid Cystic Breast cancers?

As a hybrid approach we analyzed 13 transcription factor families implicated in cancer, including the nuclear hormone receptor (NR) superfamily, across 3000 tumors from six different tumor types (Cancer Genome Atlas Research, 2014, Cancer Genome Atlas, 2012, Cancer Genome Atlas, 2012, Cancer Genome Atlas, 2015,Ahn et al., 2014). Bootstrapping approaches (Long et al., 2014) established that across cancers only the NR family was significantly down-regulated, but was neither significantly mutated nor altered by CNV(Long and Campbell, 2015). Within the NRs, only a subset of receptors (e.g. *NR3C2/MR* and *NR5A2/LRH-1*) were commonly down-regulated across all tumor types examined. By contrast, others were uniquely suppressed in only one tumor site, including *VDR* in the colon cancer (COAD) cohort; this finding may reflect the strong expression of VDR in the normal colon. VDR downregulation was not found to be driven by copy number variation or mutation and thus epigenetic mechanisms may be primarily responsible for altered expression (Long and Campbell, 2015) (Long et al., 2014).

3.1. VDR analyses at the genomic level

Arguably, VDR ChIP-seq studies are more important than transcriptomic studies as they reveal direct VDR genomic interactions, whereas transcriptomic analyses inevitably include direct and indirect VDR-mediated effects. Each VDR ChIP-Seq analysis revealed approximately 2000 to 6000 genomic loci normally distributed around transcription start sites (TSS), reflecting the binomial distributions found for other transcription factors (Tuoresmaki et al., 2014, Djebali et al., 2012). Another important finding from these studies is the dual hexameric DNA motif spaced by 3 bp, a so-called DR3 motif (Shaffer and Gewirth, 2004, Sasaki et al., 1995), is perhaps not as common as might be expected, and even found in less than a third of the genomic VDR binding sites. However, this needs to be interpreted with caution, as the low discovery frequency of DR3 type elements may in part reflect algorithm limitations for *de novo* motif discovery. Indeed if a directed position weight matrix is applied then the frequency of DR3 identification rises, but of course this also reflects the benefit of adjusting the denominator. Perhaps a global interpretation of these findings is that although the VDR does bind to DR3 type elements, it also binds to other DNA recognition elements, and may also bind in both direct (VDR-DNA) and indirect (VDR-protein-DNA) modes (reviewed in (Carlberg and Campbell, 2013)). Indeed the composite and complex nature of genomic binding sites may allow for greater biological flexibility.

Other VDR binding motifs have also been suggested (Nayeri et al., 1995), and the application of ChIP-seq approaches to nuclear hormone receptors has revealed binding site diversity and the importance of flanking regions for cofactors to be biologically important to determine function (Phan et al., 2010).

In this context, murine models of VDR action can be highly informative. For example, by focusing on biological settings where the VDR is known to play a significant role, these can be extensively analyzed in a manner that is challenging to achieve human cell systems. For example, various groups have exploited murine models to examine *in vivo* differentiation from stem cells and undertaken ChIP-Seq towards VDR and known interaction transcription factors and associated histone modifications to identify roles for how the epigenome changes during cell state changes (Meyer et al., 2016, Lee et al., 2015).

There is a probably a compelling case to be made for the re-analysis of the VDR ChIP-Seq data, from genomic alignment to differential peak calling. The rationale for re-analyses is two-part. Analyses of ChIP-Seq is not trivial in terms of statistical assumptions and the existing studies have all been analyzed in a different manner therefore there is the possibility that thresholds and cut-offs differ between studies. Secondly, the methodologies for ChIP-Seq processing are an area of active development. Most commonly, the bioinformatics community uses the R platform for statistical computing (Le Meur and Gentleman, 2012,Dudoit et al., 2003) and a range of library packages implemented in Bioconductor (Gentleman et al., 2004, Huber et al., 2015). There are multiple packages available in Biconductor that deal with the analyses of ChIP-Seq data. R and Bioconductor are both community developed and maintained and, therefore, new approaches are continually developed. Indeed, Bioconductor illustrates the combination of packages, or software libraries, that can be applied for optimal workflows for many common bioinformatic analyses.

Such a workflow has been developed for the analyses of ChIP-Seq, and the most recent approaches display a number of benefits over earlier analytical workflows (Lun and Smyth, 2015). Traditional analyses focused on identifying significant peaks, and then secondly identifying differences between these peaks, which can compound issues of false discovery. The combination of the Rsubread (Liao et al., 2014) and csaw (Lun and Smyth, 2016) library packages have a number of advantages over previous approaches, for example based around MACS(Zhang et al., 2008). Specifically, the csaw package applies a sliding window-based approach to identifying significant *de novo* genomic binding. It applies a range of filtering and normalization options and procedures to maintain statistical power. Furthermore, it is focused towards identifying regions that display differential binding under experimental conditions, such as treated and untreated and is thought to be more biologically-faithful and allows more rigorous statistical analyses in comparison to other approaches that suffer from imprecision in peak calling and therefore weaken statistical power. Importantly the window approach, which is independent of peak size, allows for a more accurate control of FDR at the level of consolidated regions and therefore is more accurate in its reporting to the user. With these considerations in mind, it is worth stressing again that, as the volume increases of both genomic data and workers analyzing the data, new protocols are continuously developed and the importance of rapid adoption of community standards is paramount.

A further opportunity available for future ChIP-Seq studies is in the judicious choice of cell line of study. Recent findings from consortia of genomic investigators such as ENCODE (Birney, 2012), RoadMap Epigenome (Roadmap Epigenomics et al., 2015) and FANTOM(Sanli et al., 2013) have developed large volumes of genomic data with some of the most intensive investigation being focused on a restricted number of cell models. For example, there are three Tier 1 cell lines in the ENCODE project including K562 cells. For K563 cells there are approximately 600 genome-wide data sets publically available, including ChIP-Seq, Methl-Seq, DNAse-Seq, RNA-Seq and other omic data. Furthermore, ENCODE have made major strides to harmonize analytical approaches for NGS data, and it is interesting to note that both ENCODE and RoadMap Epigenome have not very extensively investigated NRs in general and not specifically considered VDR. Therefore, there is an exciting opportunity once VDR ChIP-Seq is undertaken in one of these models in terms of integrative analyses (Long, van den Berg, Russell et al., 2015) that could leverage ENCODE or RoadMap Epigenome data.

For example, nuclear hormone receptors display co-operative or antagonistic gene regulation through shared interactions (Hua et al., 2009) and by virtue of co-factor sharing (Jepsen et al., 2000, Chen, 2000, Lonard and O'Malley, 2012). Their actions are also guided by pioneer transcription factors such as Forkhead box (FOX) family members (Lupien et al., 2008, Serandour et al., 2011, Sahu et al., 2013) and integrated with other transcription factors including WNT (Katoh and Katoh, 2007), SMADs (Ding et al., 2013, Zerr et al., 2014, Ito et al., 2013) and KLFs(Karp and Rassool, 2011,

Chen et al., 1993). Therefore, undertaking VDR ChIP-Seq in, for example, K562 cells has the strong potential to address many of the issues of how genomic interactions of the VDR overlaps with other TFs and co-factors.

The VDR ChIP-seq data also lend themselves to be combined with other types of publically available data to ask further questions concerning VDR function. For example, one area to emerge recently has been to examine how significant genetic variation in transcription factor binding site can relate to phenotypes and disease susceptibility. Testing the possibility that genetic variation impacts transcription factor function that underpins trait differences and disease phenotypes is analytically challenging, given the size of the datasets and again the potential for false discovery is large. Various groups have addressed this challenge; notably, both the ENCODE and Roadmap Epigenome consortia leveraged the remarkable volume of ChIP-seq data they generated and merged the binding sites with GWAS data to reveal and rank sites where SNPs appear to have a significant impact on the activity of multiple transcription factors (Roadmap Epigenomics et al., 2015, Djebali et al., 2012, Boyle et al., 2012).

However, given that VDR has not been considered in any of these consortia, we applied a statistically robust approach to integrate VDR ChIP-Seq with NHGRI GWAS SNPs, and SNPs in linkage disequilibrium, to provide novel insight into the interaction between disease/phenotype associated SNPs and VDR binding. From these analyses, we applied transcription factor motif searching and exploited other ChIP-seq data to identify significant interactions between the VDR and other transcription factors, notably NF-κB and disease traits. In this manner we identified genetic variation that was significant at the genome-wide level enriched in VDR binding sites and impacted VDR function. GWAS SNPs were identified at shared VDR and NF-κB binding regions related to immune-phenotypes. For example, we identified genome-wide significant variants at the shared site of VDR and NF-κB binding in proximity to the *Toll-like receptor 1 (TLR1)* and associated with self-reported allergy (Singh et al., 2017). This finding adds to the understanding of the previously reported pleotropic actions VDR (Szpirer et al., 1991, Wang et al., 1998, Seuter et al., 2014, Lin et al., 2013, Stoppelenburg, von Hegedus, Huis in't Veld et al., 2014, Wu et al., 2010, Liu et al., 2009, Griffin et al., 2007).

Another area of data integration is to examine how genes bound by VDR are expressed in relevant disease or phenotype data sets. Similarly, in proof of principal studies, we have focused on colon cancer for three strategic reasons. Firstly, the VDR is highly expressed in the normal colon and associated with a range of VDR-relevant functions including control of local immunity (Peterlik and Cross, 2005,Dougherty et al., 2014, Giardina et al., 2012, Pereira et al., 2011, Kaler et al., 2009,Alvarez-Diaz et al., 2009, Liu et al., 2013). Secondly, our pan-cancer analyses revealed that the VDR was commonly and significantly down-regulated, compared to other nuclear hormone receptors, only in colon cancer suggesting a functional relationship (Long and Campbell, 2015).

There is a very well-established literature supporting links between corrupted VDR signaling and colon cancer (Pereira et al., 2011, Satelli et al., 2011, Belo, van der Sar, Tefsen et al., 2013, Kim et al., 2013, Tsai et al., 2016, Palmer et al., 2004, Pena et al., 2005, Larriba et al., 2011). Our pan-cancer analyses of nuclear receptors adds to these findings, suggesting that loss of VDR-induced growth restraint may be more apparent in colon cancer than in other cancers where alterations are not apparent.

We leveraged VDR ChIP-Seq data derived in LS180 colon cancer cells from the study of Meyer and co-workers (Meyer et al., 2012). Following VDR ChIP-Seq data re-processing, using Rsubread (Liao et al., 2014) and csaw (Lun and Smyth, 2016), we examined expression of the colon-specific VDR target genes in the TCGA-COAD cohort (Cancer Genome Atlas, 2012). Clustering the tumors by expression patterns then allowed testing the relationships between expression of VDR target genes and clinical outcome. Reflecting the earlier study of Meyer et al. (Meyer et al., 2012), we identified several of the same target genes associated with VDR binding, including *DOCK1*. Expression of VDR target genes were either significantly repressed or activated in the COAD cohort, suggesting that VDR functions in both activating and repressing complexes at the basal (or physiologically-activated) state such. We reason further that when the levels of the *VDR* are reduced, as it is in this cohort, the net result is to either allow genes to escape repression or to fail to be restrained. For instance, *LGALS4* is a VDR target gene that is specific to colonic cells and is down-regulated in colon cancer, acting as a tumor suppressor (Satelli et al., 2011, Michalak et al., 2016, Solmi et al., 2004). Further analyses of these genes in terms of the association between quartile expression and disease free survival in specific patient sub-groups was undertaken. Tentatively, these analyses demonstrated the protective effect experienced by females compared to males with regards to *LGALS4* quartile expression patterns.

3.2. VDR analyses at the transriptomic level

Unbiased analyses of VDR signaling have allowed investigators to establish how it overlaps and interacts with other pathways. For example, using isogenic cell pairs with differing sensitivities to $1,25(OH)_2D_3$ signaling has identified networks that mediate anti-proliferative sensitivity towards $1,25(OH)_2D_3$. In this manner, a significant role for TGFβ signaling cross-talk with VDR was been revealed (Towsend et al., 2006, Larsen et al., 2016) and that VDR transcriptional targets could distinguish leukemia aggressiveness (Tagliafico et al., 2006).

Certainly, given the number of microarray and RNA-Seq data sets, including both coding and non-coding RNA species (Jiang and Bikle, 2014, Singh et al., 2015), it is now timely to consider meta-analyses to reveal common themes, as has been applied to disease classification (Engreitz et al., 2011, Shah et al., 2009) and more specific phenotypes (Martinez-Climent et al., 2010, Rantala et al., 2010, Lai et al., 2014).

4. Summary

Bioinformatics approaches aim to apply comprehensive statistical approaches to biological systems and to reveal novel insight. Given the high quality publically available data that is ever increasing in volume, it is timely for investigators to use these approaches to address questions concerning VDR function in health and disease. Currently, at the 20,000 ft view that considers the genome-wide level, significant roles for the VDR are identified in the regulation of the calcium signaling in bone phenotypes as well as being clearly implicated in immune system. From this perspective and with current methodologies it is harder to make a case that the VDR itself, either through loss or gain of function, is a cancer-driver or is strongly related to other phenotypes such as nutrition and diabetes.

If the perspective is changed to 2000 ft, and the question is changed to asking only what is significant within VDR signaling, then other findings can emerge. There remains considerable diversity over where the VDR binds in the genome and what are the genomic features that attract it. Opportunities exist to undertake VDR ChIP-Seq studies in cell models that have been the subject of considerable focus by genomic consortia, such as ENCODE. Data integration of VDR ChIP-Seq studies with genome-wide significant genetic variation supports a role for shared VDR and NF-κB binding at non-canonical regions, again related to immune phenotypes, but

not clearly to cancer pre-disposition.

However, such studies may lead to the possibility of revealing specific VDR-sub-cistromes that are uniquely druggable. Thus, rather than the view that the VDR alone is single driver of disease or phenotype, it is probably more accurate to begin to define the sub-cistrome actions where the VDR is significant, either by directly binding to DNA or by indirectly modifying other protein-DNA interactions. Again, these approaches will require combination and integration of omic data sets, such as ChIP-Seq for VDR and known interacting transcription factors, histone modifications and conditional RNA-Seq studies. Bioinformatics approaches will be central to such data integration. Other integration approaches can include combining VDR ChIP-Seq in cell models with patient data. For example VDR ChIP-Seq from colon cancer cell lines can be combined with RNA-Seq expression of the target genes in the colon cancer TCGA cohort. In this approach, the altered expression of VDR target genes associated with more aggressive disease and suggests that analyses of genes that are regulated by VDR, and other transcription factors, may offer an opportunity to understand how VDR signaling, rather than the VDR itself, impacts cancer phenotypes.

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