Histone modifications and chromatin organization in prostate cancer

Epigenetic mechanisms, including histone modifications, nucleosomal remodeling, and chromosomal looping, contribute to the onset and progression of prostate cancer. Recent technical advances significantly increase our understanding of genome-wide epigenetic regulation of gene expression in prostate cancer. Aberrant genomic distribution and global level of histone modifications, nucleosome repositioning at the gene promoter and enhancer regions, and androgen receptor-mediated chromosomal looping may lead to the silencing of tumor suppressor genes and activation of proto-oncogenes. In addition, androgen receptor-induced chromosomal looping facilitates recurrent gene fusion in prostate cancer. Studies in epigenetic regulation have translational implications in the identification of new biomarkers and the development of new therapies in prostate cancer.

KEYWORDS: bioinformatics ■ CHIA-PET ■ ChIP-seq ■ chromatin organization ■ epigenome ■ Hi-C ■ histone modification ■ next generation sequencing ■ prostate cancer

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Prostate cancer is the most common noncutaneous cancer in American men and the second leading cause of cancer deaths in the USA. According to NCI's SEER Cancer Statistics Review, 192,280 men will have been diagnosed with prostate cancer in the USA in 2009 [1,101]. Androgens, functioning through androgen receptors (ARs), are essential for the initiation and progression of prostate cancer [2,3]. Thus, treatment of prostate cancer through androgen-ablation therapies has been the primary treatment for advanced androgen-dependent prostate cancer (ADPC) for over 40 years. Unfortunately, after prostate cancer has progressed to a late stage, it progresses into a fatal castration-resistant disease (CRPC) [4,5].

Recent progress in epigenetic studies has had a significant impact on our understanding of mechanisms leading to the onset of prostate cancer as well as the prognosis, diagnosis and treatment of this disease [6–9]. Strictly speaking, epigenetics is defined as the study of heritable changes in genome function that occur without changes of DNA sequence [10,11]. In a more broad definition, epigenetics may involve multiple mutually interacting mechanisms, including DNA methylation, covalent modifications of histone tails and chromatin reorganization (e.g., nucleosomal remodeling and chromosomal looping) (TABLE 1) [12,13]. Since DNA methylation is the most well-known epigenetic mechanism and its function in prostate cancer has been extensively discussed [14,15], in this review, we appraise recent progress in histone modifications and chromatin reorganization and highlight their role in prostatic carcinogenesis. Furthermore, this review also provides a summary of recent advances in second-generation sequencing based technologies for genome-wide profiling of epigenetic alterations.

Technological advances: histone modifications & nucleosome positioning mapping

During the past decade, microarray-based technologies have played a major role in genome scale analysis of the epigenome. Recently, second-generation massively parallel high-throughput sequencing (HTS) technologies, which produce tens to hundreds of millions of reads in a single run, have been used extensively in epigenomic studies. Briefly, HTS-based epigenomic profiling includes chromatin immunoprecipitation (ChIP) followed by HTS (ChIP-seq) analysis of histone modifications [16–18], DNA methylation analysis such as bisulfite conversion of either the entire genome (MethylC-seq)0 [19] or a CpG island-enriched partition (reduced representation bisulfite sequencing [RRBS]) [20], and micrococcal nuclease (MNase) digestion-based nucleosomal positioning analysis [21].

These HTS-based measurements offer several advantages over hybridization-based microarray analysis. First, it is extremely sensitive. A recent study found that some biologically meaningful peaks identified by ChIP-seq were obscured

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Table 1. Proven and potential epigenetic mechanisms.

Mechanisms	Epigenetic inheritance	Ref.
DNA methylation	DNA methylation is clearly propagated through cell division	[10,11]
Histone modifications	The epigenetic inheritance of a subset of histone modifications (e.g., H3K27 methylation and H3K9 methylation) has been demonstrated. Other H3 and H4 modifications are potentially propagated through cell cycle	[10,11]
Nucleosome positioning	Nucleosome positioning is implicated in epigenetic memory	[69,71]
Chromosomal looping	The discoveries that epigenetic silencing of imprinted genes requires long-range interactions between regulatory elements suggests that chromosomal looping is a potential epigenetic mechanism	[82–84]

when the same experiment was conducted with ChIP-chip [22]. Second, it is highly reproducible. Sultan *et al.* showed that the correlation coefficients R^2 reached 0.96 between two technical replicates for HTS [23] as compared with a value of 0.73–0.90 for microarray [24].

We will next discuss ChIP-seq in detail as this technique has been widely used in histone modification and nucleosome positioning mapping [16–18,25]. The full ChIP-seq workflow comprises three steps: experiment, sequencing and data analysis. As illustrated in Figure 1, the experiment step includes *in vivo* crosslinking, chromatin isolation, fragmentation using sonication with outcome fragments approximately 200–600 bp, protein–DNA complex immunoprecipitation and reverse crosslinking, and DNA fragment purification. If the ChIP experiment aims to map nucleosome position or histone modification, MNase digestion of chromatin instead of sonication is used to localize histone signals to individual nucleosomes [16,25]. The data analysis step includes quality control, reads mapping [22,26], peak calling [27], target gene identification, consensus motif finding, comparative analysis with other ChIP-seq data and data integration (for example with RNA-seq or microarray expression data). The ultimate goal of data analysis is to present and integrate the data in a biologically meaningful way as well as to generate hypotheses, which can be tested by bench scientists.

Like many other technologies, ChIP-seq has its own limits and challenges from both an experimental and analysis point of view. In the experiment stage, for instance, there is a bias towards GC-rich fragments in both library preparation and in amplification before and during sequencing [28,29]. However, even more challenges come at the analysis stage. Such challenges exist in all current genome-wide ChIP techniques, such as ChIP-on-chip (ChIP on a microarray) and DNA adenine methyltransferase identification (DamID) [30]. The observation of tens of thousands of binding regions

in the genome raised the problems of identifying real functional binding sites from all of these candidates, and the assignment of the binding sites to their target genes. It is possible that only a subset of the binding sites is functional in a specific cell line under the specific experimental conditions. It is also likely that some binding sites are indeed nonfunctional [30]. In addition, the assignment of a specific binding site to its target gene is not always straightforward. The expedient approach to assign binding sites to the nearest known gene might give incorrect results in case of long-range regulation and undiscovered genes or alternative upstream promoters. As a result, altering the level of transcription factors in the cell may only affect the expression level of 1–10% of the potential target genes identified by ChIP [31–34]. Although currently it is not possible to accurately link a particular binding site with a specific target gene from a bioinformatics point of view, the recent advent of global chromosome conformation capture (3C) techniques (Hi-C and chromatin interaction analysis by paired-end tag sequencing [CHIA-PET]) may permit the global assignment of binding sites to their target genes [35,36]. However, a couple of challenges are associated with these technologies. First, the resolution of Hi-C remains a problem, whereby 10 million paired reads can only provide one-megabase resolution. Second, although Hi-C is performed at a low DNA concentration to favor intramolecular ligations, random collisions of DNA fragments may still happen. This will introduce considerable noise into the results and make the analysis and interpretation difficult. It is possible that CHIA-PET may detect more random collisions owing to the enrichment of ligated products on beads.

Histone modifications in prostate cancer

Histones are no longer considered to be simple 'DNA-packaging' proteins. They are subject to a large number of posttranslational modifications including acetylation, methylation,
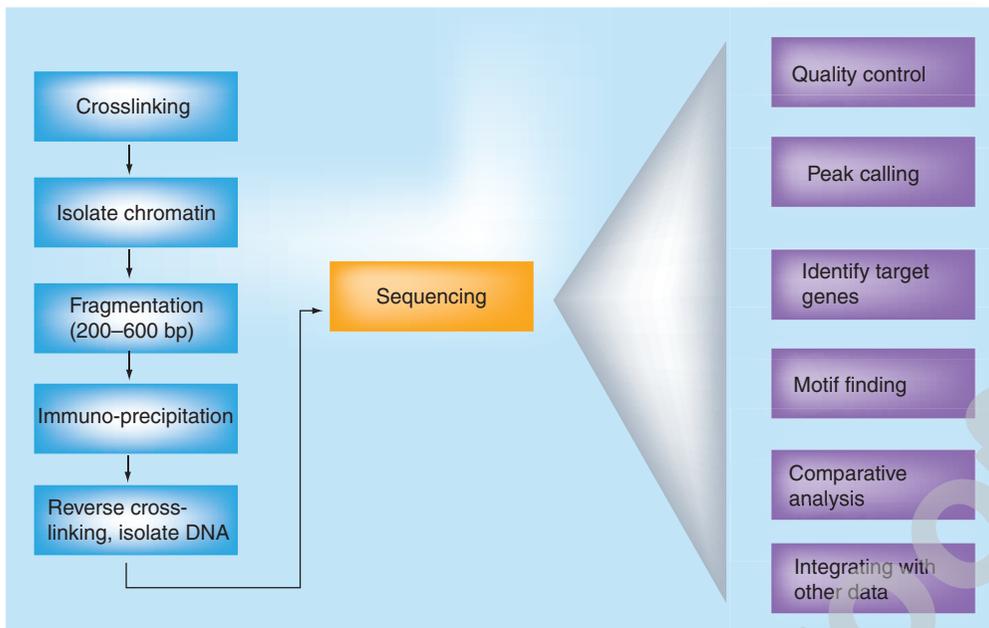
Figure 1. A typical workflow for ChIP-seq. Experimental procedures are presented in blue boxes; sequencing step is indicated in the orange box and is not fully explained; bioinformatics analysis parts are presented in purple boxes. ChIP-seq: Chromatin immunoprecipitation combined with high-throughput sequencing.

phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination and proline isomerization [37]. Among these modifications, histone acetylation and methylation are relatively well studied. Accumulating evidence indicates that the status of acetylation and methylation of specific lysine or arginine residues play crucial roles in regulating gene expression [37,38].

In general, histone acetylation is correlated with transcriptional activation and histone deacetylation is linked to gene silencing [39,40]. For example, the level of acetylated histone H3 (H3Ac) is increased at both the prostate specific antigen (PSA) enhancer and promoter upon androgen treatment, paralleling the accumulation of PSA mRNA levels in the prostate cancer cell line LNCaP [41–43]. Treatment of LNCaP cells with a histone deacetylase inhibitor trichostatin (TSA) promotes RNA polymerase II (Pol II) and H3Ac stability on PSA regulatory regions to augment AR transcription [41,42]. Histone acetylation is mediated by histone acetyltransferases (HAT) [44], several of which have been characterized as AR coactivators. For instance, CBP and p300 (KAT3A and KAT3B) are recruited to PSA regulatory regions following androgen treatment and enhance AR-mediated transcription in LNCaP cells [41,42].

In contrast to histone acetylation, histone methylation on arginine and lysine can be associated with either gene activation or repression [37,38]. For example, histone H3K9 mono-, di- and tri-methylation (H3K9me1, H3K9me2 and H3K9me3) have been linked to repression of AR target genes in LNCaP cells. Since H3K9me1, H3K9me2 and H3K9me3 are linked to repression of AR target genes in LNCaP cells, silencing of H3K9 demethylases LSD1 (KDM1), JHDM2A (KDM3A) or JMJD2C (KDM4C) will increase the levels of these repressive marks on AR target gene regulatory regions. Thus the expression AR target genes *PSA*, *TMPRSS2* and *NKX 3.1* will be decreased [45–47]. By contrast, H3K4 mono- and di-methylation (H3K4me1 and H3K4me2) are associated with AR-mediated gene activation in CRPC cell lines and tissues. H3K4me1 and H3K4me2 are significantly enriched at the AR enhancer of the proto-onco-gene gene *UBE2C* [48] in CRPC, which leads to AR binding and *UBE2C* gene expression [49].

Recent genome-wide mapping of histone modifications has confirmed some previous findings regarding the functions of specific histone modifications. For example, eighteen mapped histone acetylations are all positively correlated with gene expression in human CD4^{+} T cells [18]. Similarly, H3K4 methylations have a general correlation with gene transcription in CD4^{+} T cells [16]. Importantly, large-scale studies have provided new insight into the relationship of histone modifications and gene transcription. First, while it was well known that H3K4 tri-methylation (H3K4me3) and H3K27 tri-methylation (H3K27me3) are associated with

gene activation and repression, respectively, some genomic regions in embryonic stem cells and differentiated cells harbor both of these two marks, namely bivalent domains. These domains are correlated with low levels of gene expression [16,50–52]. Interestingly, bivalent domains are also found in the primary prostate epithelial cell line EP156T and the prostate cancer cell line PC-3. Surprisingly, the bivalent marked genes in these prostate cells are rather active [53]. Second, H3K4me3 is also detected at many silent promoters, suggesting H3K4me3 alone is not sufficient to support active transcription [54,55]. Third, different cells have different chromatin signatures of enhancers and promoters. A ChIP-on-chip analysis of H3K4me1, H3K4me2 and H3K4me3 on 44 human loci selected by the ENCcyclopedia Of DNA Elements (ENCODE) consortium in HeLa cells found that enhancers are marked by H3K4me1, whereas promoters are marked by H3K4me3. In addition, H3K4me2 is present at both enhancer and promoters in varying degrees [56]. These findings were recently confirmed at a whole-genome level in several cell lines [57]. By contrast, ChIP-seq analysis of all three states of H3K4 methylation in CD4+ T cells found that all three states are detected at some enhancers [16,18]. In LNCaP cells, while H3K4me1 is enriched primarily at enhancers and H3K4me3 is located predominantly at promoters, H3K4me2 has the most significant overlap with both enhancers and promoters [25]. Fourth, histone modifications create permissive or nonpermissive chromatin structures for gene activation or repression, but histone modification alone is not sufficient to determine gene expression level [18,55].

Increasing evidence suggests that alterations of histone modifications play important roles during prostate tumorigenesis. For example, we recently found that H3K4me1 and H3K4me2 are selectively enriched at the AR enhancers of M-phase cell cycle genes (e.g., *UBE2C* and *CDK1*) in CRPC cell models and tissues, facilitating AR up-regulation of these cell cycle genes to promote CRPC growth. Overexpression of a specific H3K4me1 and H3K4me2 demethylase KDM1 significantly decreases AR binding [49]. Similarly, another study found that increased H3K4me3 in prostate cancer cells compared with normal prostate cells is correlated with activation of genes involved in cell growth and survival (e.g., *FGFR1* and *BCL2*) [53].

Since the increased active chromatin marks like H3K4 methylations in prostate cancer facilitate activation of proto-oncogene and other genes involved in cell growth and survival, it is conceivable that increased repressive histone marks in prostate cancer lead to tumor suppressor gene silencing. Indeed, genome-wide mapping of H3K27me3 in prostate cancer cell lines and tissues revealed that H3K27me3 genomic distribution patterns change during prostate cancer progression. The specific enrichment H3K27me3 at the promoters of a large number of genes (e.g., tumor suppressor genes *GAS2*, *PIK3CG* and *ADRB2*) in metastatic prostate cancer compared with localized prostate cancer and normal prostate represses the expression of these genes, leading to prostate cancer cell growth, survival and invasion. This may ultimately cause a poor clinical outcome for prostate cancer patients [58–60]. Underlying mechanisms of an increased and broadened H3K27me3 genomic distribution in prostate cancer is attributed to overexpression of enhancer of zeste 2 (EZH2), a histone methyltransferase with substrate specificity for H3K27 [61–63]. Consistent with the role of EZH2 in establishing H3K27me3 chromatin marks, downregulation of EZH2 reactivates expression of some H3K27me3 target genes, resulting in prostate cancer cell cycle block and apoptosis [59,63]. Interestingly, a recent study found that overrexpression of EZH2 in many cancer types including prostate cancer is caused by genomic loss of microRNA-101. Overexpression of this microRNA decreases the expression of EZH2 and enrichment of H3K27me3 at its target genes promoters, leading to decreased growth of prostate cancer cells [64]. In summary, these studies suggest that EZH2 may serve as a therapeutic target, whereas overexpression of microRNA-101 may have therapeutic benefit in prostate cancer.

In addition to genomic location analysis of histone modifications in prostate cancer, immunohistochemistry (IHC) analysis of histone modifications at the level of whole nuclei in prostate cancer tissue specimens has also afforded important insights into the role in alterations of histone modifications during prostate cancer progression. It has been found that global levels of H3K4me2, H3K4me3 and histone H3 lysine 18 acetylation (H3K18Ac) are independent predictors of prostate carcinoma recurrence in patients with low-grade tumors [65,66]. Similarly, the cellular level of H3K9me2 is also associated

with disease outcome, with lower levels predicting poorer prognosis in prostate and other cancers [67]. More recently, the global levels of H3K4me1/2/3 and H3K9me1/2/3, as well as H3 and H4 pan-acetylation have been systematically investigated in nonmalignant prostate tissues and various states of prostate carcinogenesis including clinically localized prostate cancer, ADPC and CRPC. Patients with high H3K4me1 levels are more likely to suffer from tumor recurrence than patients with moderate and low staining. This observation may be explained by the fact that CRPC and ADPC patients show significantly higher levels of H3K4me1 than localized prostate cancer patients. Interestingly, H3K4me1, H3K4me2 and H3K4me3 levels are all significantly increased in CRPC compared with localized prostate cancer [68]. In summary, IHC studies of histone modifications suggest that global histone modification expression pattern changes may serve as prognostic markers in prostate cancer.

Chromatin reorganization in prostate cancer

Although epigenetic regulation of gene expression mainly include two well-known mechanisms, namely DNA methylation and histone modification, the importance of chromatin-based processes (e.g., nucleosomal remodeling and chromosome looping) in epigenetic regulation and maintenance has been increasingly appreciated [69,70].

Assembly, mobilization and disassembly of nucleosomes are key aspects of epigenetic regulation. For example, in normal cells remarkable nucleosome depletion was found in the promoter regions of a hypomethylated tumor suppressor gene *MLH1*, whereas in cancer cells the inactive hypermethylated *MLH1* promoter is associated with nucleosome occupancy in a mitotically heritable fashion. Importantly, DNA demethylation of the *MLH1* gene causes nucleosome eviction and transcriptional activation, providing strong evidence that DNA methylation-mediated epigenetic silencing of tumor suppressor genes may involve the insertion of nucleosomes into previously vacant positions [71]. In addition to its ability to control transcriptional initiation, nucleosome positioning has a profound effect on transcriptional elongation. A genome-wide analysis of histone H2A.Z ChIP-seq data and methylated DNA-seq data in human T cells identified distinct epigenetic peaks of nucleosomes

and DNA methylation enriched at both ends (i.e., just downstream of start codons and just upstream of stop codons) of protein coding units. Interestingly, elongating Pol II tends to pause near these two epigenetic ends, causing a significant reduction in elongation efficiency [72]. Such an epigenetic inhibition of Pol II elongation may facilitate the inclusion of constitutive exons during RNA splicing [73,74]. Altogether, these studies suggest that epigenetic regulation of gene expression may require specific nucleosome positioning patterns.

Since a growing body of evidence has revealed that nucleosome positioning changes correlate with alterations in gene expression, many studies have been focused on nucleosome positioning around genes, especially at transcription start sites (TSS) or in the transcribed regions. A more recent study has found that H3K4me2 marked nucleosome positioning at distal AR binding regions plays a critical role in regulating androgen-regulated genes (e.g., *PSA* and *TMPRSS2*) in prostate cancer cells [25]. In LNCaP cells, where AR is primarily located at distal regions [49,75–78], androgen treatment dismisses a central nucleosome at the AR binding site that is flanked by two well-positioned H3K4me2-marked nucleosomes, leading to AR transcription complex binding and target gene activation. The central nucleosome is more labile than the flanking ones as it contains the H2A.Z variant and a higher A/T content [25]. Although the finding that nucleosome positioning at enhancer regions influences AR activity significantly increases our understanding of how androgen regulates gene expression in prostate cancer cells, at this point it is not known which chromatin remodelers recognize H3K4me2 to cause nuclesome repositioning upon androgen treatment [79]. Addressing this issue in the future will allow understanding upstream regulatory mechanisms of androgen-mediated gene expression.

Chromosomal looping affects gene expression within the 3D context of nuclear architecture [80,81]. The discovery that epigenetic silencing of imprinted genes requires long-range interactions between regulatory elements suggests that chromosome looping is a potential novel epigenetic regulatory mechanism [82–84]. Using a GAL4 knock-in approach as well as the 3C technique that is used for detecting looping interactions between genes and their distal regulatory elements [85], Murrell *et al.* have demonstrated that the intrachromosomal interactions between the differentially methylated

regions in the imprinted genes *Igf2* and *H19* in mice are epigenetically regulated and partition maternal and paternal chromatin into distinct loops. These loops are changeable depending on their epigenetic state, enabling *Igf2* to move between an active and a silent chromatin domain [83]. Further studies have found that binding of the 11-zinc-finger protein CTCF is required for these inherited higher-order chromatin conformation [84]. Importantly, CTCF also recruits polycomb repressive complex 2 (PRC2), leading to allele-specific methylation at lysine 27 of histone H3 (H3-K27) and to suppression of the maternal Igf2 promoters [82]. Altogether these studies indicated that higher-order chromatin structure exists in an imprinting cluster, and the chromosomal loops constitute an important element in the epigenetic regulation of imprinted gene expression.

Increasing evidence suggests that chromosomal looping is important in AR-mediated gene regulation in prostate cancer. Recent genome-wide ChIP analysis revealed that most AR binding sites in prostate cancer cells are far away from the promoters of androgen regulated genes [49,75–78]. This raised the question as to how these distal AR binding sites communicate with their target gene promoters. Recent 3C studies

in prostate cancer cells supported the formation of chromosomal loops between distal AR binding sites and proximal promoters of a few target genes including *PSA*, *TMPRSS2* and *UBE2C* [41,49,77]. The recently developed genome-wide 3C assays (Hi-C and CHIA-PET) will allow us to map AR-mediated global looping in the near future [35,36].

The role of AR-mediated looping in prostate cancer is not limited to the regulation of target gene expression. In the past few years, some high-frequency recurrent gene fusions including the TMPRSS2-ETS family were identified in a majority of prostate cancer patients [86,87]. Interestingly, two recent studies found that the AR is capable of facilitating the formation of gene fusions in prostate cancer cells through inducing chromosomal looping that brings tumor translocation partners into close spatial proximity, and recruiting genotoxic stress-induced enzymes such as activation-induced cytidine deaminase (AID), which contribute to DNA breakage [88,89]. These studies convincingly challenge the prevalent concept that genomic translocations in cancer are stochastic events and the enrichment of specific translocations is a result of growth advantage selection, but also raises an important question: given that several thousands of AR binding sites have been identified from genome-wide ChIP analysis, why do these genomic translocations only occur in limited regions? One possible answer is that specific DNA methylations and histone modifications at particular AR binding sites also contribute to tumor translocations.

Conclusion

Histone modifications, nucleosomal remodeling, and chromosomal looping are important epigenetic mechanisms that regulate gene expression in prostate cancer (FIGURES 2 & 3). The change in the repressive histone mark H3K27me3 and the increased expression of H3K27me3 methyltransferase EZH2 leads to the silencing of tumor suppressor genes (e.g., *GAS2* and *ADRB2*), whereas both the genomic position alteration and protein expression of active histone mark H3K4 methylation contribute to activation of proto-oncogene (e.g., *UBE2C*). Nucleosome repositioning is not only involved in the silencing of tumor suppressor genes (e.g., *MLH1*), but also involved in the activation of genes related to prostate cancer progression (e.g., *PSA* and *TMPRSS2*). AR-induced chromosomal looping is important for AR target gene regulation and facilitates recurrent gene fusions in prostate cancer.
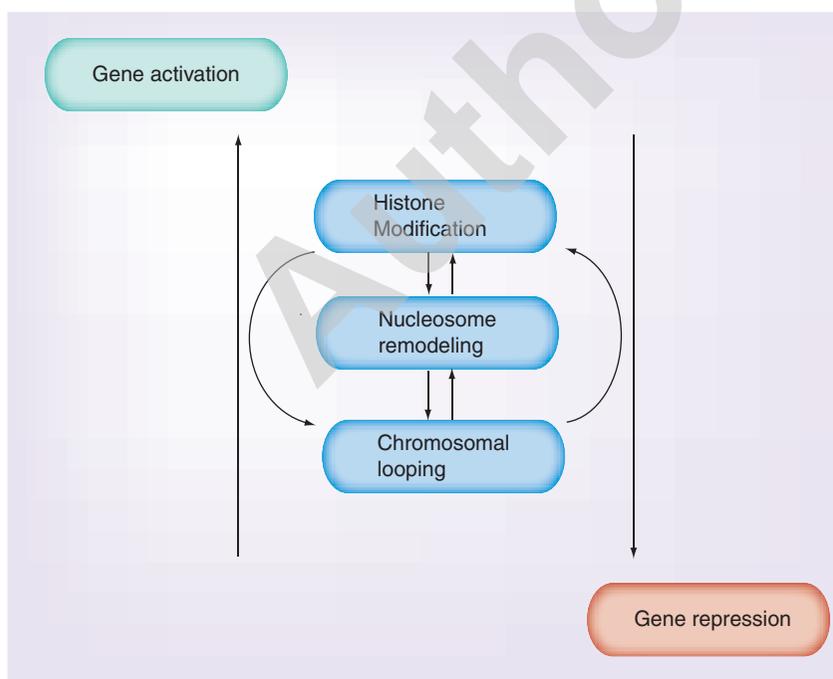
Figure 2. The interplay among histone modification, nucleosome remodeling and chromosomal looping leads to genes activation or repression in prostate cancer. For example, a histone modification may be recognized by a chromatin remodeler that causes nucleosome ejection. This may lead to a transcription factor binding, chromosomal looping and gene activation (see FIGURE 3).

Future perspective

Genome-wide approaches are now available for assessing histone modification patterns in prostate cancer. Combining genomic maps and IHC analysis of histone modifications changes in prostate cancer will allow us to use histone modifications as diagnostic and prognostic markers in prostate cancer. In addition, future studies should identify more upstream mechanisms responsible for alterations of histone modifications, which will have therapeutic benefit in prostate cancer. Nucleosome repositioning has been implicated in prostate tumorigenesis. A future challenge will be to identify upstream regulators of nucleosome repositioning. Thanks to recent technological advances of global 3C assays, this would allow global identification of direct AR target genes involved in prostate carcinogenesis in the near future. Finally future studies should integrate global epigenetic analysis (e.g., 3C assay, DNA methylation assay, nucleosome positioning mapping and histone modification mapping) to identify more molecular triggers of genomic translocations in prostate cancer. In summary, future studies in epigenetic regulation will have obvious translational implications in the identification of new biomarkers and the development of new therapies in prostate cancer.
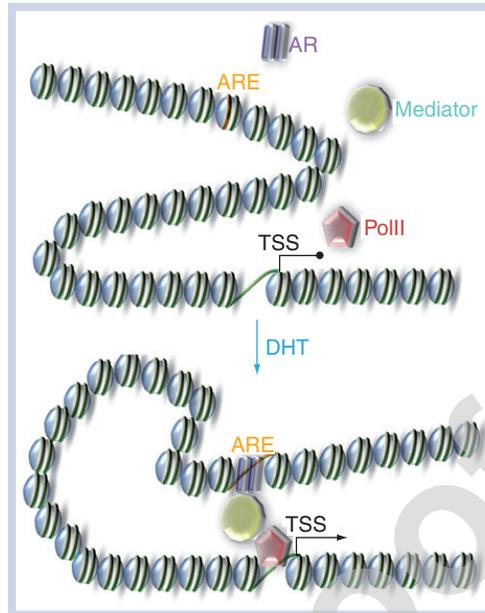
Figure 3. Androgen-induced nucleosome repositioning and chromosomal looping. In the absence of a natural androgen dihydrotestosterone (DHT), the androgen receptor (AR) does not bind to an androgen responsive element (ARE) on chromatin. DHT treatment dismisses a central nucleosome at the distal AR binding site, leading to AR transcription complex (e.g., AR–mediator complex) binding at the enhancer, RNA polymerase II recruitment to the promoter, chromosomal looping and target gene activation.
AR: Androgen receptor; ARE: Androgen responsive element; DHT: Dihydrotestosterone; PolII: RNA polymerase II; TSS: Transcription start site.

Executive summary

Technological advances: histone modifications and nucleosome positioning mapping
- High-throughput sequencing (HTS)-based measurements offer several advantages over hybridization-based microarray analysis.
- Chromatin immunoprecipitation (ChIP) combined with HTS (ChIP-seq) is a powerful technique to map histone modifications and nucleosome positioning in the genome with some limitations.

Histone modifications in prostate cancer
- Histone acetylation is generally correlated with transcriptional activation, whereas some histone methylation on arginine and lysine are associated with gene activation while others are associated with repression.
- Recent genome-wide mapping of histone modifications have afforded new insight into the relationship between histone modifications and gene transcription.
- Increasing evidence suggests that alterations in both genomic distribution and global level of histone modifications play important roles during prostate tumorigenesis.

Chromatin reorganization in prostate cancer
- In addition to DNA methylation and histone modifications, nucleosome repositioning and chromosomal looping are important epigenetic regulation mechanisms.
- Nucleosome repositioning plays a critical role in the regulation of androgen-stimulated genes in prostate cancer.
- Chromosomal looping is important for distal binding androgen receptor-mediated gene regulation and facilitates recurrent gene fusions in prostate cancer.

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