Determinants of Receptor- and Tissue-Specific Actions in Androgen Signaling

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The physiological androgens testosterone and 5α-dihydrotestosterone regulate the development and maintenance of primary and secondary male sexual characteristics through binding to the androgen receptor (AR), a ligand-dependent transcription factor. In addition, a number of nonreproductive tissues of both genders are subject to androgen regulation. AR is also a central target in the treatment of prostate cancer. A large number of studies over the last decade have characterized many regulatory aspects of the AR pathway, such as androgen-dependent transcription programs, AR cistromes, and coregulatory proteins, mostly in cultured cells of prostate cancer origin. Moreover, recent work has revealed the presence of pioneer/licensing factors and chromatin modifications that are important to guide receptor recruitment onto appropriate chromatin loci in cell lines and in tissues under physiological conditions. Despite these advances, current knowledge related to the mechanisms responsible for receptor- and tissue-specific actions of androgens is still relatively limited. Here, we review topics that pertain to these specificity issues at different levels, both in cultured cells and tissues in vivo, with a particular emphasis on the nature of the steroid, the response element sequence, the AR cistromes, pioneer/licensing factors, and coregulatory proteins. We conclude that liganded AR and its DNA-response elements are required but are not sufficient for establishment of tissue-specific transcription programs in vivo, and that AR-selective actions over other steroid receptors rely on relaxed rather than increased stringency of cis-elements on chromatin. **(*Endocrine Reviews* 36: 357–384, 2015)**

I. Introduction

Androgens orchestrate the development of male phenotype and serve as important physiological regulators in many nonreproductive tissues of both genders.

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Abbreviations: AF, activation function; AP, activating protein; AR, androgen receptor; ARB, AR-binding site; ARE, androgen response element; ChIP, chromatin immunoprecipitation; ChIP-seq, ChIP-sequencing; CPA, cyproterone acetate; CRPC, castration-resistant prostate cancer; DBD, DNA-binding domain; Dex, dexamethasone; DHT, 5α-dihydrotestosterone; ER, estrogen receptor; eRNA, enhancer RNA; GR, glucocorticoid receptor; GRE, glucocorticoid response element; LBD, ligand-binding domain; LSD1, lysine-specific demethylase 1; MENT, 7α-methyl-19-nortestosterone; MMTV, mouse mammary tumor virus; MR, mineralocorticoid receptor; PR, progesterone receptor; PSA, prostate-specific antigen; SARM, selective AR modulator; SPARKI, specificity affecting AR knock-in mice; SRC, steroid receptor coactivator; T, testosterone.

Many developmental events in males require androgens during a defined time window, whereas reproductive and nonreproductive functions are androgen-dependent throughout the life. The androgen receptor (AR), the mediator of androgen action, belongs to the nuclear receptor superfamily with specified structural domains that define their molecular actions as ligand-inducible transcription factors.

The essential function of the androgen-activated AR is to bind to its response elements at regulatory regions of AR target genes and to activate or repress their transcription in collaboration with coregulatory proteins and transcription machinery (1). The overall picture of nuclear receptor action involves, however, interplay of multiple signaling pathways in the particular cellular environment where the action is executed. Cellular steroid metabolism together with expression pattern and functional consequence of coregulators and other collaborating proteins define the context-specific transcriptional outcome. Post-transcriptional modifications alter activities of the receptors and create the chromatin landscape that regulates all DNA-templated processes. Cellular actions of androgens are further amplified through secondary effects when primary androgen target genes regulate new sets of genes or modulate other signaling pathways, for example, by inducing changes in signaling by other hormones, such as the GH (2).

This review focuses on androgen-regulated gene expression at four levels, with a particular emphasis on the potential mechanisms that are mandatory for receptor- and tissue specificity of the AR pathway (Figure 1). The four levels to be reviewed are: steroid–receptor interaction, receptor–chromatin interaction, pioneer/licensing factors in androgen signaling, and coregulatory proteins as AR modulators. This multipartite regulation forms the basis for tissue specificity in androgen signaling. Although the ligand, the receptor, and the cis-element on chromatin

Figure 1.

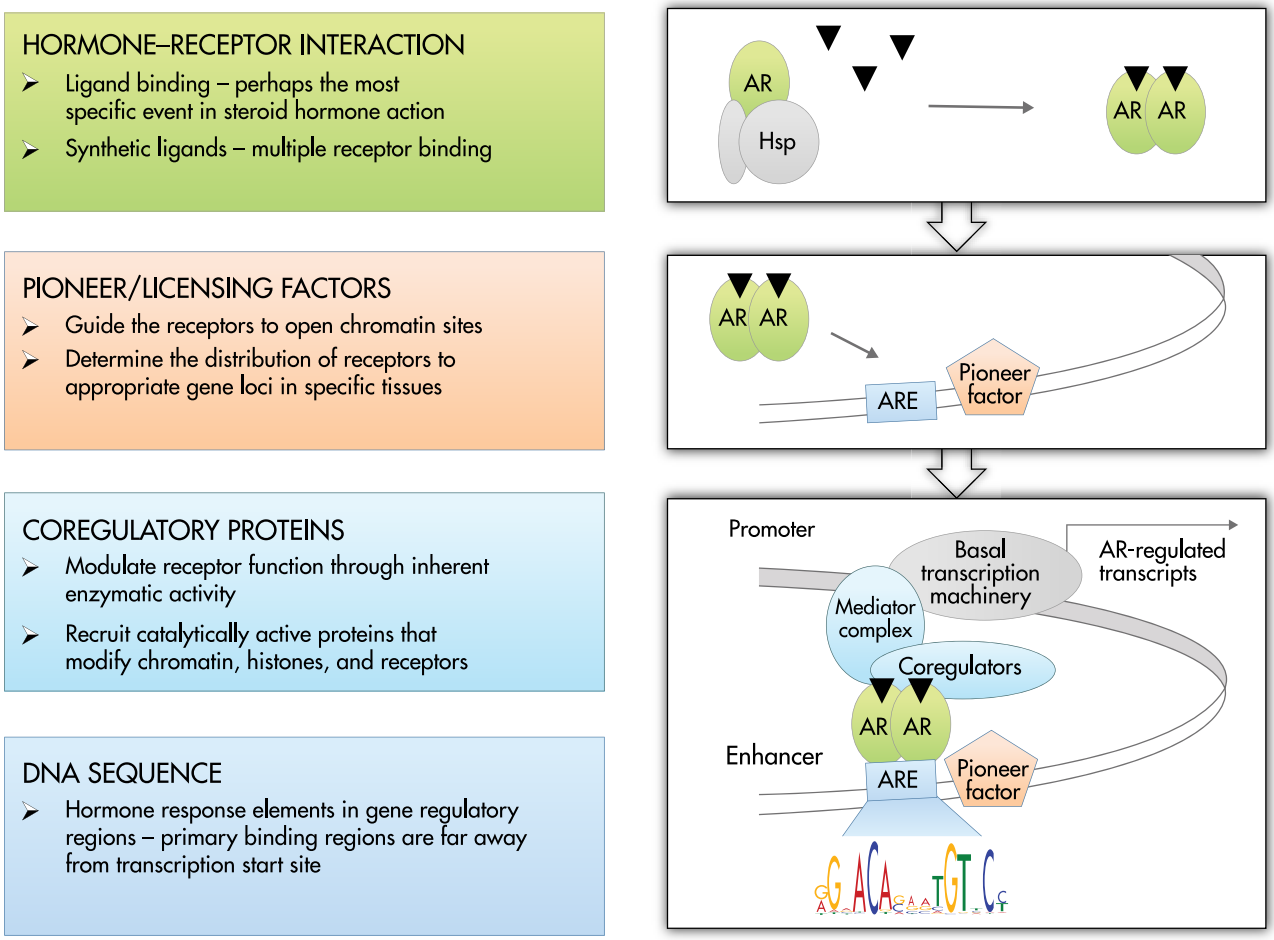


Figure 1. Multipartite physiology of the AR pathway. Each of the four regulatory levels is described in detail in the text. ARE, androgen response element; Hsp, heat shock proteins.

are all mandatory for the androgen action to take place, they are not sufficient to ensure that the regulation of the AR pathway is executed in a tissue-specific fashion and at appropriate genetic loci. The current knowledge of the extranuclear, cell membrane-initiated actions of androgens (3) in relation to receptor- and tissue-specific regulation of the AR pathway is quite rudimentary, and therefore, this review focuses on nuclear actions of androgens.

II. Steroid–Receptor Interaction

A. Physiological steroids vs cognate receptors

Under normal physiological conditions, different classes of steroids initiate distinct actions via binding to their cognate receptor proteins. The specificity of the ensuing biological response relies on a number of characteristics inherent to the steroid–receptor interaction. First, the hormone-binding specificity of all steroid receptors is fairly strict, in that the receptors bind their cognate physiological steroids with affinities usually at least one order of magnitude higher than those for other classes of steroids (4, 5). Second, the binding affinity of a given receptor is commensurate with the concentration of its physiological ligand, such that circulating steroid concentrations are close to those required for half-maximal saturation of the receptor's binding site (4). Third, physiological steroid metabolism does not usually generate metabolites that are biologically more potent than the parent compound. And finally, the formation of biologically active steroid–receptor complexes—and the ensuing biological response—is dictated by the law of mass action, and a low receptor content permits formation of functionally insufficient amounts of receptor complexes in physiological ligand concentrations (4, 6). Androgen signaling deviates to some extent from these general rules, in that testosterone (T) concentration in male serum exceeds the equilibrium binding affinity of AR by some 10-fold (7), and that conversion of T to 5α-dihydrotestosterone (DHT) in certain target tissues results in the formation of an androgen that has five to 10 times higher binding affinity for AR than the circulating hormone (4, 8).

Of all the phases in the execution of androgen signaling under physiological conditions, the interaction of AR with its cognate ligand appears to be the most specific event. Deviations from this strict specificity in steroid action occur mainly from two reasons: first, a change from a physiological to pharmacological ligand concentration; and second, modulation of the structure of the steroid that binds to the receptor. In the case of androgens, both natural and synthetic androgens in high enough doses can potentially interact with several nuclear receptor systems (4, 9). In most instances, the interaction of androgens with receptors other than AR leads to expression of biological responses characteristic of the receptor rather than the androgen. These issues are exemplified in more detail below, in the text dealing with selective AR modulators and androgens as progestins.

The human *AR* gene is expressed fairly early during fetal development (10), and likewise, T synthesis in human testis commences during the first 8–10 weeks of gestation (11). Because androgens—T and/or DHT—are required for both male sexual differentiation and masculinization of the internal and external genitalia, it has been somewhat difficult to understand how this can be accomplished in the presence of only a single *AR* gene that encodes a single AR protein. Although two AR isoforms—the full-length receptor and an amino-terminally truncated form— have been described to exist in normal human tissues (12), there is no solid evidence to indicate that they regulate distinct biological processes. Likewise, there is no clear evidence that the expression of the *AR* gene is programmed in such a fashion as to play an important regulatory role.

The development of male phenotype is an intriguing example of a tissue-specific role that physiological steroids might play. The AR-regulated genes responsible for male sexual development during fetal life must be highly sensitive to androgens. In other words, during embryonic development, locally produced T complexed to AR is sufficient to load enough holo-AR to the regulatory regions of the genes responsible for the differentiation of Wolffian ducts to epididymis, vas deferens, and seminal vesicles. By contrast, genetic networks guiding the development of accessory sex organs, such as the prostate, and masculinization of the external genitalia require loading of a high amount of the holo-AR— occupied by DHT, the formation of which is catalyzed by 5α-reductase type-2 enzyme (13)— onto the regulatory regions and thus are less sensitive to androgens. Because the formation of the androgen–AR complex follows the law of mass action, the higher circulating androgen concentrations during pubertal development and adulthood support the formation of a larger amount of holo-AR complexes without a requirement for a change in *AR* gene expression. A corollary to this notion is that in the 5α-reductase type-2 deficient patients, whose target tissues do not contain DHT, the external genitalia grow and masculinize in a holo-AR-dependent fashion only after maximal testicular T production has been achieved (14). Thus, high circulating T level is able to substitute for the absent DHT in these patients.

B. Androgens and selective androgen receptor modulators

Prior to the discovery that there is only a single AR protein, it was tacitly assumed that androgenic and anabolic effects of androgens can be dissociated, meaning that, in comparison to T, some androgens are more anabolic than androgenic. The singularity of AR, as is currently known, implies that all androgenic steroids elicit their actions through the AR protein, and that the terms anabolic or anabolic–androgenic steroids are, in fact, misnomers, and their use is no longer recommended (15). In view of this, current efforts to develop selective AR modulators (SARMs) must be based on biological principles other than the presence of more than one AR protein or tissue-specific differences in the receptor's ligand-binding affinity. In comparison to selective estrogen receptor (ER) modulators (16, 17), the progress in the development of clinically useful SARMs has clearly been lagging behind.

There are a number of clinical conditions in which tissue-specific androgen action brought about by selective androgen ligands (SARMs) could be useful, in particular, to avoid unwanted effects of male sex steroids on prostate, central nervous and cardiovascular systems, and lipids. Conditions for therapy with SARMs include, but are not limited to the following: muscle wasting (sarcopenia), osteoporosis, and frailty in elderly people; cachexia; male contraception (suppression of LH and FSH secretion); treatment of some forms of breast cancer (18, 19); hormone replacement therapy in male hypogonadism; and andropause—should the latter condition indeed exist (see Ref. 20).

A major goal in the development of SARMs has been to synthesize steroidal and/or nonsteroidal androgens that have, in relative terms, diminished efficacy to stimulate growth of the prostate gland and potentially prostate cancer development ("prostate-sparing effect"). Owing to the high 5α-reductase activity in prostate tissue, the potency of circulating T is amplified in prostate through its conversion to DHT that is five to 10 times more potent than T (7, 8). As a consequence, steroidal androgen derivatives that are not substrates of the 5α-reductase enzyme are potentially useful SARMs. For example, 7α-methyl-19-nortestosterone (MENT) (Figure 2), which is not a 5α-reductase substrate—possibly owing to the hydrophobic substituent in the B-ring—exhibits markedly increased biopotency in muscle growth and gonadotropin suppression relative to prostate growth both in rodents and in nonhuman primates (21, 22). Importantly, MENT also binds well to the progesterone receptor (PR) (9) (see below), and this property has been useful in male contraception (23).

Apart from avoiding 5α-reduction in prostate, development of SARMs has been based on assumptions that: 1) they modulate the ligand-binding domain (LBD) of AR in a manner that promotes differential coactivator interactions in a tissue-specific fashion; and 2) they do not induce interaction between amino- and carboxyl-terminal regions of AR in a way similar to that of the physiological androgens (24, 25). A corollary to these assumptions is that modifications of the AR structure by a SARM would lead to commensurate changes in transcription programs that, in turn, would be dependent on the compilations of coactivators in different tissues. Although a large number of nonsteroidal SARM-like compounds that bind to the ligand-binding pocket of AR have been synthesized and studied in various cell culture and in vivo systems (eg, Refs. 24, 26–28; see also Refs. 29–31), the progress in this field has been modest at best, especially in terms of the emergence of SARMs useful in the clinic. The same applies to small molecular compounds that potentially interact with AR in regions other than the ligand-binding pocket (32). Although a number of compounds with varying androgenic effects have entered initial clinical trials, their potential side effects as well as in vivo efficacy in humans need to be carefully assessed (33). Taken together, rational drug design for the development of novel SARMs will obviously require more comprehensive understanding of the mechanisms that are responsible for the receptor- and tissue-specific regulation of the AR pathway.

C. Modified androgens as progestins

As mentioned above, deviations from the strict binding specificity of physiological steroids to their cognate receptors occur as soon as the steroid structure is altered. With regard to the AR pathway, there is a wealth of information indicating that progestins can mimic, inhibit, and potentiate the effects of androgens (4, 34, 35). On the other hand, androgens can mimic the actions of progestins via mechanisms independent of the AR (4, 9). The best-known progestins that modify AR function are medroxyprogesterone acetate and cyproterone acetate (CPA) (Figure 2); both act as partial agonists/antagonists of the AR pathway (4, 34). Although these two C21 progestins appear to be incapable of promoting interaction between the amino and carboxyl termini of AR (34), there is no evidence to indicate that this property would lead to tissue-specific modulation of the AR pathway.

Synthetic steroidal androgens, such as MENT, 7α,17α-dimethyl-19-nortestosterone (mibolerone), and methyltrienolone (R1881) (Figure 2), bind all with high affinity not only to the AR but also to the PR (5, 36, 37). The binding affinity of mibolerone and methyltrienolone for human and rabbit PR is similar to or higher than that of the

Figure 2.

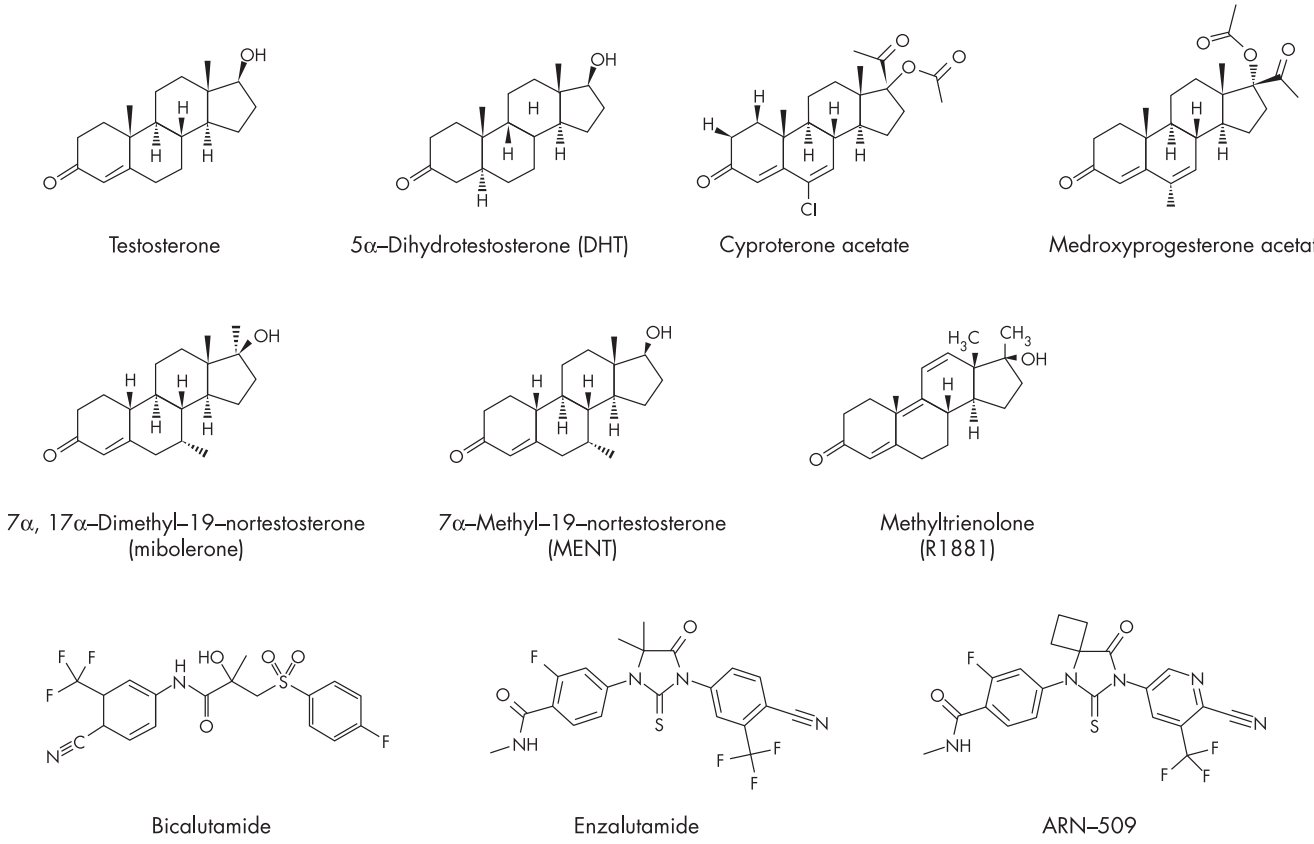


Figure 2. Structures of the two physiological androgens T and DHT, three progestational androgens (7α-methyl-19-nortestosterone, mibolerone, and methyltrienolone), two steroidal androgen agonists/antagonists (cyproterone acetate and medroxyprogesterone acetate), one first-generation antiandrogen (bicalutamide), and two second-generation nonsteroidal antiandrogens (enzalutamide and ARN-509).

cognate physiological ligand, progesterone (5, 36). Likewise, synthetic steroidal androgens are capable of eliciting biological actions in vivo, such as uteroglobin synthesis in rabbit endometrium, that are mediated by PR and inhibited by concomitant administration of an antiprogestin, but not by an antiandrogen (9, 36). In view of these data, it is important to realize that some tissue-specific actions of synthetic androgens—potentially those of some nonsteroidal SARMs as well—are, in fact, mediated by a "wrong" receptor. Likewise, the investigators using mibolerone or methyltrienolone as substitutes for physiological AR ligands ought to keep in mind the possibility that PR may mediate some of the ensuing biological responses.

In addition to PR, high doses of steroidal and nonsteroidal androgens may also interfere with glucocorticoid signaling and exert mainly inhibitory actions (5, 9, 31).

D. Antiandrogens

AR remains an important therapeutic target in the treatment of metastatic prostate cancer, and steroidal or nonsteroidal AR inhibitors—commonly known as antiandrogens, such as cyproterone acetate, flutamide, nilutamide, and bicalutamide (Figure 2)—have been used for several decades for this purpose (33). Typically, all of these compounds antagonize AR function by competing with T or DHT for binding to the LBD of AR. Antiandrogens are also potentially useful in the treatment of certain forms of breast cancer (18, 19). In the case of prostate cancer, despite initially favorable responses, most patients will develop resistance to the above-mentioned antiandrogens, resulting in the emergence of a condition termed castration-resistant prostate cancer (CRPC) that marks the lethal progression of the disease. Despite resistance to androgen deprivation therapy, CRPC cells often remain addicted to AR signaling, with the AR being able to regulate a distinct transcription program in CRPC cells (38). To overcome the resistance, a number of new AR-targeting antiandrogens have been synthesized, tested in appropriate model systems, and shown to be effective in the clinic (39, 40).

The second-generation antiandrogen enzalutamide (MDV3100; Figure 2) binds to AR with higher affinity

than previous nonsteroidal antiandrogens, reduces nuclear translocation of AR, and impairs both DNA binding and coactivator recruitment (41). A related compound, ARN-509 (Figure 2), was subsequently developed and shown to exhibit antitumor activity in CRPC patients (42). Fairly soon after the introduction of these two second-generation antiandrogens, there were reports on resistance to them that is formed either by missense mutations in the *AR* gene, leading to an amino acid substitution (F876L) in the AR LBD (43), or by formation of truncated AR variants (44, 45) (see below). Recently, phase 1–2 clinical results of ODM-201, a new orally active antiandrogen that also inhibits nuclear translocation of AR, were reported (46). An intriguing and mechanistically dissimilar antiandrogen is ASC-J9, a dimethyl derivative of the natural product curcumin, which is supposed to promote AR degradation in selective cell types as opposed to competing for LBD binding (47) and to possess better efficacy than enzalutamide or bicalutamide in suppressing prostate cancer cell invasion (48). The mechanisms by which ASC-J9 promotes AR degradation are not fully understood. Other novel antiandrogens under development include, for example, compounds that target the binding function 3 site on the surface of the AR LBD (49), the amino-terminal region of AR (50), and the DNA-binding domain (DBD) of AR (51). The extent to which these compounds will eventually contribute, when administered either alone or in combination with other drugs, to the treatment of CRPC or breast cancer patients awaits further studies.

III. Receptor–Chromatin Interaction

A. Overview of steroid receptor cistromes

The traditional view of steroid hormone signaling held that a steroid binds to its cognate aporeceptor, followed by holoreceptor translocation to the nucleus and binding to the proximal promoter region of a target gene, thereby initiating events leading to transcriptional regulation. Over the past decade, introduction of methods to study steroid receptor interaction with chromatin on a genome-wide scale using chromatin immunoprecipitation (ChIP) has enabled revision of this model and greatly advanced our understanding of gene regulation. These studies revealed that steroid receptors occupy thousands of regulatory regions within the genome— but far away from proximal promoters—and highlighted several novel features in steroid receptor signaling, such as the distal mode of regulation and the prominent role of collaborating transcription factors in transcriptional outcome.

In ChIP assay, a specific antibody against the protein of interest is used to enrich a transcription factor or another chromatin-associated protein from cross-linked chromatin. Subsequent sequence analysis of the coprecipitated DNA reveals the genomic loci of protein–DNA interaction (52). The first genome-wide technology, ChIP-on-chip, utilized genomic microarrays for hybridizing and identifying coimmunoprecipitated DNA with tiled probes designed to cover regions representative of the curated genome (53). However, it was not until the ChIP-sequencing (ChIP-seq) technology—in which the immunoprecipitated DNA is deep-sequenced using massively parallel next-generation sequencing—was introduced, when production of unbiased and quantitative digital maps for DNA-binding proteins became feasible under numerous experimental conditions (54, 55). Cistrome is a term that was coined along with the ChIP-seq technology to define the entire set of cis-acting targets of a trans-acting factor on a genome-wide scale in a given cell type. Various ChIP-seq studies over the past decade, including those of the ENCODE Consortium, have provided a comprehensive view of genomic regulatory elements, transcription factor networks, and the chromatin landscape associated with gene regulation, drawing attention to the versatility and abundance of regulatory elements throughout the genomic sequence (56–58).

ChIP studies on prostate-specific antigen (PSA) regulation suggested that AR binds primarily to the PSA enhancer rather than to the promoter region (59, 60), and the first chromosome- and genome-wide maps of ER and AR loading onto chromatin revealed that most of their binding sites localize far away from transcription start sites and proximal promoters (61–63). Numerous subsequent ChIP-on-chip and ChIP-seq studies confirmed that the distal regulation is the primary mode of action for all nuclear receptors, and that approximately 86–96% of AR-binding sites (ARBs) identified in various prostate cancer cell lines and androgen-responsive tissues are located at nonpromoter regions (38, 64–68). Chromosome conformation capture assays demonstrated further that AR-bound enhancers communicate with promoters of androgen-regulated genes, such as *PSA*, *TMPRSS2*, *FKBP51*, and *UBE2C*, by looping out the intervening DNA (60, 63, 69, 70). Loop formation is dependent on chromatin architecture, and it can be facilitated by coregulators, such as the Mediator complex, and stabilized by cohesin (69, 71). Of note, the DHT-induced chromatin looping is also believed to be responsible for the formation of androgen-responsive gene rearrangements, leading to expression of fusion proteins, such as TMPRSS2-ERG (72, 73), a recurrent feature of prostate carcinogenesis found in 50% of the prostate cancer patients (74). These rearrangements require androgen-induced co-recruitment of AR and topoisomerase IIβ to the sites of *TMPRSS2-ERG* genomic

breakpoints, followed by formation of double-strand breaks and involvement of DNA repair machinery to generate illegitimate recombinations and rearrangements between *TMPRSS2* and *ERG* (75).

Another prominent novel feature of steroid receptor signaling was discovered upon analysis of DNA sequences of the genomic loci bound by the receptors. De novo and enrichment analyses identified the presence of cis-elements for several collaborating transcription factors, such as GATA-2, FOXA1, and ETS1, within the steroid receptor binding sites, and ChIP studies confirmed co-occupancy of these factors with steroid receptors (63, 76–79). Subsequently, additional collaborating factors have been described in different contexts of transcription regulation, and versatile roles for these collaborating and/or pioneer/licensing factors have emerged in the modulation of steroid hormone action, as discussed in detail in *Section IV*.

Gene expression profiling using microarray technology—and more recently RNA-seq—is widely used to examine compilations of androgen-responsive genes under various conditions, and the reported numbers for differentially expressed transcripts upon androgen exposure range from a few hundred to up to 3000 (80–83). Meta-analysis of nine gene expression studies in androgen-treated LNCaP cells revealed that more than 1000 genes have been reported in at least two independent studies, among which a core set of over 200 genes have been shown to be androgen-regulated in more than four independent studies in this prostate cancer cell line (84). In three murine androgen-responsive tissues—prostate, epididymis, and kidney—androgens regulate expression of approximately 500–800 transcripts; importantly, a great majority of them are tissue-specific, and only a few genes are regulated in a similar fashion in all three tissues (66). Of note, in vivo ChIP-seq profiling of AR-binding events in the same tissues indicated that each of the three AR cistromes comprises approximately 10 000 to 40 000 high-confidence genomic ARBs, a remarkable majority of which are tissue-specific and commensurate with the androgen-dependent transcript profile in each tissue (66). These results underscore the context-dependent specificity of the gene regulatory landscape in the AR pathway.

B. Response elements: shared and selective for AR

The DBDs of nuclear receptors comprise two zinc fingers and a carboxyl-terminal extension region and are responsible for the receptor–DNA interaction. Several amino acids throughout the DBD make nonspecific contacts with the DNA backbone, and only a few amino acids in the so-called P box of the DNA recognition helix in the first zinc finger are responsible for the sequence-specific DNA contacts (85, 86). The sequence and structure of the DBDs are highly conserved throughout the nuclear receptor superfamily. Identical P boxes of glucocorticoid receptor (GR), mineralocorticoid receptor (MR), AR, and PR allow them to bind to similar hormone response elements—inverted repeats of the 5′-AGAACA-3′ consensus sequence with a 3-nucleotide spacer—whereas ER binds to a different cis-element due to its dissimilar P box (87).

In transient transactivation assays, AR, GR, MR, and PR are all capable of activating reporter genes through the canonical androgen response element (ARE)/glucocorticoid response element (GRE) (88). The first androgen-selective response elements identified at regulatory regions of the mouse *sex-limited protein* and the rat *probasin* genes implied that differences in the cis-element sequence contribute to the receptor-specific DNA binding (89–91). Sequence analyses combined with transactivation experiments and EMSAs with naked DNA suggested that selective AREs—that is, those that are recognized by AR but not by GR—are direct repeats of the 5′-AGAACA-3′ consensus sequence (92, 93). Specific mutations changing the sequence of these AREs toward an inverted repeat strongly enhanced glucocorticoid sensitivity of the respective enhancers (94). Crystallographic studies showed that the AR DBD dimer binds to direct repeats in a head-to-head conformation that is typical of all steroid receptors. In comparison to GR, PR, and MR, the AR DBDs make tighter contacts in their homodimerization interface due to additional hydrogen bonds introduced by serine instead of glycine residues in the second zinc finger of the AR DBD (85, 95). Mutations in the serine residue (Ser597) involved in the dimerization of the AR DBD have been described in partial androgen insensitivity patients (96–98), suggesting that the strong protein–protein interaction (dimerization) may account for different DNA-binding specificities of the receptors.

De novo cis-elements identified in the analyses of chromatin binding sites for AR, PR, GR, and MR highly resemble the canonical ARE/GRE (67, 79, 81, 99–104). However, the genome-wide distribution of binding sites, as well as transcription regulatory programs and in vivo effects of the four receptors, are distinct, which raises the question as to how steroid receptor binding specificity is determined in vivo on native chromatin. In vitro assays using naked DNA and mutated receptors demonstrated that the second zinc finger and the carboxyl-terminal extension region of the AR DBD contribute to the differential DNA binding of AR and GR in vitro (105, 106). To examine the determinants of selective AR binding in a native chromatin environment in vivo, a transgenic knock-in mouse model was generated. These mice express a chimeric AR in which the second zinc finger of the DBD is replaced with that of GR (105). These specificity affecting

AR knock-in mice (SPARKI) present a subfertile phenotype with smaller reproductive organs and differential gene expression profile in the epididymis compared to wild-type mice (105, 107).

Our in vivo ChIP-seq study revealed differential genome-wide chromatin binding between wild-type and SPARKI AR, with a significant proportion of wild-type ARBs being lost in epididymides and prostates of SPARKI mice, highlighting a subgroup of in vivo AR-binding events that are highly dependent on the second zinc finger of the receptor (108). These in vivo studies thus confirmed the existence of selective AREs and their significance for genome-wide AR-binding events and transcription programs (105, 107, 108). Analysis of the sequences specific for the wild-type AR identified a response element with a well-conserved 5′ hexamer but marginal sequence conservation of the 3′ hexamer— only a G at position 11 is highly conserved—whereas the canonical ARE/GRE (an inverted repeat of the 5′-AGAACA-3′ hexamer) was enriched among the shared ARBs, ie, the sites that both wild-type and SPARKI AR recognized equally well (108). Thus, AR-selective receptor binding in vivo is achieved through relaxed cis-element stringency rather than a distinct and strict ARE sequence, most likely due to the high-affinity protein–protein interaction at the AR DBD dimer interphase (109). Of note, the previous suggestion that the cis-element of a selective ARE is a direct repeat of the 5′-AGAACA-3′ sequence is not a general rule for AR selectivity (108). Owing to the relaxed sequence requirement for the second hexamer, a selective ARE can occasionally also resemble a direct repeat. It is nevertheless important to emphasize that AR is fully capable of binding in vivo also to canonical ARE/GRE sequences with high affinity and that the AR-selective sites should be of the most importance under the conditions where a target cell expresses other steroid receptors in addition to the AR. Figure 3 summarizes the factors ensuring the specificity in AR–chromatin interaction.

A recent study demonstrated that a single amino acid mutation in the GR DBD results in differences in DNA binding specificity and gene regulation (110). Furthermore, nucleotides flanking the 6-nt half-sites also affect receptor binding affinities (111, 112). The concept that the DNA sequence of the response elements represents merely a receptor-docking site has been challenged, owing to the reports indicating that the DNA sequence in and of itself can alter GR protein conformation and thus serve as an allosteric ligand for the receptor (112, 113). Moreover, as discussed in detail in the following sections, the local chromatin environment determines which cis-elements are available for receptor–chromatin interaction in a context-dependent manner (114).

C. The role of ligand in receptor–chromatin interaction

The physiological ligands of AR, T and DHT, bind to AR and bring about a conformational change in the LBD that facilitates nuclear localization of the receptor. Ligand binding is a prerequisite for AR–chromatin interaction, and in the absence of ligand, there is very little, if any, AR binding either in prostate cancer cell lines or in androgen-responsive tissues (66, 67). Androgen exposure elicits robust loading of holo-AR onto chromatin, along with assembly of coregulatory complexes, comprising proteins such as p160 and CBP/p300, and RNA polymerase II (59, 60, 115, 116). Dynamic assembly of AR transcriptional complex onto chromatin increases gradually after androgen exposure and peaks at around 16 hours (60). However, significant AR loading was observed within minutes after androgen exposure, with a peak at approximately 2 hours both in prostate cancer cell lines and in androgen-responsive tissues (59, 66, 67), and after 18 hours, there was a global reduction in AR occupancy compared to the 2-hour time point (117). The interaction of AR with its ligand and the subsequent holoreceptor-chromatin interaction follow the law of mass action, in that increased cellular receptor content sensitizes the AR response to lower hormone concentrations and leads to a larger number of AR-binding sites on chromatin (118, 119).

Most of the ChIP-seq studies on AR binding to chromatin have used the physiological androgen DHT or the synthetic AR agonist methyltrienolone (R1881), with exposure times ranging from a few minutes to 16 hours (summarized in Ref. 84). We have compared the effects of DHT to partial agonists/antagonists (CPA and RU486) and the antagonist bicalutamide on AR loading and AR-directed gene expression on a genome-wide scale in the LNCaP-1F5 prostate cancer cell line (67). Exposure of prostate cancer cells to the partial agonists CPA or RU486 resulted in the formation of ARBs that were quantitatively, rather than qualitatively, different from those brought about by the DHT-occupied AR (67). This agrees with previous reports showing that CPA is able to induce AR loading onto chromatin in a dose-dependent manner (59, 120). Thus, in the presence of androgens, the partial agonist/antagonist compounds attenuate AR action in two ways: 1) by competing with androgen for binding to the AR LBD; and 2) by competing as an AR complex with the androgen-occupied AR for chromatin binding sites. However, when given alone in sufficiently high doses, agonists/antagonists are capable of forming AR complexes that bring about AR-binding events qualitatively very similar to those of the agonist-occupied AR and elicit a submaximal transcriptional response. Tamoxifen-bound ER has also been shown to bind to the same loci as the estradiol-bound ER, but with a lower affinity (121). Interestingly,

Figure 3.

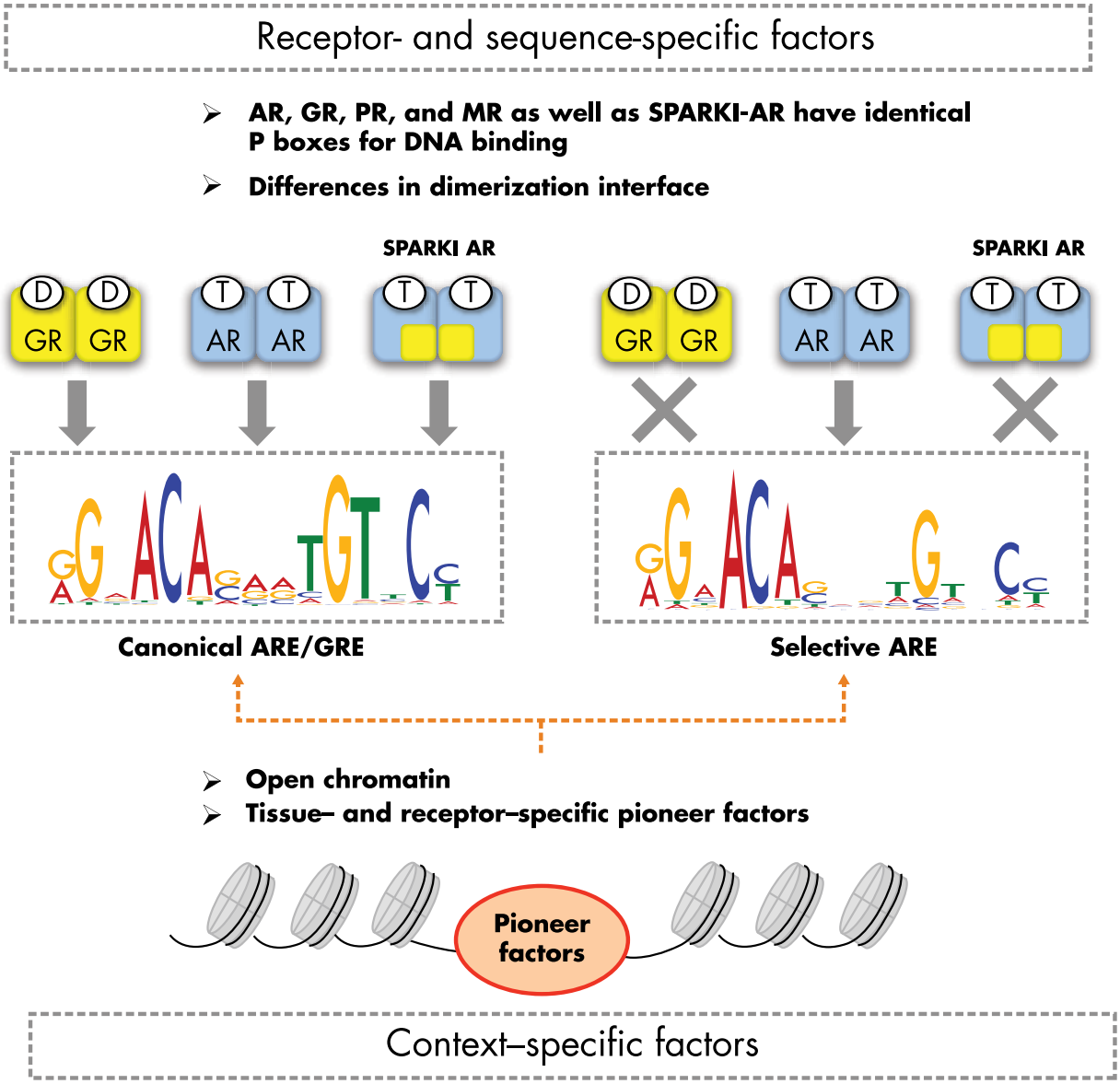


Figure 3. Determinants of AR-specific events for chromatin binding and transcriptional regulation. D, dexamethasone.

so-called endocrine disruptive compounds, to which people are exposed through food and environment, can also affect steroid receptor loading in a similar quantitative way as that described for the partial agonists/antagonists. The environmental estrogens genistein and bisphenol A were reported to regulate gene expression through ER on a genome-wide scale, although less efficiently than estradiol (122). These results suggest that steroid receptors occupied by partial agonists are potentially loaded onto binding sites with the highest affinity (ie, the strongest sites), but a full agonist is needed for a complete spectrum of chromatin–receptor interactions (67).

The AR-antagonist bicalutamide, on the other hand, brought about very limited AR loading onto LNCaP-1F5 prostate cancer cell chromatin, resembling that achieved by vehicle only (67). However, agonistic properties for bicalutamide have been reported previously by mechanisms involving either an increased cellular AR content (123) or a point mutation in the AR LBD (124). The second-generation AR-antagonist enzalutamide has higher affinity for AR than bicalutamide, and it is able to inhibit nuclear translocation and DNA binding of AR (41). Enzalutamide also prevented AR from binding to an ARE sequence on the mouse mammary tumor virus (MMTV)

enhancer in the presence of FOXA1, whereas bicalutamide was incapable of eliciting the same effect (125). However, a recent study utilizing the motif-resolution ChIP-exonuclease approach reported that agonist-liganded AR and antagonist (bicalutamide or enzalutamide)-liganded AR bind to distinct genomic loci and use different cis-elements, leading to distinct transcriptional outcomes in LNCaP prostate cancer cells (126). Curiously, unliganded apo-AR was found to bind in many instances to the same loci as the antagonist-occupied AR, albeit with a lower apparent affinity (126). Under selection pressure, prostate cancer cells are capable of inducing point mutations to the AR LBD, leading to conversion of enzalutamide to an AR agonist (127, 128) with the enzalutamide-occupied AR being loaded onto enhancer regions of AR-regulated genes (127). In addition to point mutations in the AR LBD, expression of AR transcript variants is another means for prostate cancer cells to bypass androgen blockage because these receptor forms lack the LBD and are thus constitutively active transcription factors. Expression of one of the known variants, AR-V7, is associated with enzalutamide resistance in prostate cancer patients (44), and a recent study showed a more efficient chromatin looping and *UBE2C* gene expression in cells expressing the AR variant compared to the full-length receptor (129). Thus, the variant receptor types are potentially able to substitute for the full-length AR in CRPC (130). Whether the formation of truncated AR variants devoid of the LBD is only a selection pressure-induced somatic mutation in prostate cancer cells or also takes place under physiological conditions in noncancerous tissues remains to be elucidated.

Taken together, prostate cancer cells are able to circumvent selection pressure (exposure to antiandrogens and/or androgen deprivation therapy) and eventually employ all currently available partial agonists/antagonists and full antagonists as agonistic ligands under certain conditions. Additional work is required to understand better the ways by which second-generation and upcoming antiandrogens perturb with the AR pathway and the ways by which this pathway responds to selection pressure under in vivo conditions and in cultured noncancerous and cancerous cells.

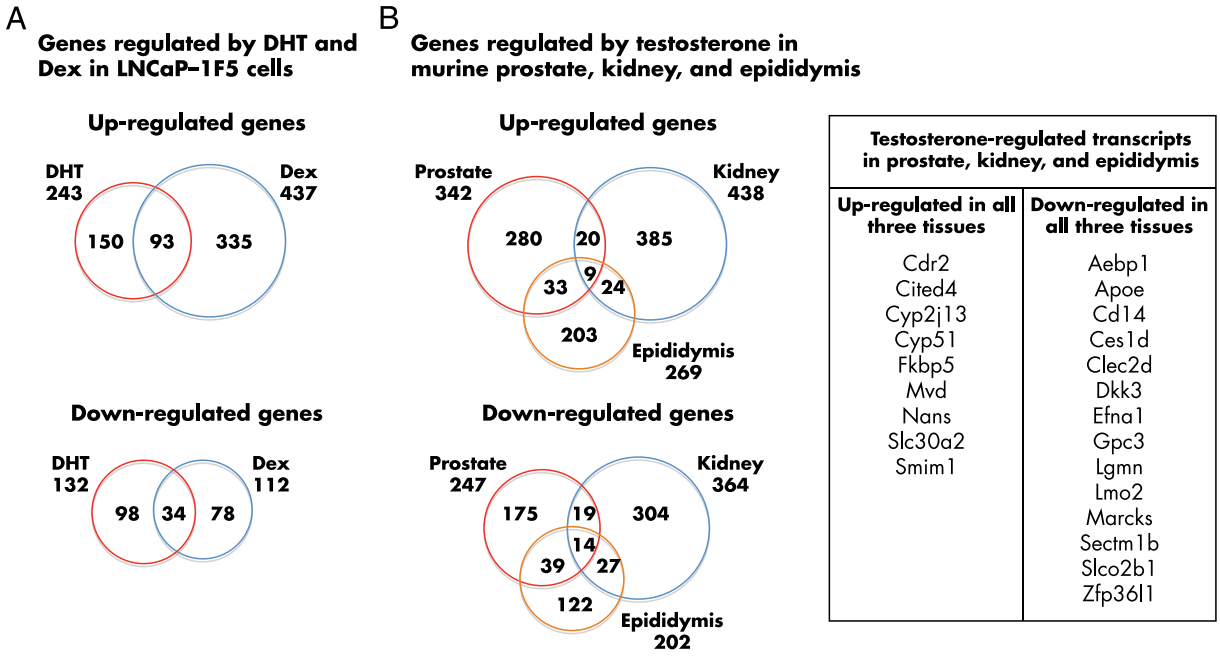
D. GR as regulator of the AR pathway

Glucocorticoids regulate inflammatory, metabolic, and survival pathways in cells through their cognate receptor, the GR. Glucocorticoid treatment is in clinical use for prostate cancer patients (131, 132), mainly to help patients deal with pain and adverse effects of chemotherapy. Glucocorticoids are currently coadministered to abiraterone-treated prostate cancer patients with the purpose of avoiding side effects from CYP17 inhibition brought about by abiraterone (133). Glucocorticoids also have tumor-suppressive effects on prostate cancer cells by inhibiting cell growth and lymphangiogenesis (134–137). However, glucocorticoids can promote prostate cancer cell proliferation by acting as the ligand for a mutated AR (138) or through IL-6 signaling (139). Moreover, the fact that AR and GR bind to similar response elements when examined using naked DNA in vitro and/or under transient transactivation conditions has raised questions about their interplay on chromatin binding in vivo.

ChIP-seq studies have shown that AR and GR are capable of binding to the same sites on chromatin (67, 79). Approximately one-half of the AR cistrome overlaps with that of GR in a prostate cancer cell line expressing both AR and GR (67), and in cell lines established from antiandrogen-resistant xenograft tumors (140). Gene expression programs of dexamethasone (Dex)-induced GR and DHT-induced AR are highly overlapping (Figure 4), and the shared AR- and GR-binding events are significantly associated with genes regulated by both androgen and glucocorticoid (67). These results imply that AR and GR occupied by their cognate ligands are capable of using the same cis-elements to regulate transcription programs, and that glucocorticoid-occupied GR can take over the androgen-occupied AR in the regulation of AR target genes (67). Furthermore, GR expression has been shown to be negatively regulated by active AR signaling in prostate tumors (141), and exposure of prostate cancer xenograft tumors to enzalutamide led to induction of GR expression in a subset of cells within just a few days (140). GR expression is also associated with enzalutamide resistance in bone marrow biopsies of prostate cancer patients (140). In another xenograft model, GR inhibition delayed castration-resistant tumor formation, and overexpression of the GR-regulated serum and glucocorticoid-regulated kinase 1 (*SGK1*) enhanced prostate cancer cell survival (142). These results imply that activation of GR signaling can maintain an active AR pathway under androgen-deprived conditions in prostate cancer, despite the previous reports on tumor-suppressive activities of GR in prostate cancer cells.

Two subsets of genes can be distinguished in the overlapping AR- and GR-regulated transcription programs: 1) DHT-dominant genes that are up-regulated more by DHT alone than by the GR-agonist Dex alone, and combined Dex and DHT exposure inhibits transcript accumulation; and 2) Dex-dominant genes that are up-regulated more by Dex alone than DHT alone, but a concomitant DHT and Dex exposure leads often to increased transcript accumulation (67). Thus, Dex-occupied GR should be considered as a partial agonist/antagonist for AR, in that liganded GR is an anti-AR in the presence of androgen but an AR ag-

Figure 4.



Testosterone-regulated transcripts in prostate, kidney, and epididymis	
Up-regulated in all three tissues	**Down-regulated in all three tissues**
Cdr2	Aebp1
Cited4	Apoe
Cyp2j13	Cd14
Cyp51	Ces1d
Fkbp5	Clec2d
Mvd	Dkk3
Nans	Efna1
Slc30a2	Gpc3
Smim1	Lgmn
	Lmo2
	Marcks
	Sectm1b
	Slco2b1
	Zfp36l1

Figure 4. Area-proportional Venn diagrams of genes up-regulated and down-regulated by DHT and Dex in LNCaP-1F5 prostate cancer cells (A) and by T in murine prostate, kidney, and epididymis (B). The transcripts regulated in vivo by T in all three murine tissues are listed separately. The data are adapted from Refs. 66 and 67.

onist in the absence of androgen. An anti-AR behavior of GR has also been reported for another cell type, the human adipocyte (143). Importantly, GR-dependent genes in GR-expressing prostate cancer specimens and in LNCaP-1F5 cells include many known oncogenes, such as *ACSL3*, *LIFR*, *NFIB*, and *BTG1* (67, 144). The interplay between AR and GR signaling is of potential clinical importance because about 30% of prostate cancers express GR, and the proportion of GR protein-expressing prostate cancers increases after androgen-deprivation therapies and in CRPC tissues (145, 146). Unlike AR, GR function is indispensable for life, which presents an inherent challenge for the establishment of GR signaling blockade aimed at treating prostate cancer patients or studying the requirement for GR function in enzalutamide-resistant prostate cancer (147). Interestingly, the pioneer/licensing factor FOXA1 can specify unique AR- and GR-binding events in a cell type-specific fashion, in that FOXA1 is important for AR in LNCaP-1F5 cells but for GR in VCaP prostate cancer cells (67), emphasizing the importance of chromatin context and collaborating transcription factors in specific hormonal response. Figure 5 summarizes current results pertaining to the interplay between AR and GR pathways in prostate cancer. Nonetheless, additional studies addressing the GR-dependent modulation of the AR pathway and its molecular determinants are required to permit better judgment between beneficial and/or adverse effects of GR signaling in prostate cancer treatment. The above results on the interplay between AR and GR signaling pathways originate mainly from experiments using cells or tissue of prostate cancer origin, and therefore, additional studies are required on other tissues and cell types that coexpress AR and GR proteins.

E. Competition between receptors for chromatin binding

Similar cis-elements of steroid receptors and overlapping chromatin-binding events among the receptors raise the question about direct competition between receptors in their loading onto chromatin. This concept was tested on the MMTV promoter array and at several endogenous loci in the mouse genome, using two receptors both activated with their cognate ligands, ie, GR and an ER variant, having identical DNA-binding affinities. Intriguingly, the two receptors did not appear to compete for loading onto same GREs, and actually at many sites, chromatin loading of the ER-variant receptor was enhanced upon GR binding (148). A similar assisted loading mechanism was observed in the context of endogenous ER and GR in a mouse mammary cell line, with no competition between the two receptors for chromatin binding on the genome-wide scale, and GR activation by Dex rather facilitated ER binding to the shared loci (149). Furthermore, there was no competition for chromatin binding events between AR and GR in the prostate cancer cell line

Figure 5.

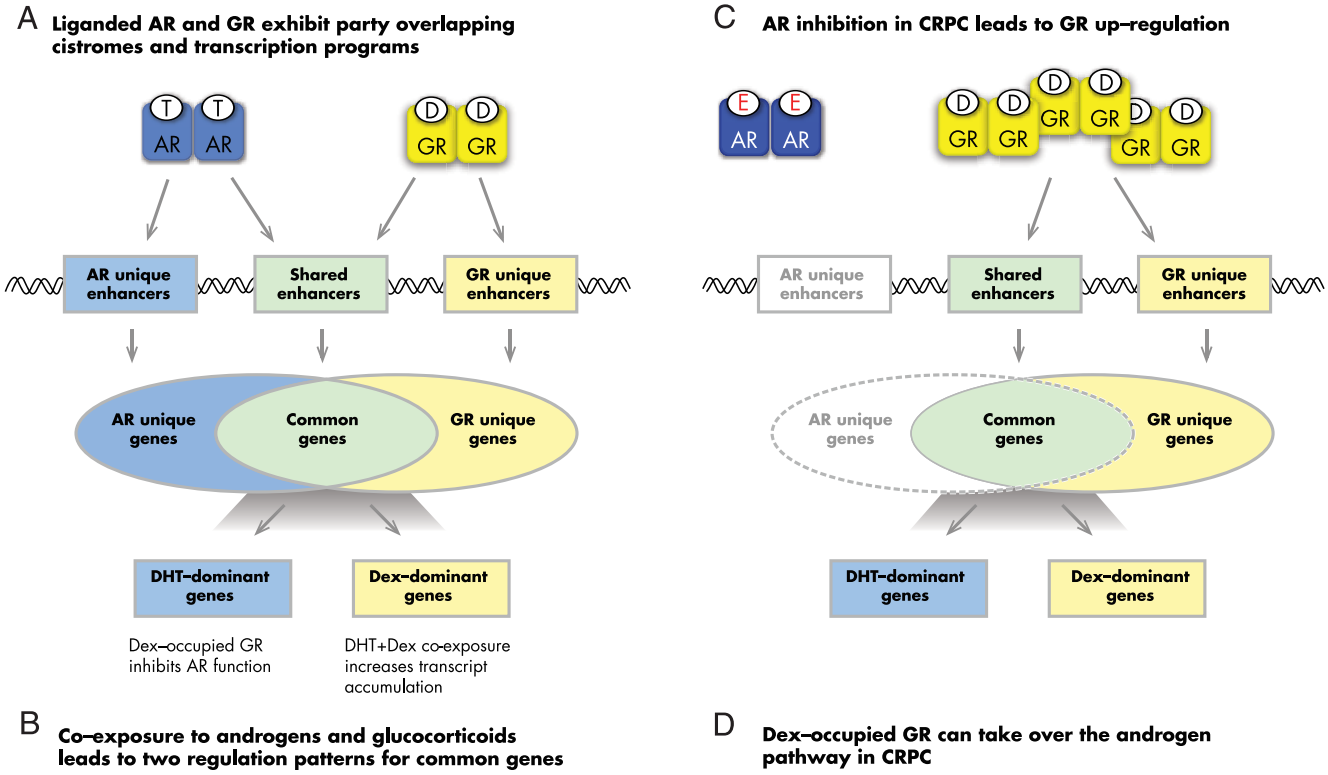


Figure 5. Interplay of AR and GR pathways in prostate cancer cells. D and Dex, dexamethasone; E, antiandrogen (eg, enzalutamide).

expressing the two receptors, but instead, their binding was increased by simultaneous exposure to the two hormones, DHT and Dex (67). Nevertheless, most ChIP-seq studies have thus far focused on receptor–chromatin interaction in the context of one receptor in a few well-characterized model systems and cancer cell lines, and further studies addressing gene regulation and the interplay of several regulatory pathways in a physiological environment are warranted to explore their role in receptor- and tissue-specific transcriptional regulation.

IV. Pioneer Factors in Androgen Signaling

A. General outline of pioneering/licensing proteins in steroid signaling

Eukaryotic chromatin serves two purposes: it organizes genome in compact three-dimensional structures and occludes gene regulatory regions from binding of transcriptional machinery, thus providing a major regulatory step in gene transcription. Chromatin structure can be modified by a multitude of mechanisms, including chemical modifications of DNA or histones, substitution of canonical histones for histone variants in nucleosomes, and repositioning of nucleosomes along regulatory DNA (150). Transcription factors typically occupy only a few percentages of their putative, computationally predicted binding sites (cis-elements) on chromatin (151, 152), and transcription factor cistromes are highly cell type- and context-specific (58). Thus, the interplay between local chromatin structure, collaborating transcription factors, such as pioneer/licensing factors, and coregulators determine the receptor-selective and tissue-specific transcriptional outcomes in vivo (114).

Pioneer/licensing factors are transcription factors that are capable of binding to compact nucleosomal chromatin. A corollary to this feature is that, by and large, their binding to chromatin precedes that of other transcription factors. Pioneer factors can have an active role in facilitating recruitment of other transcription factors by opening the local chromatin structure, but also a more passive role, in that they can be preloaded onto enhancers to enable rapid transcriptional activation (153, 154). Forkhead box (Fox)A and GATA proteins were the first reported pioneer factors. Their chromatin binding occurs before that of other transcription factors, and they are indispensable for liver differentiation program during development (155–157). The DBD of FOXA1 has a winged helix structure similar to that of linker histone, and it is thus able to

replace histone H1 on chromatin, generating a more open chromatin structure locally (158, 159). The carboxyl-terminal domain of FOXA binds directly to core histones, which in turn facilitates the opening of nucleosomal DNA structure (160). The first ChIP-on-chip studies that linked pioneer factors to steroid receptor signaling revealed high enrichment of the forkhead, GATA, and Oct motifs among the ER and AR binding sites and showed, by examining a few promoters, that depletion of FOXA1 and GATA-2 attenuated ER and AR binding to chromatin and subsequent transcriptional activation (61–63). These groundbreaking observations formulated the concept that steroid receptor binding to chromatin is regulated by the pioneer factors and paved the way for more detailed studies over the ensuing years.

On the basis of the initial studies conducted with MMTV promoter arrays, it was assumed that GR and PR bind to compact nucleosomal chromatin, recruit chromatin remodelers, and facilitate binding of other transcription factors, thus acting in a manner typical of genuine pioneer factors (161–163). However, genome-wide studies indicated that GR binds mostly to accessible chromatin, and only approximately 20% of GR binding was observed at hormone-induced remodeled chromatin (101, 164). Interestingly, genome-wide PR loading occurs at sequences organized in nucleosomes, and PR binding leads to chromatin remodeling and appearance of DNase-hypersensitive sites upon hormone exposure (99). Thus, it appears that PR could behave, at least in part, as a bona fide pioneer factor, whereas other steroid receptors, including the AR (79), rely mainly on pioneer/licensing factors to create an open chromatin environment and to expose cognate cis-elements for receptor interaction.

B. Receptor specificity of pioneer factors

FOXA1 has been studied extensively in connection of ER and AR function, and it does function as a pioneer factor for both receptors (79, 121). GATA and activating protein (AP)-2 proteins have also been implicated in ER and AR signaling (66, 165–167). Interestingly, FOXA1 has been shown to define cell type-specific binding of AR and GR to chromatin even between two prostate cancer cell lines, namely, AR in LNCaP-1F5 cells and GR in VCaP cells (67). Moreover, FOXA1 determines lineage-specific ER and AR binding in breast and prostate cancer cells, respectively (77), and differential ER and AR functions in hepatocytes (168). FOXA1 is also required for GR binding to regulatory regions of the *IL-6* gene in liver because GR binding to these sites was abolished in the livers of *FoxA1/2*-deficient mice (169). Thus, pioneer factors are not specific for a given steroid receptor per se, but rather they integrate context-specific transcriptional responses together with local chromatin environment and a particular steroid receptor (Figure 6).

One potential way to achieve receptor specificity for a pioneer factor is ligand-facilitated interaction of the receptor with the factor in question. In the case of AR, this receptor has been shown to interact in LNCaP cells with

Figure 6.

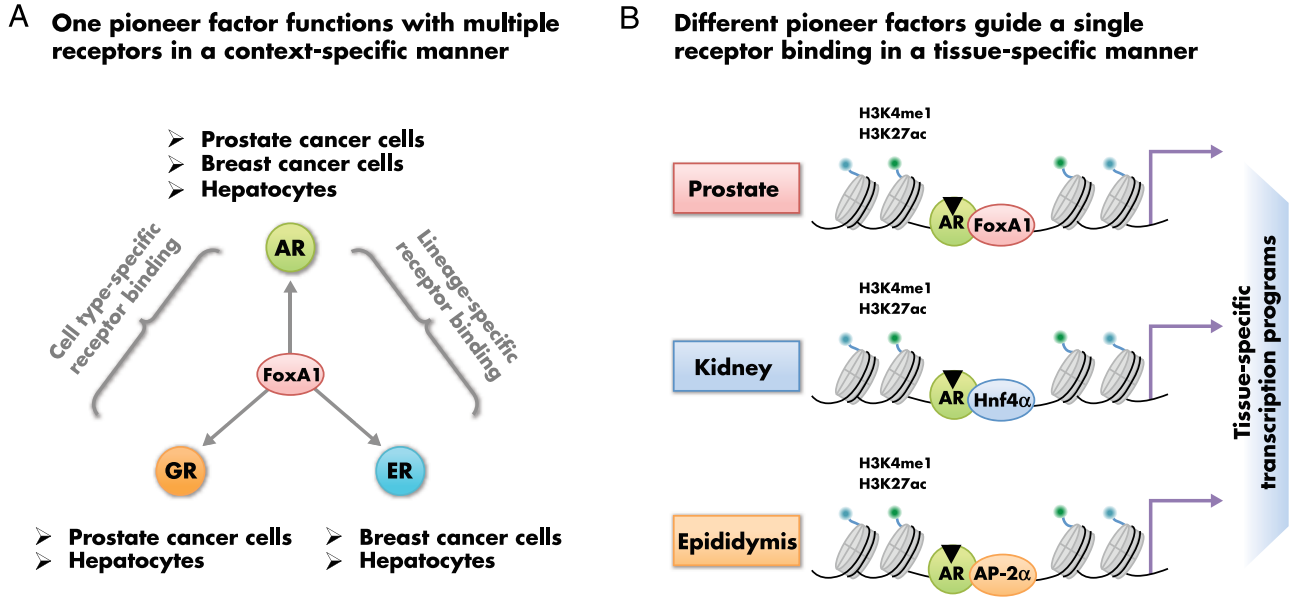


Figure 6. Context-specific functions of pioneer factors may involve partnering with multiple receptors to elicit cell type- or lineage-specific receptor binding (A) or, alternatively, a single receptor may interact with different pioneer proteins to bring about tissue-specific transcriptional outcomes (B).

the pioneer factors FOXA1, GATA2, and Oct-1 in a DHT-dependent fashion (63). Under in vivo conditions in mice, AR recruitment stabilizes binding of three pioneer factors (FoxA1, Hnf4α and AP-2α) to chromatin in target tissues (66). Likewise, chromatin binding of the respective pioneer/licensing factor enhances loading of holo-AR onto chromatin (66), implying that liganded AR interacts directly with a pioneer factor specific to a given tissue.

C. Tissue specificity of pioneer factors

Enhancers are gene regulatory elements that serve as binding platforms for lineage-specific transcription factors and sequence-specific effectors of signaling pathways, integrating information related to intracellular conditions and cellular environment to combinatorial response in gene expression patterns (57). Distal enhancers dictate cell type-specific transcriptional activation in both humans and mice, as shown by recent genome-wide studies of transcription factor cistromes and histone modifications (170, 171). Of note, the enhancer regions can be transcribed by RNA polymerase II to produce enhancer-derived long noncoding RNAs (eRNAs) (172). These eRNAs are important for AR- or ER-driven chromatin looping and transcriptional activation (173, 174) as well as for the interaction of AR and ER with the Mediator complex (173, 175). Moreover, the DNA nicking activity of topoisomerase I is a prerequisite for robust eRNA synthesis and enhancer activation (176). With regard to AR action, eRNAs are proposed to function as scaffolds that guide receptor-associated protein complexes to target chromatin and selectively regulate androgen-dependent transcription programs.

Steroid receptor cistromes mapped in a variety of cultured cancer cell lines and a few tissues in vivo show remarkable tissue specificity. About 10% of ER-binding sites and 3–14% of PR-binding sites overlap between a breast cancer cell line and osteosarcoma or endometrial cancer cell lines, respectively (177–179). AR cistromes, even in different prostate cancer cell lines, exhibit distinct, cell type-specific features (67). In three androgen-responsive mouse tissues in vivo, only 7–16% of AR-binding events were shared between prostate, kidney, and epididymis, emphasizing the high degree of tissue specificity of AR cistromes (66). Intriguingly, only a few genes were regulated by androgens in all three tissues among the hundreds of tissue-specific genes (Figure 4), underscoring the context-specific androgen action mediated by specific receptor–chromatin interactions (66). Genuine high-affinity AREs were significantly enriched among the tissue-specific AR-binding events in each of the three tissues—prostate, kidney, and epididymis (66). By contrast, cell type-specific ER-binding events have been reported to lack high-affinity ER cis-elements in cell lines of breast and uterine cancer origin (177). Importantly, regulatory regions of both androgen up-regulated and down-regulated genes under physiological conditions in vivo were enriched for high-affinity AR-binding events (66), which agrees with the findings on GR-dependent transcription programs in macrophages (180).

Cell type- and lineage-specific FOXA1 cistromes suggest that pioneer factors play an important role in the establishment of tissue-specific receptor-binding events (77, 181, 182). Genome-wide FOXA1 binding overlaps with ER in breast cancer cells and AR in prostate cancer cells, and FOXA1 binds to these shared sites before the hormone-induced receptor loading (77, 79, 121, 181, 183). Other pioneer factors reported for ERα are AP-2γ, GATA factors, and pre-B cell leukemia homeobox 1 (PBX1) (165, 166, 184–186), whereas GATA-2 and ETS family members have been linked to AR-binding events (63, 68, 78, 117, 167, 187). Pioneer factors reported for GR include FOXA1, AP-1, and C/EBP (67, 79, 100, 188). It is important to emphasize that the mere cis-element enrichment for particular collaborating protein adjacent to a receptor-binding site does not guarantee that the cognate transcription factor is indeed expressed in the particular cell type, let alone occupying those sites. Moreover, the ultimate proof of a pioneer factor's mandatory role in an AR-dependent regulation of a given target gene under physiological conditions would require the use of novel gene editing techniques to mutate the cis-element(s) of the pioneer factor (see *Section VI*). Table 1 lists the pioneer factors reported thus far for steroid receptors in various cell types with experimentally validated colocalization with each steroid receptor. Collectively, it is highly unlikely that there are distinct pioneer/licensing factors that are strictly specific for a given steroid receptor; rather, the same pioneer factor can collaborate with different receptors depending on the cell context and receptor expression (Figure 6).

Our in vivo study compared steroid receptor cistromes in three mouse target tissues under physiological conditions and elucidated the role of pioneer factors in tissue-specific regulation in androgen-dependent tissues. Distinct cis-elements for FoxA1, Hnf4α, and AP-2α were substantially enriched among the tissue-specific ARBs in prostate, kidney, and epididymis, respectively, and ChIP-seq profiling of these factors revealed significant co-occupancy with AR on chromatin of the respective tissues (66). Moreover, shared binding events between AR and tissue-specific pioneer factors colocalized with enhancers that were marked by active histone modifications, and importantly, AR loading onto these sites was precluded in the absence of the respective pioneer factor (66). Of the three

Table 1. Summary of Pioneer Factors Associated With Steroid Receptor Function in Different Cell Types

Tissue of Origin	Cell Type	Pioneer Factor	Refs.
ERα			
Mammary gland	MCF-7 and T-47D breast cancer cell lines	FoxA1	61, 77, 121, 166, 177, 216
	MCF-7	PBX1	185
	MCF-7	AP-2γ	165
	MCF-7 and T-47D breast cancer cell lines	GATA-3	166, 177, 184
Uterus	ECC-1 cancer cell line	ETV4	177
Bone	U2OS-ER osteosarcoma cell line	GATA-4	186
Liver	Hepatocyte from liver tissue	FoxA1	168
AR			
Prostate	LNCaP, LNCaP-1F5, VCaP prostate cancer cell lines	FoxA1	38, 67, 77, 79, 172, 219
	Murine prostate tissue	FoxA1	66
	LNCaP	GATA-2	63, 167
	LNCaP	ETS1	81
	LNCaP, VCaP lines, mouse prostate	ERG	68, 117, 187
Mammary gland	MDA-MB-453 ER−/AR+ molecular apocrine breast cancer cell line	FoxA1	183, 217
Epididymis	Murine epididymis tissue	AP-2α	66
Kidney	Murine kidney tissue	Hnf4α	66
Liver	Hepatocyte from mouse tissue	FoxA1	168
GR			
Prostate	LNCaP-1F5, VCaP prostate cancer cell lines	FoxA1	79, 67
Mammary gland	3134 Murine mammary epithelial cells	AP-1	188
Liver	Hepatocyte from mouse tissue	C/EBP	100

Only validated studies reporting pioneer factor occupancy at the sites shared with a steroid receptor are listed.

factors, Hnf4α is a constitutively active nuclear receptor with no previous link to AR function, whereas AP-2γ has been previously implicated in ER function (165), and AP-2α and AP-2β have been shown to co-occupy a few regions with AR on epididymal chromatin (64). It is highly likely that the above-mentioned pioneer factors are not the only ones for AR binding events in a given tissue because only one-half or less of the tissue-specific ARBs exhibited significant overlap with the respective pioneer factor binding sites. However, there was no overlap with FoxA1 and AR cistromes on renal or epididymal chromatin, emphasizing the notion that FOXA1 is not a universal, but rather is a prostate-specific pioneer factor for AR (66). A large number of FOXA1-pioneered ARBs in human prostate cancer cell lines are enriched for a composite cis-element comprising a forkhead motif and an ARE half-site (79). Intriguingly, approximately 20% of the AR-binding events unique to normal mouse prostate are also enriched for the same composite cis-element (66), suggesting that utilization of this particular DNA motif for AR signaling is specific for cells of prostatic origin. These in vivo results highlight the fact that the steroid, the receptor, and the cis-element are necessary but not sufficient for tissue-specific transcriptional regulation by androgens, and that another layer—tissue-specific expression of pioneer/licensing factors—is mandatory for the AR pathway (Figure 6).

D. Chromatin modifications and pioneer factors

Genome-wide mapping of various histone modifications and their correlation to genomic features and transcription factor-binding sites has revealed an epigenetic map encoded in histone tails (189). Acetylation is generally associated with active regulatory elements, including the modification of lysines primarily on the tails of H3 and H4 histones (190), and occupancy of the histone acetylase p300 can be used as a marker of active enhancers (191). Lysine methylation, on the other hand, plays a more versatile role in transcription-associated transient histone modifications. There are more distinct protein domain types recognizing lysine methylation than any other modification, and the methyltransferases are among the most specific histone-modifying enzymes (192). Active enhancers are characterized by monomethylation of lysine 4 in histone 3 (H3K4me1), whereas trimethylation of the same residue (H3K4me3) is predominantly found at active gene promoters (54, 193). Conversely, trimethylation of H3 lysine 27 (H3K27me3) and di- and trimethylation of H3 lysine 9 (H3K9me2 and H3K9me3, respectively), are associated with heterochromatin and enriched at transcriptionally inactive loci (194, 195). Thus, the functional consequence of histone methylation is highly dependent on the chromatin context and the modified residue.

Dimethylated H3K4 that is found at both enhancers and promoters (54, 193) defines lineage-specific FOXA1 recruitment to chromatin (77). During development, FOXA1 promotes DNA demethylation and increases H3K4 methylation (196). H3K4me2 marks AR-binding sites already in the absence of hormone, and the bimodal H3K4me2 enrichment profile after DHT exposure shows

that AR binding leads to eviction of the central nucleosome and stabilization of the flanking nucleosomes at the ARBs (197). Within the ARBs that overlap with FOXA1, central nucleosome is already evicted prior to DHT exposure, demonstrating that pioneer factors can replace nucleosomes and unmask enhancers for subsequent AR binding (198). A similar pattern in the activating histone marks has been shown for the ARBs shared with tissue-specific pioneer factors—that is, FoxA1 in prostate, Hnf4α in kidney, and AP-2α in epididymis (66).

As mentioned above, tissue-specific pioneer factors play a key role in context-dependent gene regulation, but what dictates pioneer factor binding preferences? Pioneer factors are transcription factors with their own DBDs and specific cis-elements. However, pioneer factors are also thought to function as readers of the histone code, translating epigenetic modifications into specific transcription factor binding (199), such as H3K4me2 recruiting FOXA1 binding (77). Other features associated with tissue-specific gene expression and pioneer factor occupancy include accessible chromatin (101, 200), cohesion-stabilizing tissue-specific protein–DNA complexes (201), and DNA hypomethylation (196, 202, 203). Interestingly, DNA demethylation was recently reported to regulate tissue-specific gene expression also by releasing transcript elongation block in an AR-dependent manner (204). Furthermore, there is accumulating evidence regarding the role of eRNAs in the function of active enhancers by mechanisms involving interaction with transcription factors and modulation of chromatin loops (reviewed in Ref. 205). However, understanding the full spectrum of eRNA roles in cell type-specific transcriptional regulation and pioneer factor functions awaits additional studies.

Many AR-interacting protein possess demethylase or methyltransferase activity. However, the precise interplay between H3K4 methylation, pioneer factor (eg, FOXA1) binding, and likely other mechanisms required to initiate the opening of an AR-regulated enhancer region remain to be clearly defined. For instance, methyltransferase SET9 can catalyze methylation of H3K4 and may thereby reinforce an open chromatin state in an AR-dependent manner (206, 207). Lysine-specific demethylase 1 (LSD1), on the other hand, promotes AR-dependent transcriptional activation by catalyzing demethylation of lysine 9 in histone 3 (H3K9) (208, 209), but it also has a repressive function through H3K4me2 demethylation in an androgen-dependent fashion (210). Genome-wide analyses of AR, FOXA1, and LSD1 revealed that LSD1 functions broadly as a coactivator at AR-stimulated enhancers, but it retains its H3K4me1,2 demethylase activity at these sites, suggesting that LSD1 provides a negative feedback loop to suppress gene expression in the absence of androgens (211). Furthermore, EZH2 methyltransferase of the polycomb repressive complex is a genome-wide regulator of AR-dependent gene repression (212), but it can also function as an AR coactivator in CRPC (213). Despite these intriguing findings, additional studies are required to elucidate the functional interplay between chromatin modifications and pioneer as well as other collaborating factors in tissue-specific regulation of the AR pathway.

E. Pioneer factors in hormone-dependent cancers

The role that the pioneer factors play in steroid receptor function implies that they can have clinical significance in hormone-dependent cancers. Thus far, FOXA1 is the best-characterized pioneer factor in the context of both breast and prostate cancer (214). In prostate cancer, ETS family members also have a special interest due to their involvement in recurrent TMPRSS2-driven fusion genes (74, 144), although their function in prostate cancer is not yet fully understood (215). Furthermore, several collaborating factors bind in many cases to shared loci together with AR and ER, such as FOXA1 and GATA-2 in prostate cancer cells and FOXA1 and GATA-3 in breast cancer cells, respectively (166, 167), but their functional interplay in specific steroid receptor binding needs further characterization.

In the case of ER–chromatin interaction in breast cancer cells, FOXA1 is a prerequisite for ER binding, and FOXA1 depletion leads to genome-wide attenuation of ER loading onto chromatin that decreases most ER binding events by more than 50% (121). In primary breast cancer tissue samples, forkhead motifs were particularly enriched in the ER-binding events that occurred in breast cancer patients with a poor clinical outcome (216). Interestingly, in ER-negative breast cancer cells, AR can take over ER signaling and activate the ER pathway in a FOXA1-dependent manner (183, 217).

The role of FOXA1 in AR-dependent transcription appears to be by far more complex than that in ER signaling. Depletion of FOXA1 in cultured prostate cancer cells led to a global redistribution of ARBs, including an extensive gain of novel AR-binding events (79, 172). FOXA1 defines three classes of ARBs in prostate cancer cell lines: 1) FOXA1-pioneered sites that are lost upon FOXA1 depletion; 2) FOXA1-independent ARBs; and 3) FOXA1-repressed sites that permit AR binding only upon depletion of FOXA1 (79). Interestingly, FOXA1 also elicited marked redistribution of GR-binding events in a similar fashion (79). FOXA1-depletion-dependent changes in AR occupancy were also reflected in androgen-regulated gene expression programs (79) and in the production of eRNAs (172).

In comparison to parental LNCaP cells, FOXA1 overexpression results in increased xenograft tumor size (218) and increased number of genome-wide ARBs, representing enhanced AR loading to low-affinity sites in a pattern similar to that in CRPC (219). On the other hand, AR cistromes in CRPC cell lines show androgen-independent loading of AR onto enhancers that have constitutively open chromatin structure, are often located at promoter regions, lack the ARE motif, and are independent of FOXA1 (38, 220). Moreover, in an in vivo study using a limited number of heterogeneous CRPC tissue specimens, AR cistromes were not shown to be enriched for forkhead motifs (221, 222). Nonetheless, FOXA1 was recently identified as one of the few recurrently mutated genes in prostate adenocarcinomas and CRPC samples (218, 223).

FOXA1 has a contrasting role and predictive power in breast and prostate cancers. Although forkhead motifs were highly enriched in the ER-binding events in breast cancer patients with a poor clinical outcome (216), high FOXA1 antigen level in primary cancer tissue has been shown to be a marker for good prognosis in ER-positive breast cancer, predicting longer recurrence-free and overall survival (224) and better tamoxifen response (225). By contrast, high FOXA1 antigen level in primary prostate cancer tissue was shown to predict poor prognosis and disease outcome. A large-scale tissue microarray study revealed a strong correlation between FOXA1 and AR protein levels, and high FOXA1 staining intensity was a marker for shortened time to prostate cancer-specific death (79). These results were confirmed in separate cohorts with high FOXA1 levels correlating to shorter time to biochemical recurrence (226) and higher pathological state (219). Thus, due to its prominent role in AR signaling and clear prognostic value in prostate cancer, FOXA1 presents a promising target for prostate cancer diagnostics and therapeutics.

V. Coregulatory Proteins in Androgen Signaling

A. Overview of coregulatory proteins and their functions

Due to compact nucleosome assembly of inactive chromatin, chromatin reorganization is an indispensable part of the dynamic transcriptional control by nuclear receptors, including AR. Coregulator is a protein that is recruited directly or indirectly to the genome by DNA-binding transcription factors, participating in a complex that regulates transcription of one or more genes. For the purpose of this review, pioneer/licensing proteins—also termed collaborating factors—that bind directly to DNA through a specific cis-element are not included among the coregulatory proteins. Nevertheless, the above broad definition of coregulatory proteins has resulted in a large number of proteins— over 350 for nuclear receptors— being classified as coregulators (1, 227). This vast repository of potential coregulators clearly exceeds the scope of direct interaction with a single receptor; thus, the most plausible explanation is the action of multiple factors in a sequential and/or combinatorial fashion to influence nuclear receptor-mediated transcriptional regulation. This was exemplified in MCF-7 breast cancer cells showing an ordered ERα-mediated cyclical recruitment of components of the basal transcriptional machinery and coregulators onto the *pS2* gene promoter (228). This ordered recruitment was concomitant with alterations in local chromatin structure accomplished by covalent histone modifications and nucleosome remodeling.

Coregulators ensure the transcriptional outcome by modifying chromatin structure, bridging the proximal and distal components of the transcriptional apparatus, and transducing cellular signals to the site of transcription through enzymatic modifications of histone tails. Coregulatory proteins are classified as coactivators and corepressors on the basis of their functional consequence to transcriptional regulatory processes. It should be noted, however, that this classification seems to be context-dependent. For example, steroid receptor coactivator (SRC) 2 can function as a repressor for estradiol-bound ERα in TNFα-mediated transcription (229) and histone deacetylase 1 as a coactivator for GR-mediated transcription (230). Importantly, this role reversal in coregulator action goes in tandem with the post-translational modification status of the protein, and it emphasizes the importance of coregulator dynamics exerted through this epigenetic switch (227, 231). Another way to classify coregulators is on the basis of the mode of their enzymatic activity on chromatin. Chromatin remodelers catalyze modifications of the histone–DNA interface in an ATP hydrolysis-dependent manner, leading to the loosening of tightly coiled chromatin and local chromatin environment opening for transcription factor binding, or by condensing chromatin structure and promoting gene repression (232). Histone-modifying enzymes, on the other hand, catalyze reversible covalent modifications of histone tails—for instance, acetylation, methylation, phosphorylation, ubiquitylation, and SUMOylation—although the best evidence for serving as nuclear receptor coregulators has been presented for acetylases, deacetylases, methylases, and demethylases (233). As already discussed above, combinations of histone modifications create a histone code that plays a central role in spatiotemporal regulation of DNA-templated processes (57, 234).

Histone acetylation correlates strongly with open chromatin and active enhancers (191), and many coactivator proteins are histone acetylases. Conversely, histone deacetylation is associated with an inactive chromatin state, and many corepressors possess histone deacetylase activity. Histone acetylases that have been reported to interact with AR directly and ligand-dependently, thus enhancing AR-mediated transcription, include two members of the p160/SRC gene family, SRC1 and SRC3 (1, 235–238). Moreover, SRC proteins are capable of recruiting other histone acetylases, such as p300, thus further potentiating the transcriptional activation cascade, and in vitro experiments have demonstrated a direct SRC-independent interaction between p300, CBP, and the AR (239, 240). The association of histone methylation with transcriptional status is strongly context-dependent; for example, LSD1 promotes AR-dependent transcriptional activation by H3K9 demethylation and AR-dependent gene repression through H3K4me2 demethylation (208, 210). Another demethylase, JHDM2A, catalyzes demethylation of mono- and dimethylated H3K9, interacts directly with the AR, and is recruited to regulatory elements of AR target genes in an androgen-dependent manner (241). KDM4B demethylase enhances AR-mediated transcription not only by altering histone methylation status, but also by preventing AR degradation through inhibition of ubiquitination (242). Polycomb protein EZH2, on the other hand, is involved in AR-mediated gene repression through methylation of H3K27 (117, 212), and EZH2 repression signature is linked to patient outcome in metastatic prostate cancer (243).

B. The role of ligand in coregulator recruitment

Coactivators and corepressors both possess LxxLL motifs (where L is leucine and x stands for any amino acid), also known as the nuclear receptor interaction box. Nuclear receptors bound to agonistic ligands recruit coactivators through a similar conserved LxxLL motif within their LBD (244). Ligand binding changes conformation of the LBD inducing repositioning of helix 12 and forming a hydrophobic cleft that constitutes transcriptional activation function 2 (AF-2). By contrast, corepressors such as nuclear receptor corepressor and silencing mediator of retinoid and thyroid receptors, whose recognition to nuclear receptors is mediated by "CoRNR boxes" (245, 246), preferentially interact with unliganded nuclear receptors or receptors bound to antagonists. Interestingly, the nuclear receptor corepressor and silencing mediator of retinoid and thyroid receptors may interact with both agonist-liganded AR and in presence of antagonist and partial antagonists (247). Coregulator complexes that repress transcription are generally recruited to unliganded receptors or receptors bound to inverse agonists through the same hydrophobic groove in receptor LBD, making coactivator/corepressor recruitment mutually exclusive (248, 249). A unique feature of AF-2 within the AR LBD is that it interacts predominantly with the FxxLF motif in the AR amino-terminal region and not with transcriptional coactivators, as is the case with other steroid receptors (250, 251). Thus, AF-1 within the amino-terminal region is the primary AR site for coactivator binding and, unlike other steroid receptors, constitutively active AR lacking the LBD retains transcriptional activity nearly equal to that of the full-length receptor in many but not all promoters (235, 237, 252). And as mentioned previously, AR variants comprising the amino-terminal region and the DBD of the receptor occur frequently in CRPC tissues (130), and the variants may substitute for the full-length AR in these instances.

C. Receptor- and tissue-specificity of coregulatory proteins

Similar to pioneer/licensing factors, coregulators can serve a plethora of transcription factors in a context-specific manner. For example, SRC proteins interact with AR, ER, PR, GR, and thyroid receptor (253, 254), and peroxisome proliferator-activated receptor γ coactivator-1α also interacts with AR and promotes prostate cancer cell growth (255). There are also coregulators that play a role in prostate cancer by increasing AR activity and modifying enhancer chromatin structure (1). Coregulators that are shown to be up-regulated in prostate cancer cells include, for example, p300, SRC1, and ARD1 (256–259).

Studies with tissue-selective steroid receptor modulators have demonstrated that differential expression patterns of coregulators and isoform-selective interactions with steroid receptors can modulate transcriptional outcome (1, 260). For example, tamoxifen-bound ER recruits corepressors in mammary gland cells, but the same complex is capable of stimulating gene expression in uterus, owing to high SRC1 expression levels in the latter tissue (261). There are reports showing gene-specific effects for coregulators; in these instances, coregulator depletion affects only a subset of receptor target genes (262, 263). Genome-wide mapping of chromatin loci associated with SRC proteins and ERα binding identified a subset of estradiol-regulated genes characterized by the presence of SRC3, and not the other SRC family members, that correlated with tamoxifen resistance and breast cancer prognosis (264). An in vivo study utilizing a PR activity indicator mouse crossed with knockout mice for the *SRC* genes showed that the primary PR coactivators are SRC3 in breast tissue and SRC1 in uterus (265). *SRC*-targeted mice also show differential effects on metabolic function

of several tissues (266). These examples, involving signaling systems other than the AR pathway, demonstrate that coregulators can contribute to final tissue-specific transcriptional outcomes. Nevertheless, the vast array of coregulatory proteins is also likely to have functional redundancy and overlapping actions, and therefore, clarification of their defined roles in receptor- and tissue-specific regulation of AR function awaits additional studies.

VI. Concluding Remarks and Future Directions

Transcriptional regulation mediated through regulatory elements, such as promoters and enhancers, is a combinatorial spatiotemporal process achieved by specific binding of transcription factors. In this review, we have covered different aspects of the multipartite regulation of the AR pathway with a particular emphasis on molecular determinants that govern receptor-, cell type-, and tissue-specific responses. Hormone–receptor interaction appears to be the most specific event in steroid action under physiological conditions. Interestingly, the functional steps that follow AR activation by its cognate physiological ligand—that is, holoreceptor interaction with coregulatory proteins, collaborating transcription factors, and the DNA sequence on chromatin—are highly variable and context-specific. The same pioneer factors and coregulators can interact with several steroid receptors, and multiple receptors can bind to the same cis-elements on chromatin. Despite this apparent lack of specificity, these processes ensure nonetheless distinct gene expression profiles in androgen-responsive tissues. An open chromatin environment that permits AR binding to appropriate gene loci is created in a complex interplay of context-specific pioneer factors, histone modifications, and chromatin remodeling enzymes.

ChIP-seq experiments have characterized a large number of nuclear receptor cistromes—especially those of steroid receptors—and emphasized the presence of huge variability in regulatory regions, as well as the importance of pioneer factors and collaborating proteins. However, most studies have dealt with one receptor in isolation or with a few interacting proteins. It would be important to study combinatorial rules of transcription factor occupancy to provide enhancer models operating in the context of steroid receptor cistromes in systems involving multiple receptors and clinically relevant interactions, as exemplified by the recent finding on the interplay of AR and GR pathways in prostate cancer cells (67, 140). Furthermore, enhancer regions resolved by ChIP-seq approaches are still too broad to pinpoint accurate binding site locations of nuclear receptors and their collaborating partners. ChIP-exonuclease experiments (267, 268) can be used to produce high-resolution cistromes and more comprehensive maps of receptor and pioneer factor functions to provide better understanding of the DNA motif grammar and direct or indirect cooperativity between the proteins in different tissues and cell types. Moreover, the mechanisms guiding recruitment of nuclear receptors in other systems, for example, during development and lineage specification, are still largely unknown, as are the determinants and regulators of pioneer factor expression and binding.

Recent studies on transcriptional regulation have revealed several novel concepts and regulatory mechanisms, such as eRNAs (172, 173), super-enhancer structures, topologically associating domains, and boundaries operating at the level of chromatin (269–271). eRNAs are steroid-responsive, suggesting that their formation is an early event in transcriptional regulation. Likewise, other long noncoding RNAs have been shown to play central roles in various aspects of nuclear receptor signaling, including that of AR (272). Characterization of these features for steroid receptors in cell lines, in vivo tissues, xenografts, or cancer tissue specimens has so far been carried out only to a very limited extent. Such experiments should provide important insights into the steroid receptor function and potentially define more accurately tissue-specific cistromes and transcriptomes. Limited availability of starting material often poses an obstacle in these studies, and as a consequence, they have mostly been limited to cancer cell lines. The first reports profiling ERα and AR from breast cancer (216) and prostate cancer patient material (222), respectively, are commendable but also demonstrate the need for methodological developments to harness better clarity and information out of the cistromes.

Assigning defined sets of regulatory elements to a particular transcription unit in vivo is a challenge that requires the use of new technology, such as novel genome editing tools. CRISPR-Cas9 system (273) and transcription activator-like effectors (274) will undoubtedly be used to pinpoint functional importance for the tissue-specific DNA motifs, for example, through introduction of site-specific modifications to enhancer sequences to assess their importance in the context of tissue specificity. Cis-element editing in vivo should eventually elucidate unambiguously the role and importance of a given pioneer factor for the tissue- and cell-specific regulation of the AR pathway. The transcription activator-like effector system combined with LSD1 has already been used to demethylate lysines at a specific enhancer region of the stem cell leukemia locus, leading to decreased expression of proximal genes (275). The TALEN system was recently also used to examine *AR* gene rearrangements in prostate cancer (276).

Structural biological studies will eventually lead to the description of three-dimensional structures for AR protein homodimers, together with receptor–pioneer factor and/or receptor–coregulator complexes. Recently, cryo-electron microscopy was employed to determine the quaternary structure of an active complex of DNA-bound ERα, SRC3, and p300 (277). The use of similar approaches for the AR pathway components will provide better understanding of the structural requirements for AR-specific interactions with auxiliary regulatory proteins.

Tissue-specific determinants of AR function in target tissues other that those dealt with in this review are largely unknown. With regard to muscle—an important target tissue for SARM development—there is limited genome-wide information suggesting that Mef2c, a MADS-box transcription factor, could collaborate with AR in the regulation of androgen target genes in skeletal muscle (278). Androgen regulation of hepatic metabolism (eg, drug metabolism and lipid biosynthesis) bears a clear gender difference. This is mainly due to pulsatile (male liver) vs persistent (female liver) GH secretion from the pituitary, the action of which is mediated through STAT5 signaling in hepatic cells both in rodents and in humans (2, 279, 280). Further studies are required to assess the role of direct androgen action and its potential determinants in liver.

There are reports to indicate that AR elicits non-nuclear regulation of phosphorylation events in prostate cancer cells (eg, Ref. 281). However, the current knowledge pertaining to the role of cell membrane-initiated actions of androgens in relation to receptor- and tissue-specific regulation of the AR pathway in vivo—for example, in prostate cancer tissues—is very limited (3). With regard to ERα signaling, two recent reports (282, 283) addressed this issue by using knock-in mice whose ERα palmitoylation site was mutated, preventing cell membrane attachment of the receptor. The results indicated that membrane-initiated and nuclear ERα actions have different tissue-specific regulatory roles. However, the phenotypes of the knock-in mice in the two studies were vastly different (282, 283), implying the need for additional studies to pinpoint the role of extranuclear ERα—and that of other steroid receptors—in transcriptional regulation of cell- and tissue-specific responses.

In conclusion, genome-wide studies have broadened the gene- and promoter-centric view of transcriptional regulation to comprise vast regulatory networks of tissue-specific enhancers and transcription factor binding events that are dictated by the local chromatin context. Future work utilizing existing and novel approaches is likely to reveal additional regulatory layers and clinically important features of the AR pathway.

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