Molecular Mechanisms of Androgen Receptor Function *In Vivo*

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To my family

ABSTRACT

Androgens are steroid hormones that regulate the development and function of male reproductive organs as well as physiology of many non-reproductive tissues, such as muscle, bone, liver, and kidney. Moreover, androgen signaling is involved in several pathological conditions, most common of which is prostate cancer.

In its target cells, testosterone or its more potent metabolite 5α-dihydrotestosterone regulates cellular processes by modulating gene expression through the androgen receptor (AR). Ligand-activated AR translocates to nucleus and binds to specific DNA sequences, called androgen response elements (AREs), at the regulatory regions of its target genes. AR cistromes, *i.e*., global maps of genomic AR occupancy, comprise thousands of AR-binding events primarily located at distal enhancers. AR-binding sites are characterized by distinct histone modifications, and AR recruitment is primed by pioneer factors capable of binding to compact chromatin. AR interacts with a plethora of coregulatory proteins that modify the local chromatin environment and interact with basal transcription machinery. These elements create the complex cellular landscape for androgen action.

The purpose of this thesis was to study molecular determinants of context-specific AR functions *in vivo* in murine androgen-responsive tissues. The advantage of *in vivo* studies is that – unlike in cancer cell models – androgen target cells reside within their physiological environment with an intact AR pathway. In the first part of this work, an androgen reporter mouse line with the luciferase gene under androgenic control was created. In this model, luciferase activity is a measure of AR function, and it can be used for assessing *in vivo* effects of chemical compounds on AR signaling. The androgen reporter mice were treated with genistein, a phytoestrogenic compound to which people consuming soy products are also exposed. Previous studies have implied that genistein plays a potential role in prostate cancer prevention. The results in this thesis work showed that genistein exhibits tissue-specific effects on AR signaling *in vivo*. Furthermore, genistein modulates endogenous AR-mediated gene expression in prostate, supporting its potentially beneficial role in prostate carcinogenesis.

In the second part of this work, genomic AR occupancy was examined using chromatin immunoprecipitation (ChIP) coupled with massively parallel sequencing (ChIP-seq). Distinct AR cistromes were identified in three androgen-responsive tissues: prostate, kidney, and epididymis. AR-binding events associate with tissue-specific transcription programs responsible for distinct physiological functions of androgens in these tissues. The key finding in this work was that tissue-specific AR binding is directed by divergent pioneer factors, and that previously identified forkhead box protein A1 (FoxA1) is prostate-specific rather than general pioneer factor for AR. Two novel pioneer factors for AR were identified in this study – hepatocyte nuclear factor 4 alpha (Hnf4α) in murine kidney and activating protein 2 alpha (AP-2α) in murine epididymis.

ChIP-seq was also utilized to study *in vivo* role and characteristics of selective AREs – *cis*-elements not bound by other steroid receptors. Transgenic SPARKI mice have the second zinc finger of the AR DNA-binding domain swapped with the respective part of glucocorticoid receptor, resulting in a chimeric AR unable to bind to selective AREs. A significant proportion of *in vivo* binding events of wild-type AR were not shared by SPARKI AR in prostate and epididymis, highlighting the importance of selective AREs in AR-specific functions *in vivo*. Differential receptor binding was also linked to differentially expressed genes in the epididymides of wild-type and SPARKI mice. *De novo* sequence analysis revealed that the selective AREs are characterized by decreased sequence conversation, indicating that, counter-intuitively, AR selectivity *in vivo* is achieved by relaxed rather than increased *cis*-element stringency.

In conclusion, both the AREs and the collaborating proteins contribute to precise AR-regulated transcriptional outcome in the context of native chromatin and distinct tissues. Overall, the results clarified several molecular mechanisms employed by AR *in vivo* that can potentially contribute to the development of better treatments and diagnostic tools for hormone-dependent disorders in the future.

YHTEENVETO

Steroidihormoneihin kuuluvat androgeenit säätelevät sekä miehen lisääntymiselinten kehittymistä ja toimintaa että useiden muiden kudosten, kuten munuaisten, lihasten ja luiden fysiologiaa. Normaalien fysiologisten säätelytehtäviensä lisäksi androgeenit vaikuttavat myös useiden sairauksien kehittymiseen, joista yleisin on eturauhassyöpä.

Androgeenit säätelevät kohdesolujensa toimintaa aktivoimalla tai hiljentämällä tiettyjen geenien luentaa androgeenireseptorin (AR) välityksellä. AR on ligandin (hormonin) indusoima transkriptiotekijä, joka sitoutuu kohdegeeniensä säätelyalueille tunnistaen tietyn DNA-jakson, jota kutsutaan androgeenien vaste-elementiksi. AR:n vaikutus geenien luentaan välittyy vuorovaikutuksessa muiden säätelyproteiinien kanssa, ja yhdessä nämä muuttavat kromatiinin rakennetta ja toimivat transkriptiokoneiston kanssa. Aiemmat tulokset eturauhasen syöpäsoluista ovat osoittaneet, että AR sitoutuu tuhansiin säätelyelementteihin, jotka sijaitsevat tyypillisesti kaukana geenien promoottorialueista. Ennen reseptoria näille säätelyalueille sitoutuu usein ns. pioneeritekijä, eli proteiini, jonka ajatellaan avaavan tiukkaan paketoitua kromatiinirakennetta AR:n sitoutumista varten.

Tässä väitöskirjassa AR:n toimintaa tutkittiin hiiren androgeenivasteisissa kudoksissa erilaisten hiirimallien ja modernien menetelmien avulla. Kromatiini-immunosaostukseen yhdistetyllä massiivisen rinnakkaisella DNA:n sekvensoinnilla (ChIP-seq) voidaan analysoida transkriptiotekijän DNA:n sitomispaikkoja koko genomin laajuudessa. Syöpäsoluviljelmiin verrattuna hiirikokeiden vahvuus on niiden mahdollistama AR:n vaikutusten tutkiminen fysiologisessa, monia solutyyppejä sisältävässä toimintaympäristössä.

Tässä väitöskirjatyössä valmistettiin uusi siirtogeeninen hiirimalli, jossa androgeenit säätelevät ns. raportoijageenin ilmentymistä. Raportoijageenin aktiivisuutta mittaamalla voidaan tutkia erilaisten kemiallisten yhdisteiden vaikutusta AR:n toimintaan elävän hiiren kudoksissa. Raportoijahiiret altistettiin kasviestrogeeni genisteiinille, jota myös ihmiset saavat soijavalmisteita sisältävästä ravinnosta. Aiempien tutkimustulosten

mukaan genisteiini saattaa alentaa riskiä sairastua eturauhassyöpään. Tämän työn tulokset osoittivat, että genisteiini vaikuttaa AR:n signalointiin kudosspesifisellä tavalla. Lisäksi havaittiin genisteiinin säätelevän AR-riippuvaisten geenien aktiivisuutta eturauhasessa, mikä tukee hypoteesia sen vaikutuksista eturauhassyövän kehittymiseen.

Androgeenialtistuksen aikaansaamat AR:n genominlaajuiset sitoutumisprofiilit havaittiin hyvin erilaisiksi hiiren eturauhasessa, munuaisessa ja lisäkiveksessä. AR:n sitoutumispaikat liittyivät kudoskohtaisten geenien ilmentymiseen, selittäen androgeenien erilaisia fysiologisia vaikutuksia näissä kudoksissa. Aiemmissa soluviljelytutkimuksissa on havaittu FoxA1-proteiinin toimivan AR:n pioneeritekijänä ja toiminnan säätelijänä. Yksi tämän työn tärkeistä havainnoista on, että AR:n sitoutumista ohjaavat eri pioneeritekijät eri kudoksissa ja että FoxA1:n vaikutus rajoittuu eturauhaseen. Tässä työssä tunnistettiin kaksi uutta pioneeritekijää AR:lle: Hnf4α munuaisessa ja AP-2α lisäkiveksessä.

Muut steroidireseptorit eivät sitoudu AR-selektiivisiin vaste-elementteihin *in vitro*-olosuhteissa, ja tässä työssä niitä tutkittiin *in vivo* siirtogeenisen SPARKI-hiiren ja ChIP-seq -menetelmän avulla. SPARKI-hiiren AR on kimeerinen proteiini, jonka DNA:ta sitovan osan toinen sinkkisormi on vaihdettu glukokortikoidireseptorin vastaavaan osaan, minkä johdosta SPARKI-AR ei sitoudu AR-selektiivisiin elementteihin. Merkittävä osa villityypin AR:n genominlaajuisista sitoutumispaikoista eturauhasessa ja lisäkiveksessä todettiin AR-selektiivisiksi *in vivo* -olosuhteissa. Yksi tämän työn mielenkiintoisista havainnoista on, että AR-selektiivisen DNA-sitoutumisen mahdollistaa reseptorin kyky sitoutua emäsjärjestykseltään vaihtelevampiin vaste-elementteihin kuin muut steroidireseptorit.

Yhteenvetona tässä väitöskirjatyössä osoitettiin, että androgeenien aktivoiman AR:n vuorovaikutus säätelyalueiden vaste-elementtien ja kudosspesifisten pioneeritekijöiden kanssa on edellytys fysiologisesti tarkoituksenmukaiselle geenien ilmentymiselle. Uudet havainnot AR:n toiminnan molekyylimekanismeista voivat tulevaisuudessa mahdollistaa entistä parempien hoitomuotojen kehittämisen ihmisen hormoniriippuvaisiin sairauksiin.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications that will be quoted in the text as communications I, II, and III.

I. **Päivi Pihlajamaa**, Fu-Ping Zhang, Lilli Saarinen, Laura Mikkonen, Sampsa Hautaniemi and Olli A. Jänne (2011) The phytoestrogen genistein is a tissue-specific androgen receptor modulator. Endocrinology *152*, 4395-4405.

II. **Päivi Pihlajamaa***, Biswajyoti Sahu*, Lauri Lyly, Viljami Aittomäki, Sampsa Hautaniemi and Olli A. Jänne (2014) Tissue-specific pioneer factors associate with androgen receptor cistromes and transcription programs. EMBO Journal *33,* 312–326.

III. Biswajyoti Sahu*, **Päivi Pihlajamaa***, Vanessa Dubois, Stefanie Kerkhofs, Frank Claessens and Olli A. Jänne (2014) Androgen receptor uses relaxed response element stringency for selective chromatin binding and transcriptional regulation *in vivo*. Nucleic Acids Research, in press (Epub ahead of print, PMID 24459135)

* Equal contribution

ABBREVIATIONS

AF	activation function
AIS	androgen insensitivity syndrome
AP	activating protein
AR	androgen receptor
ARE	androgen response element
ARKO	androgen receptor knockout
ATP	adenosine triphosphate
ChIP-seq	chromatin immunoprecipitation coupled to deep sequencing
CRPC	castration-resistant prostate cancer
DBD	DNA-binding domain
ER	estrogen receptor
Fox	forkhead box
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HAT	histone acetyl transferase
HDAC	histone deacetylase
Hnf	hepatocyte nuclear factor
HRE	hormone response element
LBD	ligand-binding domain
LSD	lysine-specific demethylase
MR	mineralocorticoid receptor
NR3C	nuclear receptor subgroup 3C
NTD	amino (NH_2)-terminal domain
PBX1	pre-B-cell leukemia homeobox 1
PPAR	peroxisome proliferator-activated receptor
PR	progesterone receptor
PTM	post-translational modification
SBMA	spinal and bulbar muscular atrophy
siRNA	small interfering RNA
Slp	sex-limited protein
SPARKI	specificity-affecting androgen receptor knock-in
SRC	steroid receptor coactivator
TBP	TATA-binding protein
Tfm	testicular feminization
TR	thyroid hormone receptor

INTRODUCTION

Androgens orchestrate the development of male phenotype and serve as important physiological regulators in many non-reproductive tissues. Many developmental events require androgens during a discrete time window, whereas reproductive functions are androgen-dependent throughout the life. Androgen receptor (AR), the mediator of androgen action, belongs to the nuclear receptor superfamily with distinct structural domains that define their molecular action as ligand-inducible transcription factors.

The essential function of androgen-activated AR is to bind to its response elements at the regulatory regions of the AR target genes, and to induce or repress their transcription in collaboration with coregulatory proteins and transcription machinery. The overall picture of nuclear receptor action, however, involves interplay of multiple signaling pathways in the cellular environment. Cellular steroid metabolism, as well as 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 effects 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 growth hormone.

The physiological importance of AR pathway and its role in human pathological conditions make it pertinent to study AR action. Moreover, ligand-inducible AR signaling provides an interesting pathway that potentially reveals new insights into general mechanisms and molecular determinants of transcriptional regulation. Full appreciation of the complexity of biological signaling requires *in vivo* studies in a physiological context. Vast knowledge on mouse genomics and physiology as well as sophisticated genome manipulating methods underline the benefits of the mouse as a model system. In this thesis work, state-of-the art methods have been utilized to study *in vivo* aspects of androgen-dependent transcriptional regulation in murine tissues.

REVIEW OF THE LITERATURE

1. Signal transduction and nuclear receptors

1.1 Overview of signal transduction

Development and function of multicellular organisms rely on specific and timely communication between the cells. Individual cells within an organism share the same genome, but display highly variable morphology, life span and functional characteristics. By and large, biological signaling utilizes extracellular molecules that are recognized by specific receptor proteins located on the plasma membrane or within the target cells. Autocrine or intracrine signals affect the same cell that produces the signal molecules, paracrine signals are transmitted to nearby cells, and endocrine signals, the hormones, are distributed to the whole organism *via* bloodstream. The receptor proteins bind distinct chemical entities determined by their molecular structure and activate intracellular signaling pathways leading to changes in cellular metabolism and growth. Ligands are usually small molecules such as peptides, neurotransmitters, hormones, dietary and metabolic compounds, or toxins. Agonistic ligands activate the receptor, whereas pure antagonists bind the receptor without activating it, thus preventing activation by agonist binding. Inverse agonists, on the other hand, induce responses that are opposite to the agonist effect. Under the physiological conditions, many compounds exhibit more diverse effects on receptors with a range of agonistic and antagonistic properties, depending on the cellular context. Complex interplay of various signaling pathways and intracellular conditions creates distinct cellular phenotypes during development and results in a multicellular organism capable of adaptation and responsiveness.

1.2 Intracellular signaling by nuclear receptors

A distinct class of intracellular signaling molecules, the nuclear receptor superfamily, comprises transcription factors that share structural and functional characteristics. Modular structure of nuclear receptors with separate domains for DNA- and ligand-binding enables them to act as molecular switches that recognize endocrine or metabolic signals and mediate their effects to cellular processes by binding to DNA and modulating

gene expression (Nagy and Schwabe, 2004). The human and mouse genomes include 48 and 49 genes encoding nuclear receptors, respectively, and their physiological functions encompass metabolism and energy homeostasis, as well as reproduction, development, and growth (Bookout *et al*., 2006). Classically, nuclear receptors have been divided into three categories: steroid hormone receptors (Class I), such as estrogen receptor (ER) and AR; receptors with non-steroidal ligands and with ligands that are lipid metabolites, such as thyroid hormone receptor (TR) and peroxisome proliferator-activated receptors (PPARs), respectively (Class II); and orphan receptors with no recognized ligand (Class III). However, ligands have been identified for numerous orphan receptors in recent years, and an alternative classification based on evolutionary conservation and sequence homology distinguishes six subfamilies of nuclear receptors (Germain *et al*., 2006).

The modular structure characteristics of nuclear receptors involve four distinct domains (Figure 1): amino-terminal domain (NTD, A/B domain), DNA-binding domain (DBD, C-domain), hinge region (D-domain), and ligand-binding domain (LBD, E-domain) (Aranda and Pascual, 2001). NTD is the most variable domain both in size and sequence, and it usually contains a ligand-independent transcriptional activation function (AF-1) that can operate autonomously. DBD, the most conserved domain of the nuclear receptors, makes specific high-affinity contacts with DNA using two zinc fingers and is also responsible for receptor dimerization upon activation. Hinge region connects DBD and LBD, and it usually contains the nuclear localization signal. LBD of each receptor binds its physiological ligand with high specificity due to variability in the size, shape and distribution of polar and non-polar residues within the ligand-binding pocket (Jin and Li, 2010). LBD also harbors AF-2, whose conformation and activation are strongly dependent on ligand binding.

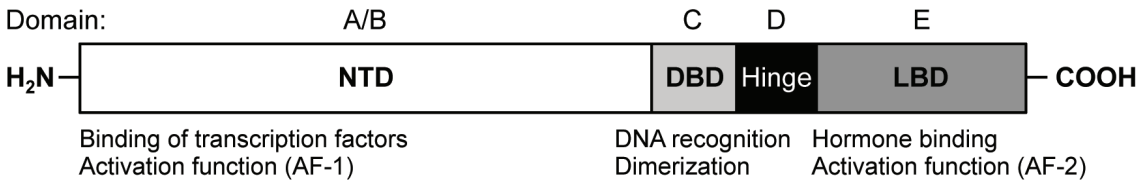

Figure 1. Functional domains of nuclear receptors with their main functions highlighted. Amino-terminal domain (NTD), DNA-binding domain (DBD), ligand-binding domain (LBD).

Unliganded nuclear receptors reside either in the cytoplasm or in the nucleus. Thus, their ligands have to be small hydrophobic compounds that are able to diffuse through the plasma membrane. Ligand binding activates the receptor and promotes its interaction with coactivator proteins followed by recruitment of basal transcription machinery, and ligand-inducible gene activation. Many Class II nuclear receptors such as TR and retinoic acid receptor are constitutively bound to chromatin and associate with transcriptional repressors in the absence of a ligand, leading to active gene repression (Germain *et al*., 2006). Unliganded steroid receptors, however, reside preferably in the cytoplasm and ligand binding induces their nuclear translocation, leading to both gene activation and repression through interaction with coactivators and corepressors, respectively. In transrepression, a nuclear receptor inhibits gene expression through other transcription factors. For example, glucocorticoid receptor (GR) suppresses inflammation by tethering to nuclear factor kappa B, thus preventing coregulator binding (Glass and Saijo, 2010). The precise transcriptional outcome achieved by nuclear receptors requires contributions from chromatin remodelers, histone and DNA modifying enzymes, collaborating transcription factors, coregulators, and basal transcriptional machinery, which will be discussed in the following chapters of this literature review.

1.3 Nuclear receptors and coregulators

Eleven alpha helices constitute the ligand-binding pocket of the nuclear receptor LBD. Helix 12 forms a mobile lid over the pocket, and its conformation is determined by the presence or absence of the ligand. Agonist binding exposes LxxLL motif (where L is leucine and x is any amino acid) for protein-protein interaction. As a result, nuclear receptor dissociates from corepressors and recruits coactivators, many of which also contains LxxLL motifs that interact with the LBD (Heery *et al*., 1997). Coactivators then modify local chromatin environment and interact with the basal transcription machinery promoting formation of the preinitiation complex at the gene promoter. Histone acetylation correlates strongly with active, open chromatin, and many coactivator proteins are histone acetylases (HAT). Conversely, histone deacetylation is associated with inactive chromatin state, and many corepressors possess histone deacetylase (HDAC) activity. To date, over 350 nuclear receptor coregulators have been reported in

the literature (Lonard and O'Malley, 2012). Typically, they form large complexes at gene regulatory regions collaborating with nuclear receptors in a cell-type and DNA-binding site-dependent manner (Rosenfeld *et al*., 2006). Moreover, classification into coactivators and corepressors seems to be context-dependent. For instance, HDAC1 that is a classical corepressor due to its histone deacetylase activity was reported to serve as coactivator for GR (Qiu *et al*., 2006), and steroid receptor coactivator (SRC)-2 was found to be an ERα-corepressor in tumor necrosis factor α-mediated transcription (Cvoro *et al*., 2006).

Compact nucleosomal assembly of inactive chromatin hinders all DNA-templated processes. Thus, chromatin reorganization is an indispensable part of the dynamic transcriptional control by nuclear receptors. Coregulators can be divided into functionally divergent classes depending on their role in chromatin reorganization (Kato *et al*., 2011). First, chromatin remodelers modify the histone-DNA interface by using the energy from adenosine triphosphate (ATP) hydrolysis, leading to loosening of tightly coiled chromatin and generating local chromatin environment open for transcription factor binding, or by condensing chromatin structure and promoting gene repression (Clapier and Cairns, 2009). The second group comprises enzymes that covalently modify histones. Various reversible post-translational modifications (PTMs) have been identified at histone tails – for instance acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation – but evidence for serving as nuclear receptor coregulators has mostly accumulated for acetylases, deacetylases, methylases, and demethylases (Kato *et al*., 2011). The coregulator proteins itself, as well as nuclear receptors, are subject to similar enzymatic PTMs that regulate their activities and function, adding another layer of complexity to the context-dependent transcriptional regulation and prospects for crosstalk with other signaling pathways.

1.4 Physiological functions of nuclear receptors

Nuclear receptors are ubiquitous in the metazoan kingdom, but absent in fungi and plants. They regulate a wide range of physiological processes from reproduction, development, and growth to nutrient uptake, metabolism, and excretion (Figure 2), and each receptor has important and nonredundant functional roles (Germain *et al*., 2006). Nuclear

receptors involved in reproductive functions include, for example, Class I receptors for sex steroids – estrogens, progestins, and androgens – that govern gender-specific traits and sexual reproduction, as well as Class III orphan receptors – steroidogenic factor-1 (SF-1) and DAX-1 (dosage-sensitive sex reversal-adrenal hypoplasia congenital critical region on the X chromosome, gene 1) – that regulate steroidogenesis and other reproductive functions both in adults and during embryonic development (Achermann *et al*., 2001). Various nuclear receptors are key regulators of metabolic pathways, such as vitamin D receptor (VDR) for dietary calcium uptake and metabolism, farnesoid X receptor α (FXRα) for bile acid metabolism, estrogen-related receptor α (ERRα) for oxidative gene expression driving mitochondrial energy utilization, and TRβ for body temperature and fatty acid and cholesterol metabolism (Bookout *et al*., 2006).

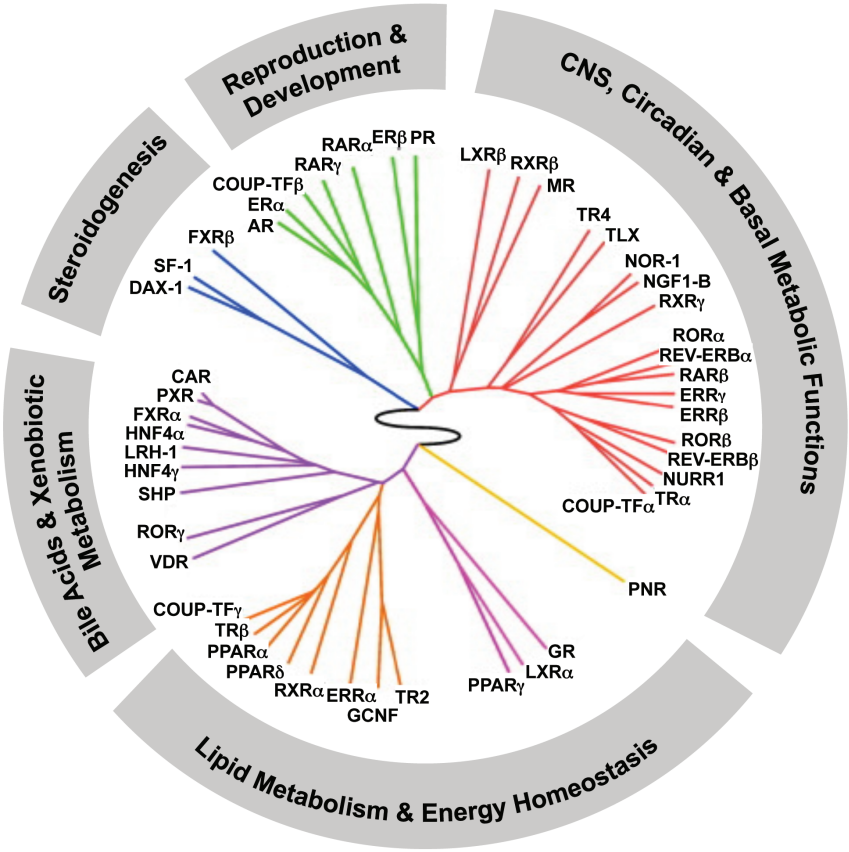

Figure 2. Expression–function relationship of nuclear receptors. Physiological functions of the 49 murine nuclear receptors identified on the basis of their expression profiles in 39 tissues. CNS, central nervous system. Reprinted from Bookout *et al*., Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network, Cell 126:789–799, 2006, with permission from Elsevier (copyright 2006).

GR is also an important regulator of metabolism promoting lipolysis and gluconeogenesis, as opposed to PPARγ that supports actions of insulin and facilitates fat storage. Both GR and PPARγ are also important anti-inflammatory factors highlighting the integration of metabolic and immune systems as a central homeostatic mechanism, dysfunction of which can lead to chronic metabolic disorders, such as obesity, type 2 diabetes and cardiovascular disease (Hotamisligil, 2006). Specific endocrine organs produce the steroid and thyroid hormones that serve as high-affinity ligands for their cognate receptors, capable of eliciting maximal physiological responses at nanomolar concentrations. However, along with the discovery of ligands for many of the "orphan" receptors, it has become clear that nuclear receptors can also function as sensors for either metabolic or environmental signals (Sladek, 2011). For instance, retinoid receptors bind vitamin A formed from exogenously obtained β-carotene, and the endogenous ligands for PPARs – various fatty acid derivatives – are metabolic signals available at micromolar range.

Important physiological roles of nuclear receptors also link them to many pathological conditions, and the ligand-binding pocket that regulates nuclear receptor functions makes them appealing targets for drug development. For instance, sex steroids are involved in pathogenesis of hormone-dependent malignancies, and compounds that antagonize estrogenic and androgenic effects are widely used to treat breast and prostate cancer, respectively. Glucocorticoids are effective immunosuppressants in various conditions, and modulators of ER and progesterone receptor (PR) can be used as hormonal contraceptives. Ligands for nuclear receptors that regulate metabolic pathways hold great promise for treating metabolic disorders. The PPARγ agonist rosiglitazone is an effective insulin sensitizer in type 2 diabetes, although the occurrence of severe side effects has limited its clinical use. Better understanding of nuclear receptor function and mechanistic insight provided by modern research methods can help in developing new drugs targeting this versatile family of transcription factors.

2. Transcription

2.1 Gene transcription and transcription factors

Genomic DNA encompasses hereditary information in two fundamental ways: transcribed regions provide template for proteins and functional RNA molecules, and regulatory DNA elements contain instructions for their appropriate expression patterns. A gene can be defined as a unit of heredity comprising the transcription unit and regulatory elements needed for producing a single protein or RNA. However, delineating a comprehensive set of regulatory elements corresponding to expression of a defined transcription unit is not straightforward and is a subject to intensive research efforts. The ENCODE Consortium has characterized functional elements in 147 cell types, and recently reported that 80% of the genome contains elements linked to biochemical functions (The ENCODE Project Consortium, 2012), highlighting the complexity and versatility of gene regulatory elements. Moreover, the cumulative coverage of transcribed regions across the human genome is about 75%, and the genes are highly interlaced with overlapping transcripts that are synthesized from both DNA strands (Djebali *et al*., 2012).

Regulatory elements of eukaryotic genes – promoters, enhancers, silencers, and insulators – ensure precise, cell type- and context-specific gene transcription. Regulatory regions contain *cis*-acting DNA elements that recruit *trans*-acting regulatory proteins, such as nuclear receptors, that bind to these elements establishing a network of gene regulation. Promoters are DNA sequences located next to transcription start site that direct RNA polymerase to initiate transcription at the correct place. Distal regulatory elements, enhancers and silencers, are defined by their functional consequence to gene regulation, and they can act independently of position or orientation, from a distance of hundreds of kilobases or even from different chromosomes (Lomvardas *et al*., 2006). Enhancers serve as binding platforms for lineage-specific transcription factors and sequence-specific effectors of signaling pathways, integrating information about intracellular conditions and cellular environment to combinatorial response in gene expression patterns (Calo and Wysocka, 2013). Distant enhancers dictate cell type-specific gene activation in both human and mice, as shown by recent genome-wide studies of transcription factor

cistromes and histone modifications (Heintzman *et al*., 2009; Shen *et al*., 2012). Insulators impede the effects of long-range regulatory elements by blocking the enhancer activity to unrelated promoters, and the vertebrate insulators are characterized by CCCTC-binding factor binding (Bell *et al*., 1999).

2.2 Basal transcriptional apparatus

All eukaryotic protein-coding genes are transcribed by RNA polymerase II, a large 12-subunit enzyme complex that is assisted by general transcription factors. Sequence-specific transcription factors bound to enhancers and promoters initiate a complex and highly dynamic process of gene activation that culminates in the formation of a preinitiation complex containing over 100 individual protein subunits. A protein complex known as mediator allows distal enhancer sites to get in contact with a gene promoter. Formation of the preinitiation complex at the core promoter is initiated by TATA-binding protein (TBP), followed by ordered assembly of other general transcription factors and RNA polymerase II. Classical *cis*-element for TBP is the so-called TATA box, but recent genome-wide analysis identified a 50-bp signature sequence at gene promoters that defines TBP binding and the preinitiation complex formation to be enriched with mostly other sequence motifs than the TATA box (Neph *et al*., 2012). The core promoter determines the point and direction of transcription initiation, and general transcription factors then orchestrate unwinding of double-stranded DNA and enable transcriptional initiation and elongation by RNA polymerase II in the context of chromatin and nucleosomes (Barski *et al*., 2007; Li *et al*., 2012a).

2.3 Chromatin structure and function

Eukaryotic DNA is tightly packaged inside the nucleus in the form of chromatin (Kornberg, 1974). A nucleosome comprises two copies of each of the canonical histone proteins (H3, H4, H2A and H2B) or their variants, and a 147-bp stretch of DNA wrapped around them. Nucleosomes are joined together by linker histones, and the string of nucleosomes is packaged to higher-order structures (Schwarz and Hansen, 1994). The compact structure of chromatin occludes gene transcription and other DNA-templated

processes, and the dynamic nature of chromatin provides an important regulatory step in making DNA accessible to these activities (Banaszynski *et al*., 2010).

Nucleosome positioning, *i.e*., the location of nucleosomes along the DNA stretch, is determined by several factors: the DNA sequence, ATP-dependent nucleosome remodelers, and transcription factors, such as activators, components of preinitiation complex, and the elongating RNA polymerase II (Bell *et al*., 2011). DNA sequence that is stiff in structure, such as homopolymers of identical bases, is resistant to nucleosome formation, whereas more a bendable sequence makes more stable contact with the histone octamer and is more likely to be involved in nucleosomal structure (Segal *et al*., 2006). Chromatin remodeling enzymes utilize energy from ATP hydrolysis to change the chromatin structure by sliding, evicting or restructuring the nucleosomes, thus contributing to nucleosome packaging and spacing as well as regulatory mechanisms of transcription (Clapier and Cairns, 2009). Other mechanisms altering chromatin structure are the use of covalent histone modifications (Li *et al*., 2007) and less stable histone variants (Banaszynski *et al*., 2010). Transcription start sites often reside in nucleosome-free regions (Segal *et al*., 2006), and especially H3.3 and H2A.Z double-variant histones-containing nucleosomes are found flanking transcription start sites of active genes (Jin *et al*., 2009). Labile H2A.Z variant is also enriched at distal enhancers, as demonstrated by a recent study in a prostate cancer cell line with the ligand-inducible AR binding leading to eviction of the central nucleosome (He *et al*., 2010).

Histone proteins possess unstructured amino-terminal tails that are subject to highly variable covalent modifications. Multiple residues in each histone can be modified, and the diversity of modifications includes acetylation, phosphorylation, methylation, deimination, *β-N*-acetylglucoseamination, adenosine diphosphate ribosylation, ubiquitylation, and sumoylation. One mechanism by which the modifications alter the chromatin structure is to change the net charge of nucleosomes. Acetylation of lysines and phosphorylation of serine, threonine, and tyrosine residues reduce the positive charge of the histones and thus loosen electrostatic interactions between histones and DNA, leading to less compact chromatin structure. Thus, histone acetylation is often associated

with transcription factor-binding sites and transcriptionally competent environment, and *in vivo* binding of the histone acetylase p300 demarcates active, tissue-specific enhancers (Visel *et al*., 2009). Secondly, combinations of histone modifications can serve as binding platforms for various non-histone proteins creating a histone code that regulates DNA-templated processes (Strahl and Allis, 2000). The map of histone modifications is brought about by enzymes that catalyze addition or removal of specific modifications in a residue-specific manner. The histone code is interpreted into meaningful biological outcome by specific proteins, such as chromatin remodeling enzymes that recognize selective combinations of modified histones and subsequently drive distinct nuclear processes (Calo and Wysocka, 2013; Li *et al*., 2007). As mentioned in Chapter 1.3, many of the histone modifying enzymes are nuclear receptor coregulators that are recruited to gene regulatory elements by hormone-dependent transcription factor binding.

Genome-wide mapping of various histone modifications and their correlation to genomic features and transcription factor-binding sites has greatly improved our understanding about the epigenetic map encoded in histone tails. Especially lysine methylation plays an important role in creating the histone code, as 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 (Bannister and Kouzarides, 2011). Active enhancers are characterized by monomethylation of lysine 4 in histone 3 (H3K4me1), whereas trimethylation of the same residue (H3K4me3) is predominantly found at gene promoters (Barski *et al*., 2007; Heintzman *et al*., 2007). Conversely, methylation of lysines 9 and 27 in histone 3 is associated with transcriptional repression (Roh *et al*., 2006). Thus, the functional consequence of histone methylation is highly dependent on the chromatin context and the modified residue. This is also demonstrated by the diverse roles of lysine-specific demethylase 1 (LSD1) in AR signaling: LSD1 promotes AR-dependent transcriptional activation by demethylating lysine 9 in histone 3 (H3K9) (Metzger *et al*., 2005), but it was recently shown to repress *AR* gene expression in an androgen-dependent fashion through H3K4me2 demethylation (Cai *et al*., 2011). Summary of covalent histone modifications and their putative roles in gene transcription is presented in Table 1.

Table 1. Covalent histone modifications involved in transcriptional regulation. Adapted from Li *et al*., The role of chromatin during transcription, Cell 128:707–719, 2007, with permission from Elsevier (copyright 2007) and from The ENCODE Project Consortium, An integrated encyclopedia of DNA elements in the human genome, Nature 489:57–74, 2012, under Creative Commons license (copyright 2012).

Modification	Putative functions
H3K4me1	Mark of enhancers and other distal elements, but also enriched downstream of TSS
H3K4me2	Mark of regulatory elements associated with promoters and enhancers
H3K4me3	Mark of regulatory elements primarily associated with promoters/TSS
H3K9ac	Mark of active regulatory elements with preference for promoters
H3K9me1	Preference for the 5′ end of genes
H3K9me3	Repressive mark associated with constitutive heterochromatin and repetitive elements
H3K27ac	Active mark; may distinguish active enhancers and promoters from inactive ones
H3K27me3	Repressive mark established by polycomb complex activity
H3K79me2	Transcription-associated mark, with preference for 5′ end of genes
H3R2,R17,R26me	Transcriptional activation
H4K20me1	Preference for 5′ end of genes
H4K16ac	Transcriptional activation
H2AK119ub	Transcriptional repression
H2BK120/K123ub	Transcriptional activation

TSS=transcription start site, me=methylation, ac=acetylation, ub=ubiquitylation

2.4 Genome-wide approaches to study transcription

Massively parallel next-generation sequencing has revolutionized the ways by which transcription can be studied. The previous gene-centric approaches concentrated on a few genes and genomic loci at a time, whereas massively parallel sequencing allows an unbiased genome-scale analysis of DNA fragments in a quantitative fashion, generating a global picture of chromatin state, histone modifications, or transcription factor binding. Chromatin immunoprecipitation (ChIP) is a method where a transcription factor or other chromatin-associated protein is enriched from cross-linked chromatin using a specific antibody (Solomon *et al*., 1988), and the DNA co-precipitated with the protein of interest is then analyzed by using region-specific primers (ChIP-qPCR), by hybridizing fluorescently labeled DNA to genomic microarrays (ChIP-on-chip) (Ren *et al*., 2000), or

by high-throughput DNA sequencing (ChIP-seq) (Barski *et al*., 2007; Johnson *et al*., 2007). Cistrome is a term for a set of *cis*-acting targets of a *trans*-acting factor on a genome-wide scale, usually determined using ChIP-seq. In addition, there are several high-throughput methods for analyzing nucleosome occupancy and DNA accessibility (DNaseI-seq, FAIRE-seq), nucleosome positioning (MNase-seq) and long-range chromatin interactions (Bell *et al*., 2011) (Figure 3). Combined with gene expression profiles generated using either array technology or RNA-sequencing, next-generation sequencing approaches provide a comprehensive view of transcriptional landscape in cells or tissues of interest under defined conditions (Zhou *et al*., 2011). The wealth of data produced by high-throughput experiments has greatly improved our understanding of transcriptional regulation. However, the present genome-wide methods study cell populations at a fixed time-point, and getting a more dynamic picture of transcriptional events remains a future challenge (Coulon *et al*., 2013). In this thesis, genome-wide methods have been utilized to study androgen-dependent transcription programs.

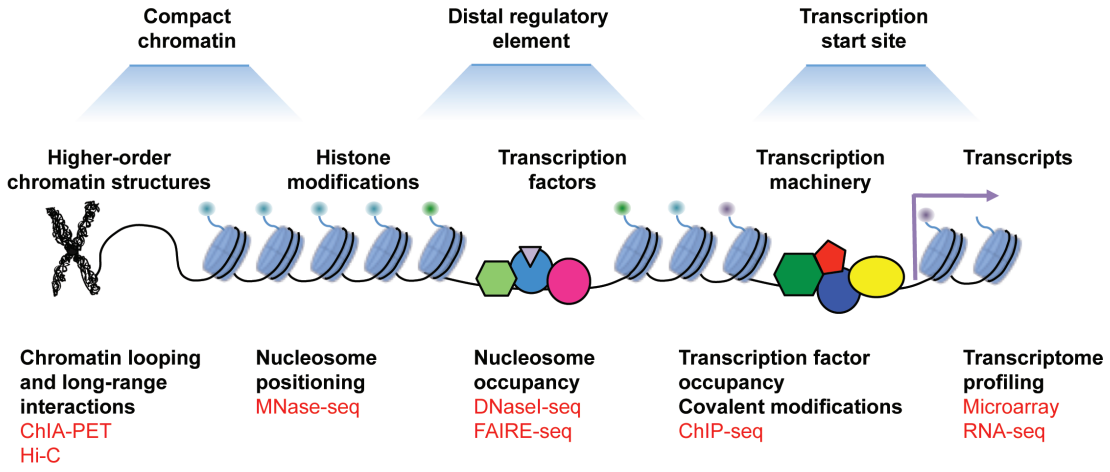

Figure 3. Chromatin structure, gene regulatory elements and genome-wide methods to study transcription (methods highlighted in red). Chromosomal DNA is protected from degradation by endonucleases, whereas linker regions are digested by micrococcal nucleases (MNase) and regulatory DNA elements are sensitive to DNase I digestion. In formaldehyde-assisted identification of regulatory elements (FAIRE) nucleosomal DNA is cross-linked and nucleosome-free accessible DNA analyzed. Genome-wide maps of transcription factor binding and chromatin modifications can be obtained using ChIP-seq with specific antibodies, and gene expression profiles by RNA-seq or microarrays. Chromosome conformation capture assay (Hi-C) reveals higher-order chromatin structures using restriction digestion and ligation of cross-linked chromatin, and high-throughput data is achieved by paired-end tag sequencing (ChIA-PET).

3. Steroid receptor-mediated signaling

3.1 Steroid hormones and receptors

Steroid hormones are small lipophilic molecules derived from cholesterol in a series of enzymatic reactions catalyzed by various cytochrome P450 and hydroxysteroid dehydrogenase enzymes (Payne and Hales, 2004). Glucocorticoids and mineralocorticoids are synthetized in adrenal cortex, and progesterone, estradiol, and testosterone mainly in gonads. Steroid hormones act as chemical messengers modulating a wide variety of physiological processes, and they exert their functions by binding with high affinity and high specificity to their cognate receptors: ERα, ERβ, PR, AR, GR, and mineralocorticoid receptor (MR). The primary mode of steroid receptor action is through genomic regulation of the target gene expression, but there is a growing body of evidence for non-genomic steroid receptor actions through cytoplasmic signaling pathways. However, these rapid effects appear to have a minor role in androgen signaling and have not yet been well-characterized. The chemical structures of steroids are very similar (Figure 4), and as lipophilic compounds, they are transferred in circulation bound to albumin and high-affinity proteins, corticosteroid-binding globulin and sex hormone-binding globulin (Hammond, 2010).

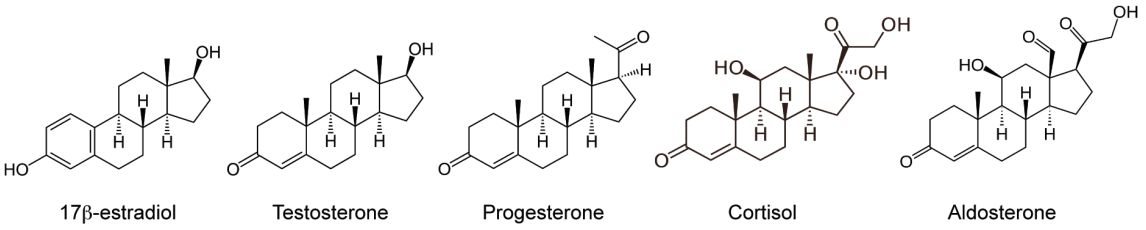

Figure 4. Chemical structures of primary physiological steroid hormones in human.

Structural and mechanistic data accumulated since cloning of the first steroid receptors, GR (Hollenberg *et al*., 1985) and ER (Green *et al*., 1986; Greene *et al*., 1986), have expanded our understanding of hormone action and gene regulation by sequence-specific transcription factors. Evolutionarily, steroid receptors appear to originate from a single ER-like ancestral receptor, and the six vertebral steroid receptor genes are products of gene duplications (Eick and Thornton, 2011). GR, MR, PR, and AR – the subclass NR3C of the nuclear receptor superfamily – have evolved to bind various 3-ketosteroids,

intermediates of estradiol synthesis, by molecular exploitation (Eick and Thornton, 2011). Estradiol, progesterone, and testosterone are critical regulators of reproductive functions through their cognate receptors, ERα, ERβ, PR, and AR. Endogenous glucocorticoids, cortisol in human and corticosterone in rodents, mediate their effects to various metabolic and inflammatory processes through GR. Mineralocorticoids, such as aldosterone, affect transepithelial sodium transport and regulate blood pressure and sodium homeostasis by increasing sodium reabsorption in the nephrons. As opposed to high ligand-specificity of other steroid receptors, MR binds both cortisol and aldosterone with a similar affinity. Mineralocorticoid selectivity is achieved by conversion of cortisol to cortisone that is unable to bind to MR in mineralocorticoid target cells by 11β-hydroxysteroid dehydrogenase type 2 (Edwards *et al*., 1988; Funder *et al*., 1988).

3.2 Steroid receptor-mediated transcription

Molecular chaperones, such as heat-shock proteins Hsp90 and Hsp70, keep cytoplasmic unliganded steroid receptors in a conformation permitting hormone binding. Ligand binding alters receptor conformation, leading to its dissociation from the chaperone complex and unmasking of the nuclear localization signal. Nevertheless, chaperones and co-chaperones are required for proper folding, trafficking, transcriptional activation, and degradation of the steroid receptors (Echeverria and Picard, 2010). Interestingly, molecular chaperones also promote disassembly of nuclear receptors from DNA regulatory elements, enabling responsiveness to changing hormone concentration (Freeman and Yamamoto, 2002).

In the nucleus, steroid receptors bind to their cognate response elements that are located primarily at distal enhancers, as demonstrated by numerous studies since the first ChIP-on-chip reports for ERα and AR (Carroll *et al*., 2005; Carroll *et al*., 2006; Wang *et al*., 2007). Multiple amino acid residues throughout the receptor DBD make non-specific contacts with the DNA backbone, whereas the sequence-specific contact with DNA is mediated by few amino acids in the P box of the DNA recognition helix in the first zinc finger (Luisi *et al*., 1991; Schwabe *et al*., 1993). The P box of GR, MR, AR, and PR comprises identical residues, and all of them bind similar hormone response elements

(HRE) – inverted repeats of the 5'-AGAACA-3' consensus sequence with a 3-nt spacer – , whereas the P box and the response element sequence of ER are different from those of the other steroid receptors (Cotnoir-White *et al*., 2011). Mutations in the first hexamer of the HRE have greater impact on receptor binding than those in the second hexamer, indicating that the first hexamer serves as a high-affinity docking site for steroid receptors (Helsen *et al*., 2012; Verrijdt *et al*., 2000). The first androgen-selective response element identified at the enhancer of the *mouse sex-limited protein* (*Slp*) gene implied that differences in the *cis*-element sequence contribute to the receptor-specific DNA binding (Adler *et al*., 1992; Adler *et al*., 1993). In transient transfection assays, AR, GR, MR, and PR are all capable of transactivating reporter gene expression through the canonical androgen/glucocorticoid response element (ARE/GRE), whereas only AR and to some extent PR bind to so-called selective AREs that resemble direct repeats of the consensus sequence (Claessens *et al*., 1996; Denayer *et al*., 2010; Schoenmakers *et al*., 1999). Interestingly, DNA can also serve as an allosteric ligand for steroid receptors, as DNA sequence has been shown to affect GR structure and function (Meijsing *et al*., 2009; Watson *et al*., 2013).

Despite their similar response elements and related protein structures, steroid receptors exert diverse physiological functions and regulate their own target genes. Various ChIP-seq studies have reported distinct binding profiles for steroid receptors in a genuine chromatin environment, yet the *cis*-elements identified thus far for AR, PR, GR, and MR highly resemble the canonical ARE/GRE (Ballare *et al*., 2013; Grøntved *et al*., 2013; John *et al*., 2011; Lain *et al*., 2013; Massie *et al*., 2011; Rubel *et al*., 2012; Sahu *et al*., 2011; Sahu *et al*., 2013; Ueda *et al*., 2014). Thus, the determinants of *in vivo* binding specificity of the NR3C family steroid receptors are still enigmatic, and multiple *cis*-elements within the context of one enhancer can contribute to the specificity and extent of the physiological response (Robins, 2005; Verrijdt *et al*., 2002). Moreover, potential crosstalk between different steroid receptors expressed in the same cell types has recently gathered growing interest. About 50% of AR-occupied enhancers in prostate cancer cells can also be bound by GR, and GR is able to modulate the AR-signaling pathway (Arora *et al*., 2013; Sahu *et al*., 2013). Dexamethasone-liganded GR elicits redistribution of ER

binding on a genome-wide scale in murine mammary gland epithelial cells (Miranda *et al*., 2013), demonstrating molecular interplay between steroid receptor pathways. Furthermore, estradiol- and progesterone-induced transcriptional programs are largely overlapping in bovine liver, suggesting that these hormones have partly redundant roles in this tissue (Piccinato *et al*., 2013).

Transcription factors typically occupy only few percent of their putative binding sites (*cis*-elements), highlighting the importance of factors other than the DNA sequence for specific chromatin binding (Joseph *et al*., 2010; Kaplan *et al*., 2011). Moreover, many nuclear proteins are highly mobile, with the residence time on DNA from ten seconds to one minute for steroid receptors, as judged by live single-cell microscopy with fluorescent probes (McNally *et al*., 2000; van Royen *et al*., 2011). This suggests that individual components of transcriptional protein complex exchange rapidly, although the productive complex at the regulatory element stays longer (Karpova *et al*., 2008). Finally, interplay of local chromatin structure, collaborating transcription factors, and coregulators determines the receptor-selective transcriptional outcome *in vivo* (Wiench *et al*., 2011b).

3.3 Pioneer factors for steroid receptors

Arrangement of DNA into nucleosomal chromatin occludes regulatory elements from transcription factor binding. Pioneer factors are a special class of transcription factors that are able to bind to condensed chromatin and prime target loci in the genome for other factors to bind (Zaret and Carroll, 2011). The first pioneer factors, forkhead box (Fox)A and GATA proteins, were reported to precede other transcription factors in chromatin binding and to be indispensable for liver differentiation program (Gualdi *et al*., 1996; Lee *et al*., 2005; Watt *et al*., 2007). The FoxA proteins belong to the Fox family of winged helix transcription factors, and their unique properties can facilitate DNA binding of other transcription factors. The DBD of FoxA proteins resembles that of a linker histone, allowing them to bind to nucleosomal DNA (Cirillo *et al*., 1998; Clark *et al*., 1993), and their carboxyl-terminal domain binds directly to core histones facilitating the opening of nucleosomal DNA structure (Cirillo *et al*., 2002). FoxA1 recognizes a specific *cis*-

element on DNA. However, pioneer factors are also thought to function as readers of the histone code, translating epigenetic modifications into specific transcription factor binding (Magnani *et al*., 2011b). Moreover, FoxA1 also has a transactivation domain that can recruit coregulators to modify local chromatin environment (Zaret and Carroll, 2011). The role of FoxA1 in steroid receptor function has been highlighted by multiple recent genome-wide studies, especially for ERα function in breast cancer cells and AR function in prostate cancer cells (Augello *et al*., 2011). Characteristic features of genome-wide FoxA1 binding are the significant overlap with ERα and AR cistromes and the binding to these sites prior to hormone-induced receptor loading (Eeckhoute *et al*., 2006; Hurtado *et al*., 2011; Lupien *et al*., 2008; Robinson *et al*., 2011a; Sahu *et al*., 2011). In breast cancer cells, FoxA1 depletion leads to decreased ERα binding and attenuated estrogen-dependent gene transcription (Carroll *et al*., 2005; Hurtado *et al*., 2011). Interestingly, in prostate cancer cells, FoxA1 plays a more diverse role and reprograms AR binding on a genome-wide scale: only a subset of AR-occupied enhancers is pioneered by FoxA1, and a significant proportion of new AR-binding events appear upon FoxA1 depletion (Sahu *et al*., 2011). Other pioneer factors reported for ERα are activating protein (AP)-2γ, GATA factors, and pre-B-cell leukemia homeobox 1 (PBX1) (Eeckhoute *et al*., 2007; Magnani *et al*., 2011a; Miranda-Carboni *et al*., 2011; Tan *et al*., 2011; Theodorou *et al*., 2013), whereas GATA-2 and ETS family members have been associated with AR binding (Chen *et al*., 2013; Chng *et al*., 2012; Massie *et al*., 2007; Wang *et al*., 2007; Wu *et al*., 2014; Yu *et al*., 2010).

Early studies on the mouse mammary tumor virus promoter suggested that GR and PR are able to bind compact nucleosomal DNA, thus functioning as pioneer factors themselves (Perlmann and Wrange, 1988; Sun *et al*., 1983). However, genome-wide studies have revealed that GR binds predominantly to accessible chromatin (John *et al*., 2008; John *et al*., 2011) and identified other proteins functioning as pioneer factors for GR, including FoxA1 (Sahu *et al*., 2011), AP1 (Biddie *et al*., 2011), and C/EBP (Grøntved *et al*., 2013). Nevertheless, a recent mapping of the PR cistrome and chromatin structure in breast cancer cells reported that functionally active PR-binding events reside in nucleosomal DNA and that ligand-induced PR binding initiates chromatin remodeling,

providing genome-wide evidence for PR functioning as a pioneer factor (Ballare *et al*., 2013). AR has not been reported to bind nucleosomal DNA, but a significant subset of AR-binding events in prostate cancer cells is not affected by FoxA1 depletion (Sahu *et al*., 2011), suggesting involvement of other factors in creating the open chromatin conformation. Thus, although the functional importance of pioneer factors in steroid receptor signaling is well-established, our understanding of comprehensive mechanistic insights into the interplay of receptors, pioneer factors and chromatin modifications is still very limited.

3.4 Tissue-specific functions of steroid receptors

Steroid receptors facilitate specific transcriptional responses at different stages of development and in various cell types and tissues. Steroid hormones circulate in bloodstream, and are thus available for all tissues in the body. The first determinant for cell type- and tissue-specificity of hormone action is the expression of the cognate receptor. Transcript variants have been identified for all steroid receptors, and the different isoforms can potentially modulate tissue-specificity of responses. For example, PR-A isoform is essential for uterine and PR-B for mammary gland development in the mouse (Mulac-Jericevic *et al*., 2003; Mulac-Jericevic *et al*., 2000), but most human target tissues express the two isoforms at similar levels (Scarpin *et al*., 2009). GR expression levels are under the control of nine alternative untranslated first exons, and alternative splicing at the 3' end of GR mRNA leads to three protein isoforms (Oakley and Cidlowski, 2011). Of the two estrogen receptors, both of which are expressed in many target tissues, ERβ often has repressive effect on ERα transcriptional activity *in vivo,* for example, in uterus (Weihua *et al*., 2000). Nevertheless, although protein isoforms can modulate steroid receptor-mediated responses, other mechanisms are needed to ensure full spectrum of their tissue-specific functions.

A plethora of coregulatory proteins can contribute to tissue-specific transcriptional responses. Studies with tissue-selective steroid receptor modulators have demonstrated how differential expression patterns and isoform-selective interactions with steroid receptors can modulate transcriptional outcome (Smith and O'Malley, 2004). For

example, tamoxifen-bound ER recruits corepressors in mammary gland cells, but is able to stimulate gene expression in the uterus due to high SRC-1 expression levels (Shang and Brown, 2002). Genome-wide mapping of genomic loci associated with SRC proteins and ERα binding identified a subset of estradiol-regulated genes characterized by the presence of SRC-3, and not the other SRC family members, that correlated with tamoxifen-resistance and breast cancer prognosis (Zwart *et al*., 2011). An *in vivo* study utilizing a PR activity indicator mouse crossed with knockout mice for SRCs showed that the primary PR coactivators are SRC-3 in breast tissue and SRC-1 in uterus (Han *et al*., 2006). Other cellular proteins are likely to contribute to tissue-specific transcriptional regulation as well, for example, a majority of nuclear envelope proteins capable of organizing intranuclear landscape and affecting nuclear functions exhibit tissue-restricted expression patterns (de Las Heras *et al*., 2013).

The ligand, the receptor, and coregulators are mandatory for steroid hormone-mediated gene regulation, but they are not sufficient for determining the landscape of receptor occupancy and subsequent transcriptional outcome. Cell type-specific transcription factor binding is often associated with specific histone modifications (Barrera *et al*., 2008; Heintzman *et al*., 2009), DNA methylation status (Wiench *et al*., 2011a), and accessible chromatin (John *et al*., 2011; Tang *et al*., 2011). Genome-wide mapping of transcription factor cistromes has revealed that the distal regulatory elements define cell type-specific gene regulation in human and mouse (Heintzman *et al*., 2009; Shen *et al*., 2012). Interestingly, distinct enhancers also drive expression of common genes in different cell types (Kieffer-Kwon *et al*., 2013) and the ENCODE project revealed a vast diversity of enhancers in the human genome (The ENCODE Project Consortium, 2012). However, the determinants ensuring favorable chromatin structure and the factors governing receptor recruitment to these specific enhancer sites are still elusive, despite the technological advancements in mapping the chromatin structure, histone modifications, and transcription factor occupancy.

Recent genome-wide studies have suggested that pioneer factors might play a role in the tissue-specific receptor binding (Eeckhoute *et al*., 2009) and have proposed mechanisms,

such as covalent histone modifications (Lupien *et al*., 2008) and DNA hypomethylation, governing pioneer factor binding to tissue-specific loci (Serandour *et al*., 2011; Xu *et al*., 2007), and cohesin stabilizing tissue-specific protein-DNA complexes (Faure *et al*., 2012). Cell type-specific ERα binding was reported to associate with differential gene regulation in MCF-7 breast cancer cells and U2-OS osteosarcoma cells ectopically expressing ER (Krum *et al*., 2008) as well as in breast and endometrial cancer cell lines (Gertz *et al*., 2013). 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 (Sahu *et al*., 2013). Moreover, FoxA1 determines lineage-specific ER and AR binding in breast and prostate cancer cells, respectively (Lupien *et al*., 2008) and differential ER and AR function in hepatocytes (Li *et al*., 2012b).

Most steroid receptor cistromes have thus far been analyzed in few well-characterized cancer cell lines, and the tissue-specific cistromes representing physiological conditions are very limited. Moreover, many ChIP-seq studies from intact tissues or cancer cell lines that have suggested that pioneer factors dictate cell type-specific cistromes, have reported only enriched *cis*-elements within the receptor-bound regions, not taking into account whether or not these elements are actually occupied by the cognate factor, such as the recent PR cistromes suggesting differential *cis*-element enrichment around PR-binding events in the mammary gland and uterus (Lain *et al*., 2013; Rubel *et al*., 2012; Yin *et al*., 2012). Table 2 summarizes the pioneer factors reported for steroid receptors in various cell types thus far, but a comprehensive view of the ways by which pioneer factors contribute to tissue-specific transcriptional outcome is still lacking. Thus, *in vivo* studies under physiological conditions are of prime importance, which has been one of the primary scopes of this thesis.

Table 2. Summary of pioneer factors associated to steroid receptor function in different cell types. Only validated studies reporting putative pioneer factor occupancy at shared sites with steroid receptors are listed.

Receptor	Tissue of origin	Cell type	Pioneer factor	References
ERα	Mammary gland	MCF-7 and T-47D breast cancer cell lines	FoxA1	Carroll *et al.,* 2005 Lupien *et al.,* 2008 Hurtado *et al.,* 2011 Ross-Innes *et al.,* 2012 Theodorou *et al.,* 2013 Gertz *et al.,* 2013
		MCF-7	PBX1	Magnani *et al.,* 2011
		MCF-7	AP-2γ	Tan *et al.,* 2011
		MCF-7 and T-47D breast cancer cell lines	GATA-3	Eeckhoute *et al.,* 2007 Theodorou *et al.,* 2013 Gertz *et al.,* 2013
	Uterus	ECC-1 cancer cell line	ETV4	Gertz *et al.,* 2013
	Bone	U2OS-ER osteosarcoma cell line	GATA-4	Miranda-Carboni *et al.,* 2011
	Liver	Hepatocyte from liver tissue	FoxA1	Li *et al.,* 2012b
AR	Prostate	LNCaP, LNCaP-1F5, VCaP prostate cancer cell lines	FoxA1	Lupien *et al.,* 2008 Wang *et al.,* 2009 Sahu *et al.,* 2011 Sahu *et al.,* 2013 Wang *et al.,* 2011
		LNCaP	GATA-2	Wang *et al.,* 2007 Wu *et al.,* 2014
		LNCaP	ETS1	Massie *et al.,* 2011
		LNCaP, VCaP lines, mouse prostate	ERG	Yu *et al.,* 2010 Chng *et al.,* 2012 Chen *et al.,* 2013
	Mammary gland	MDA-MB-453 ER-AR+ molecular apocrine breast cancer cell line	FoxA1	Robinson *et al.,* 2011a Ni *et al.,* 2011
	Liver	Hepatocyte from mouse tissue	FoxA1	Li *et al.,* 2012b
GR	Prostate	LNCaP-1F5, VCaP prostate cancer cell lines	FoxA1	Sahu *et al.,* 2011 Sahu *et al.,* 2013
	Mammary gland	3134 murine mammary epithelial cells	AP1	Biddie *et al.,* 2011
	Liver	Hepatocyte from mouse tissue	C/EBP	Grøntved *et al.,* 2013

4. Androgen receptor

4.1 *AR* and its regulation

Androgens regulate cellular functions through AR and the *AR* gene is expressed in various tissues and cell types, including Leydig and Sertoli cells in testis, both epithelial and stromal compartments of accessory sex organs, such as prostate and seminal vesicles, and many cell types in non-reproductive tissues, such as skeletal muscle, bone, fat, and skin (De Gendt and Verhoeven, 2012). Expression of the human *AR* is governed by a TATA-less promoter (Faber *et al*., 1991; Tilley *et al*., 1990), and many transcription factors have been implicated in *AR* expression, including CREB, Myc, c-Jun, Sp1, Foxo3a, and LEF1 (Shiota *et al*., 2011). Recently, it was reported that AR represses its own transcription by recruiting LSD1 to an enhancer at second intron of *AR* (Cai *et al*., 2011). Normal splicing of *AR* exons 1–8 results in 10.6-kb mRNA species with a 2.7-kb open reading frame. A naturally occurring splice variant for *AR*, termed AR45, is produced from an alternative first exon and yields a 45-kDa receptor isoform in which the entire NTD is replaced by seven novel amino acids (Ahrens-Fath *et al*., 2005). Ectopically expressed AR45 is a negative regulator of AR signaling, but its functional significance *in vivo* remains elusive (Dehm and Tindall, 2011).

Cloning of the *AR* cDNA and chromosomal localization of the *AR* gene to Xq11-12 (Chang *et al*., 1988; Lubahn *et al*., 1988) initiated the molecular characterization of AR protein function and mapping of variations in the *AR* gene and its expression (Brinkmann *et al*., 1989). Subsequent studies have shed light into the molecular pathogenesis of clinical conditions, such as prostate cancer and androgen insensitivity syndrome (AIS), although the connection of these disorders to androgens was established already more than 50 years ago. Mutations in the *AR* gene disrupting the functional domains of the protein lead to AIS with characteristics of normal serum androgen levels but subnormal signs of androgen action. The severity of the symptoms reflects the defects in AR protein, and most reported mutations affect the LBD of AR (Jääskeläinen, 2012). Androgens promote proliferation of normal and malignant prostatic cells, and androgen deprivation therapy is often used to inhibit growth of prostate tumors. Advanced stages of prostate

cancer are characterized by AR-dependent but castration-resistant tumor growth that is often achieved through *AR* mutations or overexpression (Knudsen and Kelly, 2011). AR splice variants with truncated carboxyl terminus found in prostate cancer cells generate a ligand-independent receptor (Dehm *et al*., 2008; Guo *et al*., 2009; Hu *et al*., 2009; Sun *et al*., 2010). Recently, *AR* gene rearrangements modeled using transcription activator-like effector nuclease (TALEN) genome engineering showed that constitutionally active, truncated AR is able to drive transcriptional program similar to that of the full-length receptor, highlighting a mechanism for androgen-independent growth of castration-resistant tumors (Nyquist *et al*., 2013).

4.2 Structural and functional domains of androgen receptor

The modular structure of AR protein comprises the typical functional domains of steroid receptors. NTD of AR is encoded by exon 1 of *AR*, exons 2 and 3 encode the first and second zinc fingers in the DBD, respectively, and exons 4–8 are responsible for the hinge region and the carboxyl-terminal LBD (Figure 5A). AR is subject to PTMs that alter its transcriptional activity and contribute to the stability of the protein (Gioeli and Paschal, 2012). So far, five different modifications and 23 modifiable residues have been reported (Figure 5B).

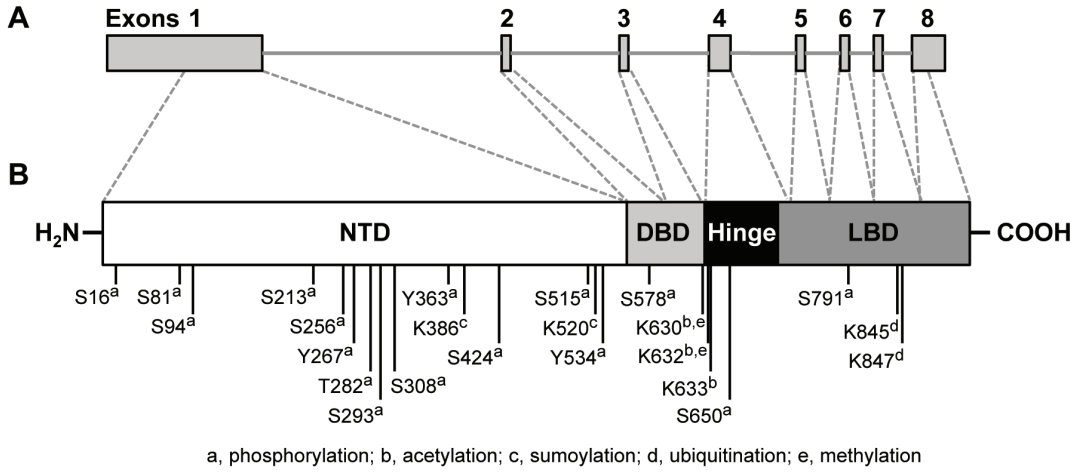

Figure 5. Eight exons of the *AR* gene (A) encode four functional domains of the AR protein (B). Amino acid residues with reported post-translational modification are indicated. Adapted and reprinted from Gioeli & Paschal, Post-translational modification of the androgen receptor, Molecular and Cellular Endocrinology 352:70–78, 2012, with permission from Elsevier (copyright 2012).

NTD is the least conserved of the AR functional domains, but it harbors conserved areas responsible for specific functions, namely, the ligand-dependent interaction with LBD (Ikonen *et al*., 1997) and the AF-1 (Palvimo *et al*., 1993). General mechanism of nuclear receptor activation by ligand binding induces repositioning of helix 12 and forming a hydrophobic cleft that constitutes AF-2. A unique feature of AF-2 within the AR LBD is that it predominantly interacts with the FxxLF motif in the AR NTD and not with transcriptional coactivators as in other steroid receptors (Dubbink *et al*., 2004; van de Wijngaart *et al*., 2012). Thus, AF-1 is the primary site for coactivator binding in AR and, unlike other steroid receptors, constitutively active AR lacking LBD retains transcriptional activity nearly equal to that of the full-length receptor (Alen *et al*., 1999; Bevan *et al*., 1999). NTD also contains majority of the phosphorylation sites within AR. Phosphorylation is the most abundant of the AR PTMs, and it affects protein-protein interactions in the proximity of the phosphorylated site, modulating interaction with coregulators and AR transcriptional activity (Gioeli and Paschal, 2012). Another PTM targeting the NTD is sumoylation, *i.e*., conjugation of small ubiquitin-like modifiers (SUMO) to lysine residues. Mutation analyses have revealed that sumoylation of AR primarily at lysine 386 attenuates AR transcriptional activity (Kaikkonen *et al*., 2009; Poukka *et al*., 2000). NTD of human AR also harbors polymorphic polyglutamine and polyglycine tracts of varying length. The length of polyglutamine tract alters AR transcriptional activity (Mhatre *et al*., 1993), and it is likely to play a role in pathogenesis of prostate cancer (Robins, 2012).

Highly conserved AR DBD contains two zinc fingers, each possessing four cysteines tetrahedrally coordinated to a Zn^{2+} ion. The amino acids in the α-helix of the first zinc finger (P-box) recognize nucleotides in the major groove of the DNA-binding element, whereas D-box within the second zinc finger forms the dimerization interface (Luisi *et al*., 1991; Shaffer *et al*., 2004) (Figure 6). In addition to the palindromic ARE/GREs recognized by all NR3C family steroid receptors, AR binds to selective AREs that are not recognized by GR, as discussed in Chapter 3.2 (Denayer *et al*., 2010). Comparison of crystal structures of AR and GR DBDs revealed that the spatial positioning of the four amino acid residues that are different in the dimerization interfaces of AR and GR DBDs

allow AR to make stronger DBD-DBD contacts (Shaffer *et al*., 2004), potentially contributing to AR binding to selective AREs (Helsen *et al*., 2012). Biochemical analyses have implicated also the first 12 amino acids of the hinge region, the so-called carboxyl-terminal extension, in selective AR binding (Schoenmakers *et al*., 1999). Interestingly, lysines within the carboxyl-terminal extension are the only acetylation and methylation sites reported for AR (Fu *et al*., 2000; Gaughan *et al*., 2002; Gaughan *et al*., 2011; Ko *et al*., 2011). These PTMs enhance LBD-NTD interaction and transcriptional activation of AR, whereas serine phosphorylation in the hinge region increases its nuclear export (Gioeli and Paschal, 2012).

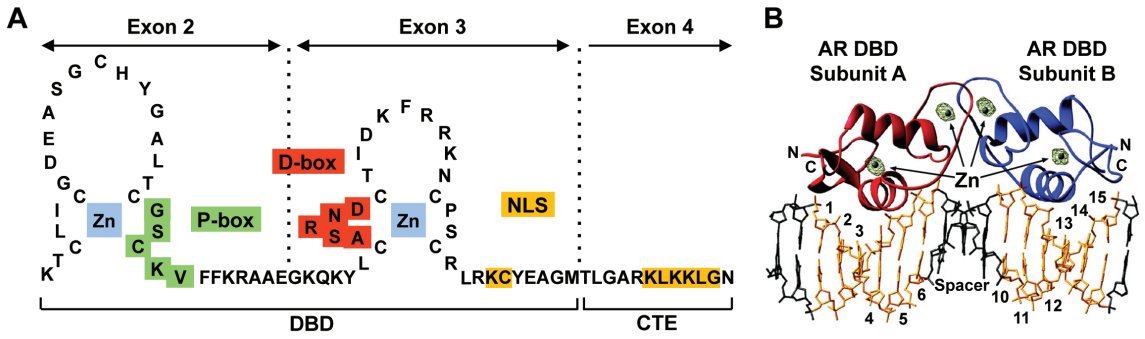

Figure 6. A. Schematic presentation of AR DBD and carboxyl-terminal extension (CTE). B. Crystal structure of the AR DBD dimer bound to a selective ARE. Numbers 1–6 indicate the nucleotides in the first hexamer and 10–15 in the second hexamer. Reprinted from Shaffer *et al*., Structural basis of androgen receptor binding to selective androgen response elements. PNAS 101:4758–4763, 2004. Copyright (2004) National Academy of Sciences, U.S.A.

4.3 Androgen receptor-mediated gene regulation

Androgen-induced dissociation from the heat shock complex, receptor dimerization and translocation to nucleus initiate AR-dependent transcriptional regulation. The bipartite nuclear localization signal of AR involves two clusters of amino acids located in the second zinc finger of the DBD and the hinge region (Figure 6) (Zhou *et al*., 1994), and its interaction with importin-α facilitates AR translocation to the nucleus upon ligand binding (Cutress *et al*., 2008). Early ChIP studies at regulatory elements of androgen-induced genes in the *kallikrein* cluster characterized dynamic assembly of agonist-induced transcription complex with AR, coactivators, such as p160 and CBP/p300 proteins, and RNA polymerase II (Kang *et al*., 2004; Shang *et al*., 2002). AR-coactivator complex occupancy gradually increases peaking at 16 h after androgen-treatment, and

both chromatin looping and polymerase tracking were identified as a mechanism for enhancer-promoter interaction within the *prostate-specific antigen (PSA,* also known as *kallikrein-related peptidase 3)* gene (Wang *et al*., 2005). Looping has also been reported for the regulatory regions of other androgen-induced genes, such as *TMPRSS2* (Wang *et al*., 2007), *FKBP5* (Makkonen *et al*., 2009), and *UBE2C* (Chen *et al*., 2011). According to the looping model, the mediator complex facilitates the long-range communications (Malik and Roeder, 2010), and MED12 was reported to mediate the enhancer-promoter interaction in an androgen-induced fashion (Wang *et al*., 2011).

The first chromosome- and genome-wide maps of AR binding using ChIP-on-chip technology revealed the distal enhancers as the primary AR loading sites and identified several collaborating transcription factors, namely GATA-2, FoxA1, and ETS1, co-occupying these sites (Jia *et al*., 2008; Lupien *et al*., 2008; Massie *et al*., 2007; Wang *et al*., 2007). Subsequent high-resolution cistromes revealed that the genomic landscape of AR binding encompasses thousands of binding events (Yu *et al*., 2010), characterized the functional relationship of AR and FoxA1 with FoxA1 having both pioneering and repressive effects on AR binding (Sahu *et al*., 2011; Wang *et al*., 2011) and identified distinct chromatin modification, namely H3K4me2, marking active enhancers prior to AR recruitment (He *et al*., 2010) (Figure 7). Gene expression profiling studies have reported hundreds of androgen up- and down-regulated transcripts and identified multiple cellular pathways, such as anabolic metabolism, as androgen targets (Jin *et al*., 2013; Massie *et al*., 2011). Differential recruitment of coregulators directs transcriptional signal to local chromatin conformation, and over 200 coregulators implicated in AR function give rise to the diversity of responses and integration of signals from various cellular pathways (Heemers and Tindall, 2007; van de Wijngaart *et al*., 2012).

The coactivators involved in AR-dependent gene activation include chromatin remodelers, such as BAF57, histone acetyltransferases, such as SRC-1, and histone methyltransferases, such as SET9 (Bevan *et al*., 1999; Gaughan *et al*., 2011; Link *et al*., 2005). As discussed in Chapter 2.3, histone acetylation is associated with transcriptionally active enhancers, whereas histone methylation is involved in both gene

activation through methylation of H3K4 and gene repression through methylation of H3K9 and H3K27. Many methyltransferases and demethylases have been associated with AR-dependent gene activation and/or repression, such as LSD1 that is able to demethylate both H3K4 and H3K9 (Cai *et al*., 2011; Cai *et al*., 2013; Metzger *et al*., 2005). However, determinants of LSD1 function and subsequent gene activation or repression are largely elusive. Furthermore, both FoxA1-pioneered and FoxA1-independent AR-binding events are characterized by H3K4me2 marks (He *et al*., 2010; He *et al*., 2012; Sahu *et al*., 2011), which raises an intriguing question about the mechanistic interplay between histone methylation and pioneer factors for the provision of chromatin environment favorable to AR binding.

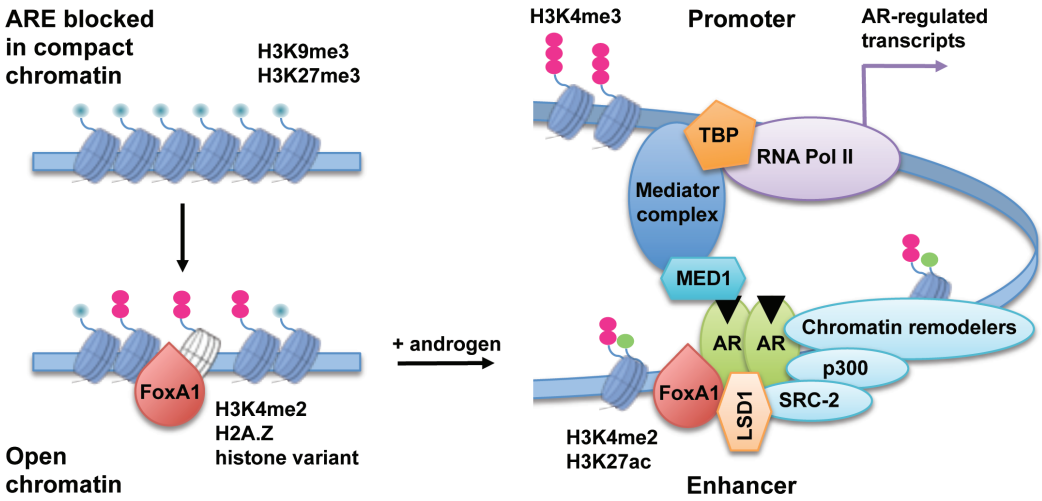

Figure 7. Characteristic events of androgen-dependent gene activation. FoxA1 represents a pioneer factor that primes chromatin for AR binding. Demethylase LSD1 and acetylases SRC-2 and p300 are examples of coregulators recruited to enhancers by AR, but other factors not shown in the figure are also involved in functional transcriptional complex. The spheres representing histone modifications are color-coded as follows: blue for repressive marks such as H3K9me3 and H3K27me3, and pink and green for activating marks H3K4me and H3K27ac, respectively.

The mechanisms of gene repression are likely to involve both protein-protein interactions as well as direct AR binding to regulatory elements of repressed genes and recruitment of corepressors such as NCoR and SMRT, resulting in histone deacetylation (Grosse *et al*., 2012). Both androgen up- and down-regulated genes are associated with AR- and FoxA1-binding sites (Sahu *et al*., 2011), but recruitment of corepressors HDAC1, 2, 3 and EZH2 was reported to occur specifically to a subset of AR-binding loci shared with ERG (Chng

et al., 2012). Moreover, a direct repressive mechanism has been described with AR-dependent recruitment of LSD1 in repressing *AR* expression (Cai *et al*., 2011) and EZH2-mediated H3K27 trimethylation for number of repressed genes (Zhao *et al*., 2012).

4.4 Androgen receptor ligands and exogenous modulators

The major physiological androgens are testosterone and 5α-dihydrotestosterone (DHT). Testosterone is synthetized from cholesterol in testis under the control of hypothalamic-pituitary axis. Hypothalamus secretes gonadotropin-releasing hormone that stimulates production of luteinizing hormone in anterior pituitary that, in turn, promotes testosterone synthesis in the Leydig cells of testis. Negative feedback-loop regulates testosterone synthesis, in that low circulating steroid levels release the hypothalamus and pituitary from feedback inhibition. Other physiological sources of androgens include the ovaries in females and the adrenal cortex in both genders. In many target tissues, such as prostate and skin, 5α-reductase catalyzes conversion of testosterone to a more potent androgen, DHT, that has 5-10 times higher affinity for AR compared to that of testosterone. In some tissues, testosterone can be also aromatized to estradiol.

Androgen deprivation therapy is used to suppress transcriptional activity of AR in prostate cancer patients. Androgen deprivation can be achieved by decreasing testosterone levels by surgical or pharmacological castration, or by inhibiting AR function in the target cells by antagonistic compounds that compete with testosterone or DHT for AR binding. The first antagonist developed for AR, cyproterone acetate, has a steroidal structure, and it also has agonistic effects on AR function (Wilding *et al*., 1989). ChIP-seq profiling of AR cistromes in prostate cancer cells demonstrated that AR-binding patterns are very similar in response to DHT and cyproterone acetate (Sahu *et al*., 2013). The non-steroidal antiandrogens flutamide and bicalutamide can also exert agonistic effects in the presence of high cellular AR content (Chen *et al*., 2004). Novel compounds targeting the AR pathway are abiraterone acetate, a CYP17 inhibitor that blocks androgen production (Attard *et al*., 2008), and the second generation antiandrogen enzalutamide that binds to AR with higher affinity than bicalutamide and inhibits receptor translocation to the nucleus (Tran *et al*., 2009).

Distinct androgen effects in various tissues have prompted the search for selective androgen receptor modulators. Activation of the AR pathway in muscle and bone would be beneficial in conditions involving loss of muscle mass, such as age-related frailty syndrome and cachexia, but androgens also play a role in the development of prostate hyperplasia and carcinoma. Binding of androgen receptor modulators to the ligand-binding pocket of the AR LBD may cause subtle differences in receptor conformation when compared to the DHT-activated AR, thus potentially leading to differential interaction with coregulators, other transcription factors, and AREs (Haendler and Cleve, 2012). This is supported by conformational differences in crystal structures of the AR LBD complexed with different ligands, namely cyproterone acetate, bicalutamide, LGD2226, and *N*-aryl-hydroxybicyclohydantoin-based compounds (Bohl *et al*., 2005; Bohl *et al*., 2007; Sun *et al*., 2006; Wang *et al*., 2006). Several compounds with varying androgenic and anabolic effects have entered clinical trials, but potential side effects as well as *in vivo* efficacy in humans need to be assessed (Haendler and Cleve, 2012).

Natural compounds or industrial chemicals with abilities to modulate nuclear receptor action, the so-called endocrine disruptors, have recently gathered interest due to their possible roles in risk and prevention of hormone-dependent disorders (Diamanti-Kandarakis *et al*., 2009). For instance, bisphenol A, a plasticizer used in polycarbonate products, has been associated with adverse effects, such as altered behavior and asthma in children (Rochester, 2013). Although the extent of health risks associated with bisphenol A exposure are still under debate, increased public awareness and scientific evidence have resulted in banning of polycarbonate plastics in baby bottles in many countries. Several endocrine disruptors show antiandrogenic properties, including fungicides vinclozolin and procymidone, and plasticizers bisphenol A and phthalates (Luccio-Camelo and Prins, 2011). Some environmental compounds, such as soy phytoestrogens, have been associated with attenuated prostate cancer risk in several cohort and case-controlled studies (Jian, 2009; Yan and Spitznagel, 2009). Better understanding of molecular mechanisms elicited by endocrine modulators is needed to correctly assess their potential health risks or benefits. Especially *in vivo* studies are warranted to complement epidemiological risk analysis and *in vitro* cell culture-based assays.

5. Androgen signaling *in vivo*

5.1 Effects of androgens on male sexual function

Sex hormones are not crucial to the life of an individual, but they are absolutely necessary for the reproduction and survival of metazoan species. The essential role that androgens play in male physiology is demonstrated by the feminine phenotypes of 46,XY-males with AIS, characterized by undescended testis producing testosterone but feminine external genitalia and secondary sexual features (Jääskeläinen, 2012). Genital ridges in mammalian embryo are capable of differentiating into testes or ovaries. Importantly, male sex determination depends on the expression of the Y-chromosomal *sex-determining region Y* (*SRY)* gene that induces differentiation of Sertoli cells and formation of testis. Testosterone and DHT are responsible for masculinization of the embryo by directing the development of genital organs in concert with other cellular signaling pathways (Wilhelm and Koopman, 2006). Testosterone production by murine fetal Leydig cells is luteinizing hormone-independent (Zhang *et al*., 2001), but in human chorionic gonadotropin is required for initiation of testosterone production (O'Shaughnessy *et al*., 2006).

Starting from puberty, androgens promote the development of male secondary sexual characteristics, such as growth of larynx, resulting in deeper voice, and male pattern hair growth. Likewise, maturation and function of reproductive organs is highly androgen-dependent. Testosterone is an essential regulator of spermatogenesis through AR expression in Sertoli cells and peritubular myoid cells – but not in germ cells–, as elucidated by cell-type specific AR knockout (ARKO) mouse models (De Gendt *et al*., 2004; Holdcraft and Braun, 2004; Tsai *et al*., 2006; Welsh *et al*., 2009). Epididymis is the organ where sperm maturation takes place, and highly androgen-responsive accessory sex glands, seminal vesicle and prostate, secrete fluids and compounds that contribute to sperm functionality. In these tissues, AR is expressed both in stromal and epithelial compartments, and it can thus elicit various effects on tissue morphology and secretory functions (De Gendt and Verhoeven, 2012). Epididymis is a tubule with four distinct segments responding differently to castration and testosterone-treatment in terms of

cellular proliferation and gene expression patterns (Hamzeh and Robaire, 2009; Sipilä *et al*., 2006). In prostate, AR ablation in stromal cells results in defective epithelial proliferation (Yu *et al*., 2011), whereas epithelial cell-selective ARKO mice display hyperproliferation and poor differentiation of prostatic epithelium (Simanainen *et al*., 2007; Wu *et al*., 2007).

5.2 Androgen action in non-reproductive tissues

Androgens have anabolic actions in many non-reproductive tissues, including bone, fat, and skeletal muscle. Androgens stimulate both linear bone growth and skeletal maturity, finally leading to closure of epiphyseal plates and termination of growth. Testosterone affects bone both by direct AR activation and through ER after testosterone aromatization. Furthermore, growth hormone and insulin-like growth factor 1 contribute to growth, as both sex steroids and growth hormone markedly increase during puberty, and their actions are mutually amplified in the control of growth, increase in muscle mass, and mineralization of the skeleton (Callewaert *et al*., 2010). Osteoblast-selective ARKO mouse demonstrated that androgens keep promoting bone health throughout the life by regulating bone resorption (Notini *et al*., 2007). In muscle, testosterone increases muscle size and strength in both young and elder men (Bhasin *et al*., 1996; Bhasin *et al*., 2005). Testosterone increases protein synthesis in skeletal muscle leading to hypertrophy (Ferrando *et al*., 1998), but it also induces proliferation of skeletal muscle progenitor cells (Sinha-Hikim *et al*., 2003). Myocyte-selective ARKO models have highlighted distinct responses to AR ablation between different muscle types, in that the weight of highly androgen-responsive levator ani but not the limb muscles was decreased by the ablation (Chambon *et al*., 2010; Ophoff *et al*., 2009). Better understanding of testosterone actions in bone and muscle would benefit in the quest for developing selective AR modulators for clinical use.

Also other tissues, such as liver and kidney, are androgen-responsive, and exhibit sexual dimorphism in their physiology and under pathophysiological conditions (Chang *et al*., 2013). Hepatocytes of both genders express AR, and AR expression is increased in liver carcinoma (Eagon *et al*., 1991). The incidence of hepatocellular carcinoma is lower in

women than men (Ferlay *et al*., 2010), and a recent *in vivo* ChIP-seq study demonstrated that FoxA pioneer factors mediate both the protective effects of ER and the deleterious effects of AR to the pathogenesis of liver carcinoma in mice (Li *et al*., 2012b). In rodent kidneys, AR regulates many well-studied target genes, and AR expression has been located to proximal tubules of the renal cortex by studies on the expression of *kidney androgen-regulated protein (Kap)* (Meseguer and Catterall, 1990) and *ornithine decarboxylase 1 (Odc1)* (Crozat *et al*., 1992). Androgens promote cellular hypertrophy and polyamine synthesis in murine kidney (Catterall *et al*., 1986; Tovar *et al*., 1995), and testosterone-induced AR has been implicated in the pathogenesis of hypertension in rats (Reckelhoff *et al*., 2000). However, the role that androgens play in human kidney remains elusive. Furthermore, although compilation of physiological effects of androgens are well known in several target tissues, the molecular mechanisms of these effects are just beginning to emerge, and further studies with state-of-the-art approaches are required.

5.3 Androgen-related diseases

Androgen insensitivity syndrome (AIS) and Kennedy's disease (spinal-bulbar muscular atrophy, SBMA) are rare conditions inherited in an X-linked recessive manner. Both are caused by mutations in *AR*; AIS by missense or nonsense mutations mostly in the LBD resulting in impairment or complete loss-of-function of AR, and SBMA by expansion of polymorphic CAG repeat in the NTD beyond 40 repeats, leading to AR protein with neuronal toxicity (Matsumoto *et al*., 2013). Currently, there is no effective treatment for AIS or SBMA. AR has been implicated in many cancers (Chang *et al*., 2013), and it can serve as a molecular mechanism to bypass ER signaling in breast cancer (Ni *et al*., 2011; Robinson *et al*., 2011a). However, prostate cancer is by far the most common AR-related disease, and AR signaling plays a significant role in all stages of this disease.

Prostate cancer is the second-most commonly diagnosed cancer worldwide among men, with almost 900,000 new cases every year (Ferlay *et al*., 2010). Its advanced forms are associated with high mortality rates, and at present lack curative treatment. Androgen signaling is essential not only for normal prostate development, but also for the growth of prostatic malignancies (Lonergan and Tindall, 2011). Prostate cancer is a heterogeneous

disease often characterized by the loss of *NKX3-1*, *PTEN*, and *retinoblastoma* genes (Shen and Abate-Shen, 2010). The most common genomic rearrangement found in about a half of prostate cancers is the *TMPRSS2:ERG* fusion, resulting in androgenic control of the expression of oncogenic ETS transcription factors (Tomlins *et al*., 2005). Moreover, exome sequencing of prostatic tumors have identified recurrent mutations in *SPOP*, *FOXA1*, and *MED12*, as well as in genes for several chromatin and histone-modifying enzymes (Barbieri *et al*., 2012; Grasso *et al*., 2012), and a recent study identified a prostate cancer predisposition allele associated with a stronger binding of the HOXB13 transcription factor resulting in altered *RFX6* expression (Huang *et al*., 2014).

Androgen signaling is central to all stages of prostate cancer, and androgen deprivation therapy is used to suppress tumor growth. In castration-resistant prostate cancer (CRPC), AR signaling is reactivated through multiple mechanisms, such as AR deregulation, intracrine androgen synthesis, AR mutations and alternative splicing, and alterations in PTMs and coregulatory pathways (Knudsen and Kelly, 2011). Genome-wide AR profiles in CRPC cell lines have shown a set of binding loci occupied by AR in the absence of androgens (Decker *et al*., 2012; Wang *et al*., 2009). The first ChIP-seq study reporting AR-binding events in clinical CRPC tissues showed only a moderate overlap with AR cistromes present in prostate cancer cell lines, demonstrating the heterogeneity of the disease (Sharma *et al*., 2013). The role of FoxA1 in AR signaling has been extensively studied in various prostate cancer cells over the past decade (Augello *et al*., 2011). Importantly, FoxA1 protein expression in prostate cancer tissue was identified as a poor prognostic marker for disease progression (Sahu *et al*., 2011; Robinson *et al*., 2013). ETS transcription factors have also been intensively studied, in particular after the identification of the TMPRSS2:ERG fusion protein, and an integrated repressive pathway involving AR, ETS factors and polycomb protein EZH2-mediated histone methylation has been described (Yu *et al*., 2010; Zhao *et al*., 2012). Recent transgenic mouse models underline the role of another ETS factor involved in gene fusions, ETV1, in modulating AR signaling (Baena *et al*., 2013; Chen *et al*., 2013). At present, prostate specific antigen (PSA) is the only widely-used biomarker for prostate cancer progression, but the novel

findings are anticipated to provide a better classification of prostatic tumors according to their molecular subtypes in the years to come.

5.4 Mouse models to study AR

Various *in vivo* approaches have contributed to our understanding of androgen-regulated AR action. Castration allows for observing the effects of androgen withdrawal, and subsequent ligand-treatment can be used to modulate the AR pathway. Mice do not express the enzymatic pathway to synthesize androgens in the adrenals, and castration of male mice leads to complete androgen ablation (van Weerden *et al*., 1992). A mouse model with a phenotype closely resembling AIS was reported in 1970, and this testicular feminization (Tfm) mouse line was used to delineate androgen action in the development of male phenotype (Lyon and Hawkes, 1970) and in sexual dimorphism of extragenital tissues (Bardin and Catterall, 1981). Subsequently, transgenic full ARKO mice have been developed, and similar to Tfm males, ARKO mice display a female appearance with small intra-abdominally located testes as their only internal reproductive organs (Matsumoto *et al*., 2003; Yeh *et al*., 2002). Furthermore, cell-type specific ARKO mouse models have provided more detailed mechanistic insights into AR function in different cells and organs, as described in Chapters 5.1 and 5.2.

In addition to knockout models, knock-in approaches have been used to study AR action. Knock-in models to study prostate cancer and SBMA, generated by swapping the murine AR NTD to its human counterpart and introducing polyglutamine tracts of varying lengths (Albertelli *et al*., 2006; Robins, 2012), reported neuromuscular pathology in males with the longest CAG repeat of 113 triplets (Yu *et al*., 2006). Short polyglutamine tract of 12 repeats, on the other hand, was associated to earlier development of prostate cancer when these knock-in mice were crossed with the TRAMP prostate cancer mouse model (Albertelli *et al*., 2008). In a different knock-in approach, swapping of the second zinc finger of AR to that of GR created a chimeric receptor capable of binding to classical but not selective AREs (Schauwaers *et al*., 2007; Schoenmakers *et al*., 1999). The transgenic males with this chimeric AR, termed specificity affecting AR knock-in (SPARKI), have smaller reproductive organs than their wild-type littermates (Schauwaers

et al., 2007). SPARKI males are subfertile, owing to the impaired sperm maturation in the epididymis (Kerkhofs *et al*., 2012). Comparison of genome-wide binding profiles for SPARKI and wild-type AR *in vivo* was one of the aims of this thesis.

Transgenic mice expressing a reporter gene driven by hormone-responsive enhancers can be used to elucidate nuclear receptor function in live animals *in vivo* (Maggi and Ciana, 2005). A few models reporting for AR action in prostate have been described with the reporter gene expression being driven by human *PSA* or *KLK2* promoters (Iyer *et al*., 2005; Lyons *et al*., 2006; Xie *et al*., 2004). AR activity indicator mouse, however, harbors a modified AR with the GAL4 DBD driving LacZ expression (Ye *et al*., 2005). Cross-breeding of this mouse line with knockout models for AR coregulators showed that AR activity in the testis is decreased by the loss of SRC-2, whereas ablation of SRC-1 surprisingly increases AR activity (Ye *et al*., 2005). Reporter mice are promising tools for elucidating spatio-temporal patterns of AR activity *in vivo* in a noninvasive fashion, but the full extent of their potential remains to be established. Several transgenic mouse models have also been developed for studying prostate cancer, mostly with targeted expression of oncogenes or disruption of tumor suppressor genes (Wu *et al*., 2013).

The AR signaling pathway is highly conserved in mammals. Within AR proteins, DBD and LBD are identical in human and mouse, the hinge region harbors few different amino acids, and the NTDs differ by 15% in protein sequence (Robins, 2012). In general, conservation of transcription factor cistromes has been reported for mouse and human (Hemberg and Kreiman, 2011), although huge plasticity in the distal regulatory elements also results in different binding patterns and regulatory pathways among species (The ENCODE Project Consortium, 2012; McLean *et al*., 2011; Odom *et al*., 2007). Nevertheless, many functional targets and DNA binding preferences are conserved (Schmidt *et al*., 2010), and in many regards, the mouse is an optimal model system with sophisticated genome manipulating tools and vast amount of data available on mouse strains, mutant phenotypes, and disease models (Keane *et al*., 2011). In conclusion, both wild-type mice and transgenic mouse models provide valuable information about physiological processes including androgen action and gene regulation.

AIMS OF THE STUDY

The purpose of this study was to elucidate molecular mechanisms of AR action under physiological conditions *in vivo*. Wild-type mice as well as two transgenic mouse models were studied using modern approaches, including *in vivo* imaging, gene expression profiling, and ChIP-sequencing. The specific aims were the following:

Aim 1 To develop and characterize a transgenic androgen reporter mouse line.

Aim 2 To study the effects of the phytoestrogenic isoflavonoid genistein on AR signaling *in vivo*.

Aim 3 To elucidate AR cistromes and androgen-induced transcription programs in three androgen-responsive tissues – prostate, kidney, and epididymis – and to delineate the mechanisms of tissue-specific gene expression.

Aim 4 To examine the significance of AR DNA-binding domain in and of itself in determining the rules for *in vivo* chromatin binding in two androgen-responsive tissues, epididymis and prostate, by utilizing the SPARKI transgenic mouse line.

MATERIALS AND METHODS

1. Mouse models and treatments

1.1 Transgenic mouse models

To produce the androgen reporter mouse line, *Slp*-HRE2-TATA-Luc construct (Verrijdt *et al*., 2000), a kind gift from Dr. G. Verrijdt (University of Leuven, Belgium), containing four AREs (*Slp*-HRE2) of the mouse *Slp* gene (Adler *et al*., 1993), *thymidine kinase* minimal promoter, and TATA-box driving the expression of the *luciferase* reporter gene was used. The 3-kb matrix attachment region (MAR) insulator sequence from the chicken *lysozyme* gene (Phi-Van *et al*., 1990), kindly provided by Professor W. H. Strätling (University of Hamburg, Hamburg, Germany), was subcloned to the 5' and 3′ ends of *Slp*-HRE2-TATA-Luc to generate the reporter construct pGEM3Z-*Slp*-HRE2-TATA-Luc-5′MAR-3′MAR. The final construct was verified by restriction enzyme digestions and DNA sequencing. The pGEM3Z-*Slp*-HRE2-TATA-Luc-5′MAR-3′MAR construct was digested with *Sma*I to remove plasmid DNA. The 8.4-kb DNA fragment was gel-purified and injected into zygotes of FVB/N mice using standard pronuclear injection techniques, and the embryos were transferred into pseudopregnant females. Genotyping was performed by PCR with primers detecting the reporter construct (communication I).

SPARKI transgenic mouse model was designed in the group of Professor Frank Claessens (University of Leuven, Leuven, Belgium) and produced in the laboratory of Dr. Johan Auwerx (Institute of Genetics and Molecular and Cellular Biology, Strassbourg, France). The details of the procedure have been previously published (Schauwaers *et al*., 2007). In short, the second zinc finger of the AR BDB was swapped to the respective part of the GR DBD by creating a targeting vector in which exon 3 of *Ar* was replaced with exon 4 of *Gr*. Morula aggregation of the targeted ES cell clone with C57BL/6 blastocysts produced a chimeric male mouse. Transgenic female offspring from chimeric breeding was crossed with a C57BL/6 CMV-CRE male to achieve male offspring with a mutant AR allele without the neomycin cassette. The mice were tested for the presence of wild-type or mutated AR by PCR analysis.

1.2 Mice and hormonal treatments

All the experiments were carried out using 8–12-week-old male and female mice. In communication II, wild-type males of ICR (CD-10) outbred strain were used. In communications I and III, transgenic mice heterozygous to reporter construct and harboring the chimeric AR (SPARKI) along with their wild-type littermates were used, respectively. Mice were orchiectomized under medetomidine-ketamine general anesthesia through a lateral incision to scrotum. Hormonal treatments were started four days after castration using subcutaneous injections. The androgen dose and the 5-d duration of testosterone and genistein administration were chosen on the basis of the previous work on androgen action in murine tissues (Kontula *et al*., 1984; Melanitou *et al*., 1987) and to assure that steady-state conditions were reached. Summary of treatments in different experiments is shown in Table 3.

Table 3. Hormonal treatments of mice in the *in vivo* experiments of communications I, II, and III.

Study	Compound	Vehicle	Dose / body weight	Exposure	Animals	Experiment
I	Testosterone	Oil	30 mg / kg	24 h	Castrated males	*In vivo* imaging, *ex vivo* luciferase measurement
	Testosterone	Oil	30 mg / kg	5 d	Castrated males, intact females	*Ex vivo* luciferase measurement
	Genistein	Oil 2% DMSO	10 mg / kg	5 d	Castrated males, intact males and females	*Ex vivo* luciferase measurement, gene expression profiling, AR immunoblotting
	Estradiol	Oil 0.5% EtOH	10 μg / kg	4 d	Intact males	Gene expression profiling
II	Testosterone	Oil	30 mg / kg	2 h	Castrated males	Chromatin immunoprecipitation
	Testosterone	Oil	30 mg / kg	12, 24 h 3 d	Castrated males	Gene expression profiling
III	Testosterone	Silastic implant	1 mg / kg	7 d	Castrated males	Gene expression profiling

All *in vivo* experiments in communications I and II were performed according to the guidelines for animal experiments at the University of Helsinki and under license from appropriate Finnish Review Board for animal experiments, and in communication III with approval of the Katholieke Universiteit Leuven ethical committee.

1.3 *In vivo* imaging and *ex vivo* luciferase assays

In vivo luciferase activity was monitored using IVIS Imaging System (Caliper LifeSciences, Hopkinton, MA). Mice were injected with the luciferase substrate luciferin (4.5 mg / mouse in 100 µl of PBS, ip). Imaging was carried out 10 min post-injection in dark light-tight chamber with a sensitive charge-coupled device camera under medetomidine-ketamine anesthesia. The photon counts were registered for 1 min and converted to a pseudocolored image and superimposed on a photographic image of the mice using Living Image Software (Caliper LifeSciences).

For protein isolation and *ex vivo* luciferase assay, tissues were flash-frozen in liquid nitrogen immediately after harvesting and stored at −80°C. Frozen tissues were homogenized with Ultra Turrax (Ika, Staufen, Germany) in 300 µl of tissue lysis buffer containing 100 mM K_2PO_4 (pH 7.8), 1 mM dithiothreitol, 4 mM EGTA, and 4 mM EDTA with a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Homogenates were centrifuged for 30 min at 16,000 × *g* (4°C), and supernatants were used for protein concentration and luciferase activity measurements. Luciferase activity was assayed from duplicate samples (20 µl) using Luminoskan Ascent luminometer (Thermo Fisher Scientific, Waltham, MA) and 80 µl of luciferin solution (Promega, Madison, WI). Protein concentrations were measured using BioRad Reagent (BioRad Laboratories, Hercules, CA) according to the manufacturer's instructions. The luciferase activities were expressed as relative light units (RLU) per µg of protein. Statistical analyses were performed using Student's *t* test.

2. Cell lines and *in vitro* experiments

2.1 Cell lines and culture procedures

Immortalized epithelial cell line from caput epididymis (mE-Cap28 cells) has been established from GPX5-Tag1 transgenic mice (Sipilä *et al*., 2004), and was a kind gift from Dr. Petra Sipilä (University of Helsinki, Finland). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Life Technologies, Carlsbad, CA) in the presence of 10% fetal bovine serum (FBS, Thermo Fisher) and antibiotics (penicillin and streptomycin). HeLa cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM containing 4.5 g/l glucose, 4 mM L-glutamine, and penicillin (100 IU/ml)-streptomycin (100 μg/ml) (Sigma-Aldrich, St. Louis, MO), and supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA). Murine proximal tubule epithelial cells (MRPTEpiCells) were purchased from ScienCell Research Laboratories (Carlsbad, CA) and were cultured in the growth medium provided by the manufacturer.

Primary culture of proximal tubule epithelial cells from murine kidney was prepared as described previously (Asadi *et al*., 1994). Kidneys were harvested in ice-cold Hank's balanced salt solution (Gibco) and decapsulated. Cortices were dissected and minced with scalpel, followed by treatment with collagenase I from *Clostridium histolyticum* (Gibco) in the presence of 0.5% BSA (Fraction V) for 45 min at room temperature. Tubule suspension was filtered through wire mesh and centrifuged for 2 min at 60 x *g*. The pellet was washed with and resuspended in the growth medium consisting of 1:1 DMEM/F-12 (Gibco) supplemented with 0.015 M $NaHCO_3$ (Gibco), 0.1 mM Na-pyruvate (Gibco), 2.5 mM NaCl, 5 μg/ml bovine insulin (Sigma-Aldrich), 5 μg/ml human transferrin (Sigma-Aldrich), 50 nM hydrocortisone (Sigma-Aldrich), 1.2 ng/ml epidermal growth factor (Gibco), and 10% charcoal-stripped FBS (Thermo Fisher). Tubules were allowed to sediment by gravity in growth medium for 10 min. The supernatant was removed, and the sediment was resuspended and allowed to sediment for 10 min. Sedimentation was repeated four times, and the final sediment was plated on culture dish. The presence of tubules in the final sediment was confirmed by microscopic evaluation.

2.2 Cell culture experiments

For ChIP assays, mE-Cap28 and primary kidney cells were seeded in their growth media supplemented with 10% charcoal-stripped FBS. After three days, cells were treated with 100 nM testosterone or vehicle for 2 h. Transactivation experiments were carried out as previously described (Verrijdt *et al*., 2002) in HeLa cells transfected with AR or GR expression plasmid (Scheller *et al*., 1998), luciferase reporter plasmid containing four copies of the ARE sequences (communication III), and β-galactosidase expression plasmid (Stratagene, La Jolla, CA). Luciferase and β-galactosidase activities were measured after a 24-h exposure to 10 nM methyltrienolone (Perkin Elmer, Waltham, MA) or dexamethasone (Sigma-Aldrich).

2.3 Small interfering RNA (siRNA) transfection

siRNA transfections were performed to deplete AP-2α from mE-Cap28 cells. Cells were seeded in DMEM containing 10% charcoal-stripped FBS, and 70–80% confluent cells were transfected with siRNA SMARTpools targeting AP-2α mRNA or non-targeting control (Thermo Fisher) with Dharmafect-1 transfection reagent according to the manufacturer's instructions. AP-2α mRNA and protein levels were used to monitor depletion efficiency and ~80% reduction was observed at 72 h after transfection. ChIP assays were performed at 72 h post-transfection using 2-h testosterone treatment (100 nM). The siRNA sequences are shown in communication II.

2.4 Electrophoretic mobility shift assay (EMSA)

AR and GR DBDs were expressed as glutathione S-transferase fusion proteins (Schoenmakers *et al*., 1999). Double-stranded oligonucleotides containing ARE sequences (communication III) along with *Slp*-MUT and *Slp*-HRE control sequences (Denayer *et al*., 2010) were labeled with [α-^{32}P]dCTP and incubated with purified AR DBD or GR DBD in 10 mM HEPES (pH 7.9), 2.5 mM $MgCl_2$, 0.05 mM EDTA, 8% glycerol, 50 mM NaCl, 2.5 ng/µl poly (dIdC), 1 mM dithiothreitol, and 0.05% Triton X-100 for 20 min on ice. DBD-bound and unbound probes were resolved on a 4% polyacrylamide gel and bands visualized with STORM-840 PhosphorImager (GE Healthcare, Buckinghamshire, UK).

3. Gene expression profiling

3.1 RNA isolation, cDNA synthesis, and qRT-PCR

Tissues were harvested and stored in RNALater (Qiagen GmbH, Hilden, Germany). Total RNA was isolated from the tissues using RNeasy Mini kit and RNeasy Lipid Tissue Midi kit (Qiagen) according to the manufacturer's instructions. cDNA synthesis was carried out using 1–2 μg of total RNA with Superscript III First-Strand Synthesis System (Invitrogen) or Transcriptor High Fidelity cDNA synthesis kit (Roche) using random hexamers. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using SYBR Green mastermix (Roche) and 1 μM forward and reverse primers in total volume of 20 μl. The results were calculated with LightCycler 480 Software (Roche) using standard curve and normalized to 18S rRNA levels. Student's *t* test ($p<0.05$ or $p<0.001$) was used to calculate the statistical significance of differences in gene expression in different experimental groups. The primer sequences are provided in communications I, II, and III.

3.2 Microarray experiments

The RNA integrity values of the samples were > 8, as determined using Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). Direct hybridization assay was performed for 500 ng of each total RNA sample (n = 3–4 biological replicates per group) using Illumina TotalPrep RNA Amplification kit (Ambion, Austin, TX) according to the manufacturer's protocol. Biotin-labeled cRNA was hybridized to expression chips using MouseWG-6 v2 Expression BeadChip kit (Illumina Inc., San Diego, CA). After staining with streptavidin-Cy3 (GE Healthcare), chips were scanned using iScan System (Illumina) at the Biomedicum Functional Genomics core facility. Data analysis was performed using Anduril software (Ovaska *et al*., 2010) and Genomic region operation kit (Ovaska *et al*., 2013) together with "R" software and Bioconductor "lumi" package (http://www.r-project.org/, http://www.bioconductor.org). Raw intensity values were normalized independently between arrays for each sample using quantile normalization. The median values of sample replicates were used to calculate differentially expressed genes between the treatment groups with cut-off values of fold-change ≥1.5 and a p-value < 0.05

(Student's *t* test). Unsupervised hierarchical clustering and heat maps were generated using Cluster and TreeView (Eisen *et al*., 1998).

3.3 Immunoblotting

Proteins were extracted from frozen tissues and protein concentrations determined as described in Chapter 1.3. Cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.8), 150 mM NaCl, 15 mM MgCl2, 5 mM EDTA, 0.5% Nonidet P-40, 0.3% Triton X-100, and complete protease inhibitor set (Roche). Fifty μg of total protein from tissues or from mE-Cap28 and primary kidney cells was resolved on a 7.5% or 10% SDS-PAGE (BioRad) and transferred onto enhanced chemiluminescence (ECL) membrane (GE Healthcare). The specific antibodies used in immunoblotting are described in communications I and II. The immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and visualized with ECL reagent (GE Healthcare) and were scanned and quantified by using the ImageJ program in communication I.

3.4 Immunohistochemistry

Prostate, kidney, and caput epididymis tissues were collected from intact male mice and fixed in 4% paraformaldehyde at 4°C overnight, dehydrated, and embedded in paraffin. Five-μm sections were dewaxed with xylene, rehydrated, and treated with 2% hydrogen peroxide for 15 min to block the endogenous peroxidase activity. For antigen retrieval, the slides were boiled in 10 mM sodium citrate (pH 6.0). The blocking was performed in 1% bovine serum albumin and normal goat or horse serum (Vector Laboratories, Burlingame, CA), and incubation with primary antibodies (communication II) was carried out in 1:200 dilution at 4°C overnight. Biotinylated anti-rabbit and anti-mouse IgG secondary antibodies (Vector Laboratories) were used in 1:200 dilution, and the immune complexes were visualized using the Vectastain Elite ABC and DAB substrate kits (Vector Laboratories). Counterstaining was carried out using Mayer's hemalum solution (Merck, Darmstadt, Germany). The slides were dehydrated and mounted using Permount (Fisher Chemicals, Fair Lawn, NJ).

4. Genome-wide analysis of transcription factor binding

4.1 Chromatin immunoprecipitation (ChIP) and ChIP-qPCR

In ChIP assays, testosterone- or vehicle-treated cells or tissues from intact or testosterone- or vehicle-treated castrated male mice were used. Testosterone-treatment of 2 h was used for both cells (100 nM) and mice (1 mg in 100 μl mineral oil, ip). Cells were cross-linked in 1% formaldehyde for 10 min at room temperature, washed with ice-cold PBS and scraped in Farnham lysis buffer as previously described (Sahu *et al*., 2011). Cell pellet was resuspended in 300 μl RIPA buffer containing protease inhibitors and sonicated to 100–500-bp chromatin fragments with Bioruptor (Diagenode, Liege, Belgium). Fresh tissues were minced with a scalpel, and frozen tissues were pulverized before 20 min cross-linking (1% formaldehyde, room temperature). Cross-linking was stopped by using 0.125 M glycine for 5 min at room temperature, followed by two washes with ice-cold PBS. The cross-linked tissues were homogenized in hexylene glycol buffer containing 1 M hexylene glycol, 0.1 mM $MgCl_2$, 5 mM EGTA, 1 mM PIPES, 2 mM dithiothreitol, and complete protease inhibitor set (Roche) using Ultra Turrax (Ika), and the homogenate was filtered through nylon net (Isomaa *et al*., 1982). To isolate crude nuclear fraction, the filtered homogenate was centrifuged, and the pellet was resuspended in 500 μl RIPA buffer. Chromatin was sonicated to yield fragments of 100–500 bp in size using a micro-tip sonicator (Misonix Inc., Farmingdale, NY).

For a single immunoprecipitation reaction, 100 μl chromatin from cells or tissues was used. Antibodies conjugated to magnetic Dynabeads (Invitrogen) were incubated at 4°C with sonicated chromatin overnight, followed by five washes in LiCl wash buffer and reverse cross-linking at 65°C for 15 h. Immunoprecipitated DNA was purified using Qiaquick PCR purification kit (Qiagen) and eluted in 100 μl of elution buffer. In ChIP-qPCR, immunoprecipitated and input DNA was amplified using SYBR Green Mastermix (Roche) and specific primers, and the results were shown as % of input values. Details of the antibodies and primer sequences are provided in communications II and III.

4.2 ChIP-sequencing

Immunoprecipitated DNA samples were processed for library preparation according to Illumina's instructions as described previously (Sahu *et al*., 2011), by pooling three to four immunoprecipitates for each library. In brief, the DNA samples were blunt-ended and ligated to sequencing adapters. Adapter-ligated DNA fragments (size range, 150–300 bp) were excised from agarose gel and purified with QIAquick Gel Extraction kit (Qiagen). Isolated DNA was amplified by PCR (20 cycles), and the purified DNA library was sequenced using Genome Analyzer II (Illumina) at Biomedicum Functional Genomics core facility. ChIP-seq reads were filtered using the Illumina chastity filter during the base-calling process and the reads were aligned to reference mouse genome (mm9) using Bowtie, without any mismatches. ChIP-seq experiments were performed in biological duplicates, and only reproducible peaks were used for further analyses.

4.3 Bioinformatics analyses of ChIP-seq data

ChIP-seq peak calling and differential peak calling were performed using MACS version 1.4, MACS2 (Feng *et al*., 2012; Zhang *et al*., 2008) and HOMER (Heinz *et al*., 2010) algorithms. The overlap analysis, CEAS analysis, genome-wide correlation, motif analysis, and tag density maps were performed using Cistrome: an integrative platform for transcription regulation studies (Liu *et al*., 2011). In communication II, *de novo* and motif enrichment analyses for top-enriched 5,000 AR ChIP-seq peaks unique to each tissue were performed using SeqPos motif tool in Cistrome, and the results were sorted on the basis of z-score. *De novo* motifs were identified using TOMTOM motif comparison tool in MEME Suite (Gupta *et al*., 2007). Find Motif algorithm in HOMER analysis suite (Heinz *et al*., 2010) was used for motif scanning among all AR-binding sites in each tissue, and for *de novo* analysis in communication III along with MEME Suite (Bailey *et al*., 2009). Data visualization was carried out using Integrative Genomics Viewer (Robinson *et al*., 2011b).

RESULTS AND DISCUSSION

1. Generation of the androgen reporter mouse model (Communication I)

1.1 Generation of the transgenic reporter mouse model

Generation of transgenic estrogen reporter mouse lines ten years ago demonstrated the potential of using *in vivo* imaging to study steroid receptor function in live mice (Ciana *et al*., 2003; Lemmen *et al*., 2004). In this thesis work, the first androgen reporter mouse suitable for *in vivo* imaging in multiple tissues was described – the previous models for AR were either designed for reporter expression in prostate only or used LacZ reporter that needs to be detected with staining rather than imaging (Iyer *et al*., 2005; Lyons *et al*., 2006; Xie *et al*., 2004; Ye *et al*., 2005). The androgen reporter construct containing luciferase reporter gene driven by four copies of the AR-selective *Slp*-HRE2 response elements (Verrijdt *et al*., 2000) and thymidine kinase minimal promoter was integrated to mouse genome by pronuclear injection technique. MAR-insulator sequences (Phi-Van *et al*., 1990) were introduced to the construct to minimize potential position effects of the genomic integration site on reporter gene expression. In this model, luciferase expression is a measure of androgen action *via* AR. Luciferase is a well-suited reporter for *in vivo* studies due to its low background activity in mammals in bioluminescent imaging and easily quantifiable and sensitive enzymatic assay for *ex vivo* measurements (Ottobrini *et al*., 2006). The progeny of fourteen founder animals that had the reporter construct integrated into their genome was screened for luciferase expression in different tissues. The mouse line with the greatest number of androgen-responsive tissues reproducible expressing the *luciferase* reporter gene was selected for further studies.

1.2 Validation of the androgen reporter mouse

Luciferase expression was detected in several tissues in intact male and female mice of the reporter mouse line, including skeletal muscle, brain, and lung. The highest values for luciferase activity were observed in skeletal muscle in females and prostate in males. Testosterone administration induced reporter gene activity, as judged by *in vivo* imaging and *ex vivo* luciferase measurements from testosterone-treated castrated males and intact

females when compared to vehicle-treated controls (Figure 8). Interestingly, reporter activity in lungs of castrated males was androgen-induced, and this observation led to further characterization of AR action in lung and identified lung as a novel androgen target tissue (Mikkonen *et al*., 2010).

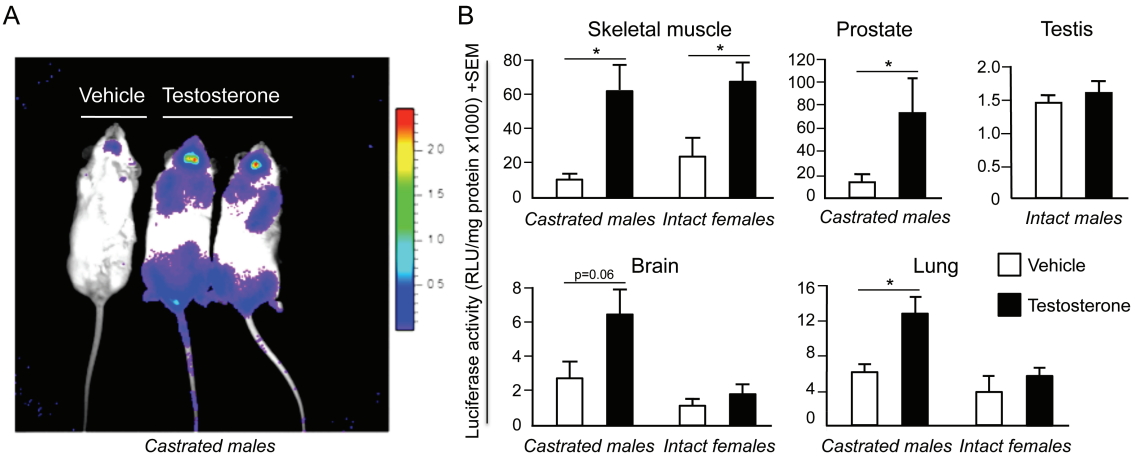

Figure 8. Androgen-induced luciferase activity measured *in vivo* using sensitive charge-coupled device camera (A) and by enzymatic *ex vivo* assay (B) from tissues of testosterone-treated castrated males and intact females.

Despite the insulator sequences flanking the reporter construct, luciferase expression was not detected in some known androgen-target tissues, such as liver and kidney. Random genomic integration after pronuclear DNA injection might result in transgene localization at a site where the local genomic environment is not favorable for luciferase expression or its androgen regulation. Recently, another androgen reporter mouse line was described (Dart *et al*., 2013). In this case, the reporter construct was targeted to the *hypoxanthine phosphoribosyltransferase (Hprt)* gene locus that provides an open chromatin environment for targeted transgene expression (Bronson *et al*., 1996). This latter mouse line showed a broader range of tissues with detectable luciferase activity, attesting to the effect of genomic environment for transgene expression. Nevertheless, our reporter mouse showed robust, reproducible, and androgen-responsive reporter expression in several important AR-target tissues, including prostate, skeletal muscle, testis, and brain, and it was well-suited for studying tissue-specific *in vivo* effects of chemical compounds or other conditions of interest.

2. Genistein as a novel androgen receptor modulator (Communication I)

2.1 Genistein affects reporter gene activity in a tissue-specific manner

Genistein, a polyphenolic phytoestrogen present in soy, is a bioactive compound with estrogenic effects in cells (*e.g*., Chang *et al*., 2008) and tissues (*e.g.,* Moggs *et al*., 2004; Thomsen *et al*., 2006). Considerable amount of genistein is found in circulation of individuals consuming soy products, up to 2.5 μM in plasma of soy-based formula-fed infants (Setchell *et al*., 1997). Genistein is an interesting compound in terms of cancer prevention due to its effects on steroid hormone signaling and epidemiological evidence for low incidence of hormonal cancers in soy-consuming Asian countries (Jian, 2009).

Androgen reporter mice were exposed to five-day treatment with genistein to examine its *in vivo* effects on AR signaling at a dose relevant in humans on a soy-rich diet. In the presence of physiological androgens, genistein decreased luciferase activity in prostate, brain, and testis without affecting AR protein amount, when compared to intact vehicle-treated mice. However, under androgen-deprived conditions, genistein had an agonistic effect on AR function in prostate and brain (Figure 9A). Reporter activity in skeletal muscle and lung was unaffected by genistein treatment, demonstrating the tissue-selective nature of genistein action. According to an *in silico* docking model, genistein fits to the AR ligand-binding pocket (Wang *et al*., 2010); however, there are conflicting reports on genistein bioactivity in androgen reporter model systems *in vitro* (Bovee *et al*., 2008; Takahashi *et al*., 2004; Takeuchi *et al*., 2009). Our results provided evidence for genistein modulating AR signaling under *in vivo* conditions in a tissue-specific manner.

2.2 Genistein affects endogenous gene expression profile in prostate

Genistein effect on expression profiles of endogenous genes was assessed using gene expression microarray. With a fold change ≥ 1.5 ($p < 0.05$), 146 and 307 transcripts were differentially expressed in response to genistein treatment in prostates of intact and castrated mice, respectively. In intact prostate, two thirds of transcripts regulated by genistein were also androgen-dependent, strongly supporting the notion that genistein affects gene expression by modulating AR activity. In most cases in intact prostate,

genistein effect on gene expression was opposite to that of androgen (Figure 9B). By contrast, genistein had more androgen-like effects in the prostates of castrated males (Figure 9B). Genistein is also able to affect estrogen signaling, but a greater proportion of genistein-regulated genes in murine prostate overlap with AR transcriptional program compared to that of ER, implying that the effects of genistein on androgen signaling in mouse prostate are unlikely to involve ER activation. Interestingly, genistein-treatment has been shown to bring about thousands of ERα-binding events in breast and uterine carcinoma cell lines (Gertz *et al*., 2012). Delineation of genistein-induced AR cistrome in murine prostate would complement the transcription profiles obtained in the present study. Although genistein can also modulate growth factor receptor functions, inhibition of receptor tyrosine kinases requires much higher circulating genistein levels than the one used in the present study (Akiyama *et al*., 1987).

In conclusion, genistein elicits both agonistic and antagonistic effects on AR-regulated gene expression. Of note, a recent study reported that AR mutations change the agonistic/antagonistic effects of genistein in prostate cancer cell lines (Mahmoud *et al*., 2013), which might explain previous controversial results in studies using cell lines. The strength of our study is, however, the use of physiological *in vivo* environment with multiple cell types within the normal tissue structure together with the intact AR pathway containing endogenous coregulators and collaborating factors.

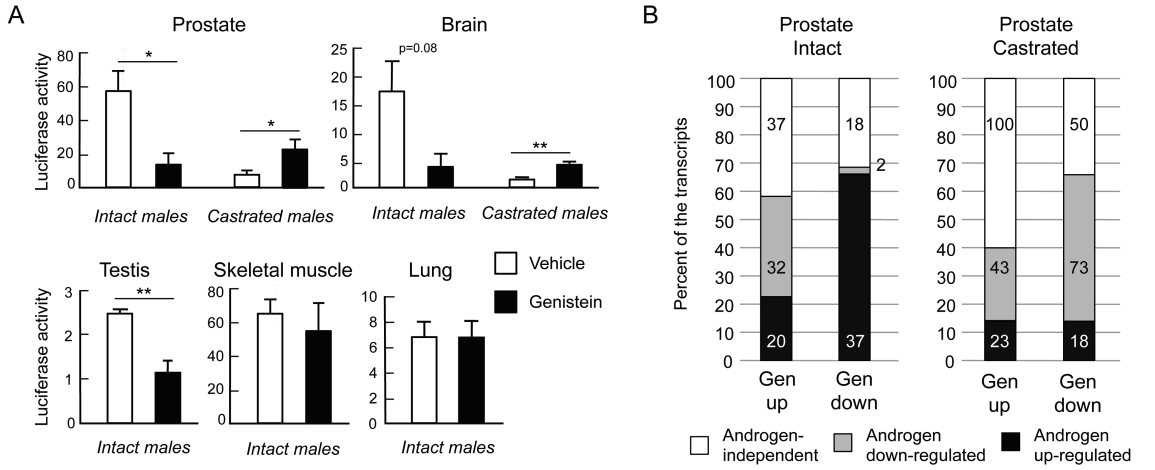

Figure 9. The effect of genistein on androgen-induced luciferase activity measured by enzymatic *ex vivo* assay in five different tissues of castrated and intact males (A) and on endogenous gene expression profiles in prostates of castrated and intact males (B).

3. Characteristics of the AR pathway *in vivo* (Communications II and III)

3.1 *In vivo* AR cistromes are highly tissue-specific

Seminal papers using chromatin immunoprecipitation coupled with high-throughput sequencing from mouse tissues have demonstrated the power of ChIP-seq in studying tissue-specific transcription factor binding and chromatin modifications *in vivo* (Shen *et al*., 2012; Visel *et al*., 2009). However, despite numerous AR cistromes reported for prostate cancer cell lines, only few genome-wide AR binding profiles in tissues have been described. The first *in vivo* AR cistrome was reported for mouse epididymis (Hu *et al*., 2010), and subsequently, AR binding has been examined in the context of murine liver and prostate carcinogenesis (Chen *et al*., 2013; Li *et al*., 2012b) and in primary myoblasts (Wyce *et al*., 2010). Recent study also described AR binding profiles in prostate cancer patient samples (Sharma *et al*., 2013), but comprehensive analyses of AR cistromes and transcriptional programs under physiological conditions have been lacking.

In this thesis work, AR cistromes were determined for three androgen-responsive tissues – prostate, kidney, and epididymis – in castrated and androgen-treated male mice utilizing the ChIP-seq approach. High-quality data of *in vivo* AR occupancy was obtained with excellent reproducibility of two biological replicate samples and robust androgen-induced binding profiles. With stringent analysis parameters (false discovery rate < 2%, peak present in two biological replicates), 10,171 AR chromatin binding events in prostate, 14,062 in kidney, and 22,598 in caput epididymis were detected. Importantly, there was no significant chromatin AR binding in the absence of androgens. AR cistromes are highly tissue-specific: only a small proportion (7–16%) of AR-binding events were shared between all three tissues, and the majority of the binding events were unique to a given tissue (Figure 10A). The highest number of overlapping binding events was observed between the two reproductive tissues, prostate and epididymis. Previous studies reporting cell type-specific cistromes for ERα and PR have been based on cultured cancer cell lines (Gertz *et al*., 2013; Krum *et al*., 2008; Yin *et al*., 2012). AR cistromes obtained in our study under physiological conditions in live animals confirm that steroid receptor cistromes are highly tissue-specific.

3.2 AR regulates distinct cellular pathways in different tissues

Androgen-regulated transcripts in prostate, kidney, and epididymis were determined by microarray analysis at three different time intervals after testosterone exposure – 12 and 24 hours and 3 days – to obtain a comprehensive view of the AR transcriptome. At each time point, hundreds of differentially expressed transcripts were identified when castrated testosterone- and vehicle-treated mice were compared (fold change ≥ 1.5, $p < 0.05$). Importantly, AR regulates distinct transcription programs in different tissues: the majority of both up- and down-regulated transcripts are unique to each tissue, and only a few common genes are similarly regulated in all three tissues (Figure 10B). Interestingly, unsupervised hierarchical clustering revealed that there are transcripts up-regulated by androgen in one tissue, but down-regulated in another (Figure 10C), attesting to the context-specificity of androgen-dependent transcriptional regulation.

Enrichment analysis of gene ontology categories was performed for differentially expressed gene categories after a three-day testosterone-treatment. Various metabolic pathways are under androgenic control especially in kidney. Importantly, androgen-induced transcripts in prostate and epididymis but not in kidney are over-represented for genes involved in cell cycle regulation and mitosis. This agrees with the previous notion that androgen exposure increases hypertrophy but not hyperplasia of renal proximal tubule cells (Bardin and Catterall, 1981). In prostate, on the other hand, both epithelial and stromal AR contribute to the epithelial proliferation, as judged from the phenotypic changes in cell type-specific ARKO mice (Simanainen *et al*., 2007; Wu *et al*., 2007; Yu *et al*., 2011).

Androgen-regulated transcripts in each tissue were mapped to the AR-binding events. The majority of the differentially expressed genes in each tissue and at every time point have at least one AR-binding site within ±100 kb of the transcription start sites, confirming androgen-regulation of these transcripts and validating the accuracy of the ChIP-seq data. Both up- and down-regulated genes in each category could be mapped to the nearest AR-binding event statistically more often than stably expressed genes after testosterone exposure (Figure 10D).

3.3 Selective AREs guide AR binding to specific enhancers *in vivo*

All NR3C family steroid receptors – AR, GR, MR, and PR – bind to and transactivate *via* the classical androgen/glucocorticoid response element (AGE/GRE), an inverted repeat of the 5'-AGAACA-3' sequence, but the so-called selective AREs can only be bound by AR and not by GR (Denayer *et al*., 2010). Previous studies have shown that AR and GR bind to shared loci on native chromatin in cancer cells (John *et al*., 2011; Sahu *et al*., 2011; Sahu *et al*., 2013). Ligand-occupied GR is able to modulate the AR pathway and maintain the expression of AR-regulated genes under androgen-deprived conditions (Arora *et al*., 2013; Sahu *et al*., 2013). This phenomenon is of clinical importance since cancer cells of prostate cancer patients on AR-suppressing drugs are potentially able to bypass the AR blockade through GR action. Importantly, more than half of prostate carcinomas from patients treated with androgen deprivation therapy express GR (Yemelyanov *et al*., 2012), and glucocorticoids are currently administered to abiraterone-treated prostate cancer patients to avoid side effects from CYP17 inhibition (Attard *et al*., 2012).

The chimeric AR with its second zinc finger replaced with that of GR binds only to classical AREs (Schoenmakers *et al*., 1999). Knock-in mice with this chimeric AR, termed SPARKI mice, have smaller reproductive organs than the wild-type littermates and are hypofertile (Kerkhofs *et al*., 2012; Schauwaers *et al*., 2007). In the present study, the importance of the receptor DBD for chromatin binding *in vivo* was studied by examining the SPARKI AR cistrome and by comparing it to that of wild-type AR in murine epididymis and prostate. Both tissues showed distinct binding profiles for wild-type and SPARKI ARs (Figure 10E), and there are three categories of AR-binding events *in vivo*. Shared loci are bound by both SPARKI and wild-type AR with a similar affinity. Wild-type AR-preferred binding events – 55 and 50% of the epididymal and prostatic AR cistromes, respectively – comprise the AR-selective enhancers *in vivo*, and SPARKI AR is recruited to these sites with lower affinity (Figure 10E). Surprisingly, there are also SPARKI AR-preferred loci that wild-type AR binds with blunted affinity. Although mechanistic determinants for enhanced recruitment of SPARKI AR to this subset of loci

remain elusive, they are likely due to the local chromatin environment and/or differential interactions with coregulators (Wiench *et al*., 2011b).

Importantly, differential AR binding *in vivo* was associated with significant changes in transcription programs. According to gene expression microarrays, there are 219 androgen-dependent transcripts in epididymis with differential expression in SPARKI mice compared to wild-type mice (fold change ≥ 1.5, $p < 0.05$) (Figure 10F). In conclusion, selective AREs play a significant role in genome-wide AR binding in two reproductive tissues, prostate and epididymis, agreeing with the hypofertile phenotype of the SPARKI males (Schauwaers *et al*., 2007).

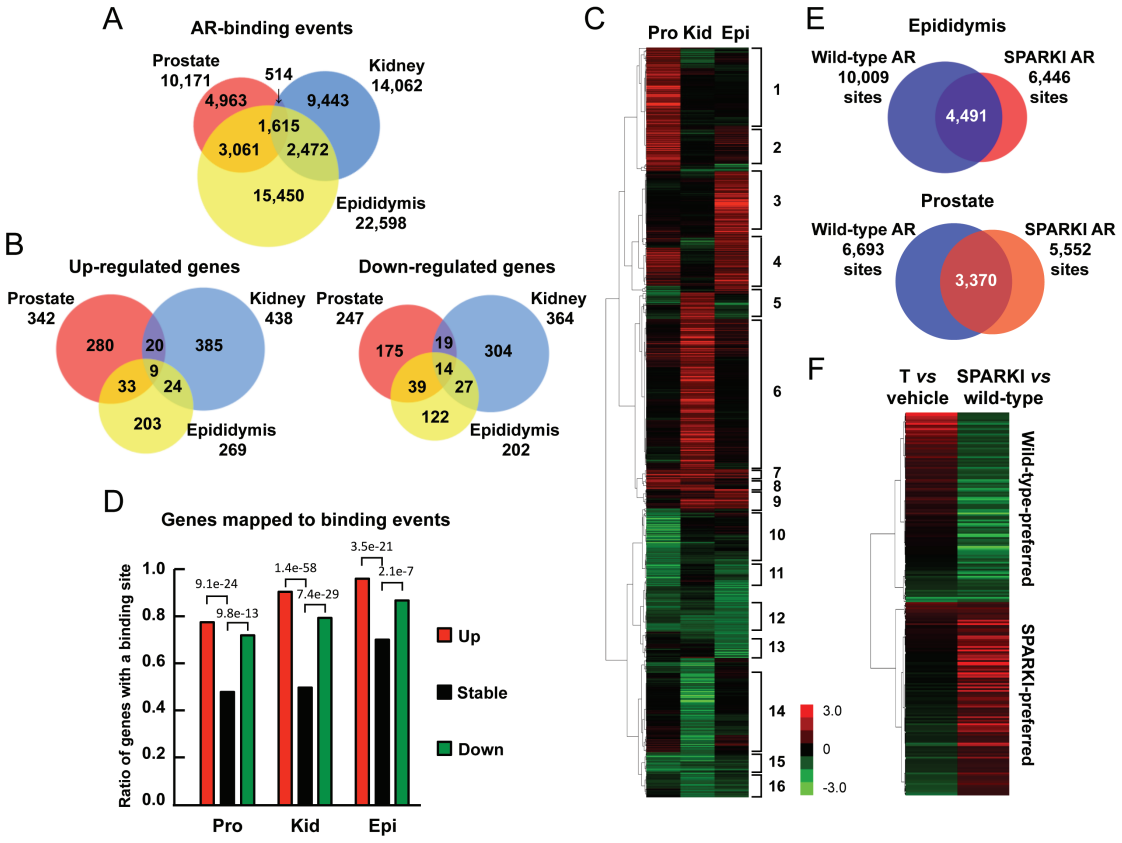

Figure 10. A. Area-proportional Venn diagram of AR-binding events in prostate, kidney, and epididymis after a 2-h androgen treatment. B. Transcripts up- and down-regulated in prostate, kidney, and epididymis after a 3-day androgen exposure. C. Unsupervised hierarchical clustering of androgen-regulated transcripts in prostate, kidney, and epididymis. D. Mapping of androgen-regulated genes to AR-binding events within ±100 kb from transcription start sites. E. Area-proportional Venn diagrams of AR-binding events in prostate and epididymis of wild-type and SPARKI mice. F. Unsupervised hierarchical clustering of androgen-regulated and differentially expressed transcripts in epididymis of wild-type and SPARKI mice.

4. Determinants of context-specific AR binding (Communications II and III)

4.1 Distinct pioneer factors guide tissue-specific AR binding *in vivo*

To investigate the cellular determinants for tissue-specific AR binding, *de novo* and motif enrichment analyses were performed for all prostate-unique and top 5,000 of kidney and epididymis-unique AR-binding events. Interestingly, *de novo* analysis identified distinct *cis*-elements enriched among AR binding events: FoxA1 in prostate, hepatocyte nuclear factor 4 alpha (Hnf4α) in kidney and AP-2α in epididymis, suggesting that these factors bind to the sites shared with AR in a tissue-specific manner. Indeed, ChIP-seq carried out for these transcription factors in prostate, kidney and epididymis revealed significant overlap for AR and FoxA1 cistromes in prostate, AR and Hnf4α cistromes in kidney, and AR and AP-2α cistromes in epididymis (Figure 11A). Of note, there is no FoxA1 binding in kidney and epididymis nor Hnf4α binding in prostate and epididymis (Figure 11B). A small number of AR-binding events overlap with AP-2α binding in prostate and kidney, but the number is considerably lower than the overlapping sites with FoxA1 or Hnf4α, respectively. ARE *cis*-elements were also found to be significantly enriched among the tissue-specific AR-binding events in prostate, kidney, and epididymis. The situation appears to be different in the case of ERα, with cell type-specific ER cistromes having only weak ER-response elements in breast and uterine cancer cell lines (Gertz *et al*., 2013).

In order to serve as pioneer factors for AR, tissue-specific collaborating factors should occupy the enhancers already prior to androgen-induced AR binding. ChIP-seq from the tissues of castrated males confirmed that FoxA1 in prostate, Hnf4α in kidney, and AP-2α in epididymis bind to chromatin in the absence of androgen and potentially prime it for subsequent AR binding (Figure 11B). Furthermore, a greater proportion of enhancers shared by AR and FoxA1 in prostate, AR and Hnf4α in kidney, and AR and AP-2α in epididymis reside in an open chromatin environment marked by H3K4me1 and K3K27ac histone modifications than the AR only sites, further attesting to the functional significance of these shared sites as transcriptionally active enhancers (Figure 11C). Immunohistochemical antigen staining confirmed that the tissue-specific collaborating

factors, that is, FoxA1 in prostate, Hnf4α in kidney, and AP-2α in epididymis, are expressed in the same cell types as AR.

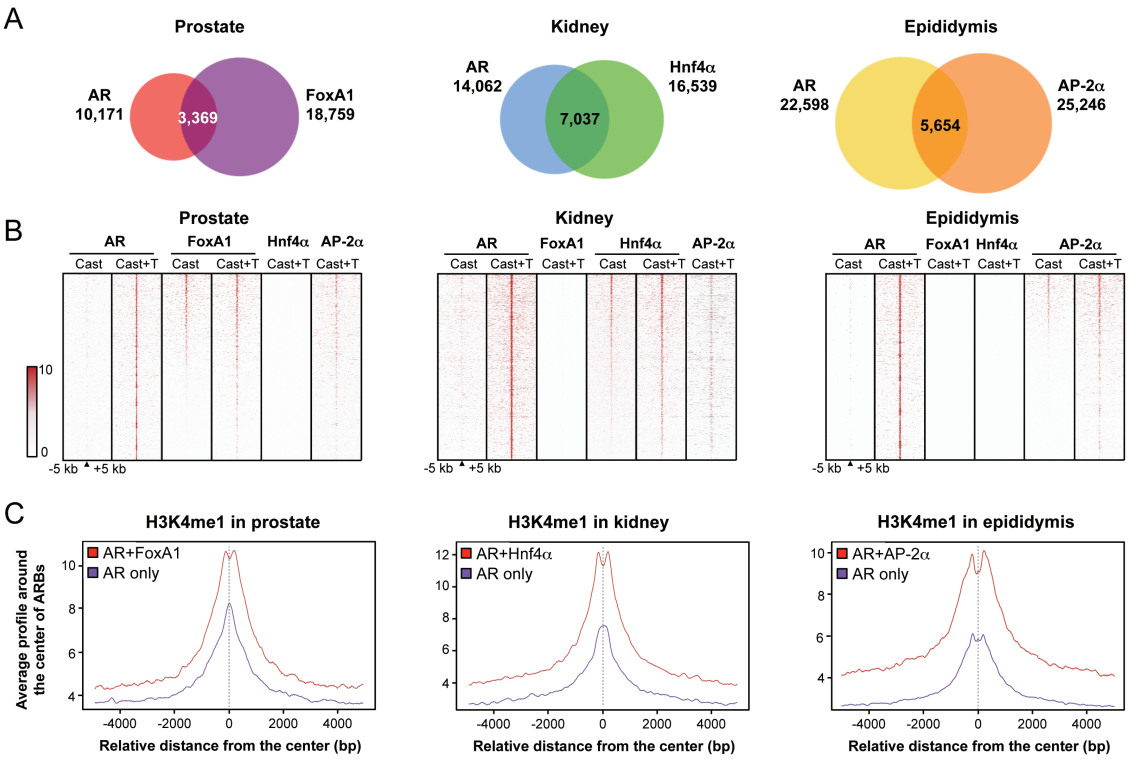

Figure 11. A. Area-proportional Venn diagrams of AR-binding events with tissue-specific pioneer factors FoxA1 in prostate, Hnf4α in kidney, and AP-2α epididymis after 2-h androgen treatment. B. Tag density maps of AR, FoxA1, Hnf4α, and AP-2α in prostate, kidney and epididymis. C. Average tag profiles of ChIP-seq tags for H3K4me1 histone modification in shared AR-FoxA1 and AR only sites in prostate, in shared AR-Hnf4α and AR only sites in kidney, and shared AR-AP-2α and AR only sites in epididymis.

The pioneering role for FoxA1 is well-established in prostate cancer cell lines (Lupien *et al*., 2008; Sahu *et al*., 2011), and our *in vivo* results from murine prostate confirm that observation. Other pioneer factors suggested for AR in prostate cancer cell lines include GATA and ETS factors (Chen *et al*., 2013; Chng *et al*., 2012; Massie *et al*., 2007; Wang *et al*., 2007; Wu *et al*., 2014; Yu *et al*., 2010). Our results revealed two novel pioneer factors for AR, Hnf4α in kidney and AP-2α in epididymis, and underline that FoxA1 is not a universal, but rather a prostate-specific pioneer factor for AR. To confirm further that AR binding is dependent on these novel factors, immortalized murine epididymal cell line depleted of AP-2α using specific siRNA, and primary epithelial cells from renal proximal tubules inherently devoid of Hnf4α, were used in ChIP assays. Attenuation of

AR binding was observed under these depletion conditions at the loci shared by AR and Hnf4α and AR and AP-2α in these renal and epididymal cell lines. AP-2γ was recently identified as pioneer factor for ER in breast cancer cells (Tan *et al*., 2011). AP-2γ is not expressed in murine epididymis, but another member of the same protein family, AP-2β, may also play a role in epididymal AR binding (Hu *et al*., 2010). Hnf4α is a constitutively active nuclear receptor that has not been previously linked to AR function. However, Hnf4α is reported to be required for small heterodimer partner (SHP) binding, and liver-specific depletion of Hnf4α results in reduction in H3K4 trimethylation (Kir *et al*., 2012). Interestingly, Hnf4α depletion has more prominent effects on gene expression in liver of male mice compared to females (Holloway *et al*., 2008), but its role in AR signaling in tissues other than kidney remains to be elucidated.

The idea that distinct pioneer factors dictate cell type-specific steroid receptor cistromes has been suggested on the basis of the results acquired from the cancer cell lines (Krum *et al*., 2008). However, overlapping steroid receptor and pioneer factor cistromes have been produced mostly from breast and prostate cancer cell lines for ER and AR, respectively (*cf*. Table 2). Although collaborating factors other than FoxA1 have been discovered, many of these novel factors are also linked to steroid receptor function in the same cell types, such as GATA-3 and AP-2γ for ERα function in breast cancer cells (Gertz *et al*., 2013; Tan *et al*., 2011; Theodorou *et al*., 2013) and GATA-2 and ETS factors for AR function in prostate cancer cells (Massie *et al*., 2011; Wu *et al*., 2014). Furthermore, several studies that have suggested differential pioneer factor involvement have merely reported differential *cis*-element enrichment around receptor-binding events, not taking into account whether the suggested factors actually bind to these sites, or are even expressed in the cell types in question (Lain *et al*., 2013; Rubel *et al*., 2012; Yin *et al*., 2012). In summary, this thesis work on androgen receptor binding in multiple tissues and under physiological conditions *in vivo* emphasizes the role of distinct pioneer factors for tissue-specific AR cistromes (Figure 12). This concept is likely to apply to other steroid receptors as well. However, pioneer factors for AR in tissues other than those investigated in the present work, and for other steroid receptors in their multiple target tissues remain to be discovered.

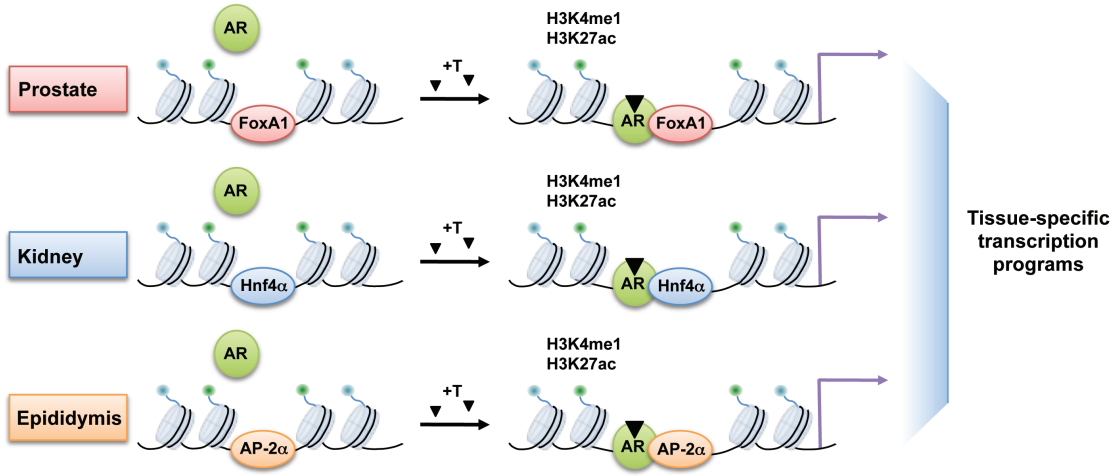

Figure 12. Schematic representation of the role of distinct pioneer factors in tissue-specific AR binding and transcriptional regulation.

4.2 Selective AREs exhibit relaxed sequence stringency *in vivo*

In vivo AR cistromes revealed three categories of AR-binding events: wild-type AR-preferred, SPARKI AR-preferred, and shared sites bound by both receptors with similar affinity. In order to study the DNA sequence of the *cis*-elements enriched for these three categories, *de novo* motif search was performed. As expected, canonical ARE/GRE, an inverted repeat of the 5'-AGAACA-3' hexamer, was enriched among the shared AR-binding sites, *i.e.*, the sites that both wild-type and SPARKI AR are able to bind (Figure 13). A similar motif was also present among the SPARKI AR-preferred sequences. Strikingly, the *cis*-element enriched among wild-type AR-preferred sequences in both prostate and epididymis showed very weak sequence conservation in the second hexamer (Figure 13). These results suggest that AR-selective receptor binding *in vivo* is achieved through relaxed *cis*-element stringency rather than any exact ARE sequence. Thus, the previous concept that DNA sequence of selective AREs is a direct repeat of the 5'-AGAACA-3' sequence (Denayer *et al.*, 2010; Helsen *et al.*, 2012; Verrijdt *et al.*, 2000) is not a general rule of AR-selectivity. However, due to the relaxed sequence requirement for the second hexamer, a selective ARE can potentially also resemble a direct repeat. Interestingly, the middle nucleotide in the spacer displays sequence conservation and enrichment of an adenine over other nucleotides among shared and SPARKI AR-preferred sites in prostate (Figure 13B). The importance of the spacer sequence was

recently demonstrated in a report showing that the nucleotides in the GRE spacer affect GR DBD conformation and its DNA-binding affinity (Watson *et al*., 2013).

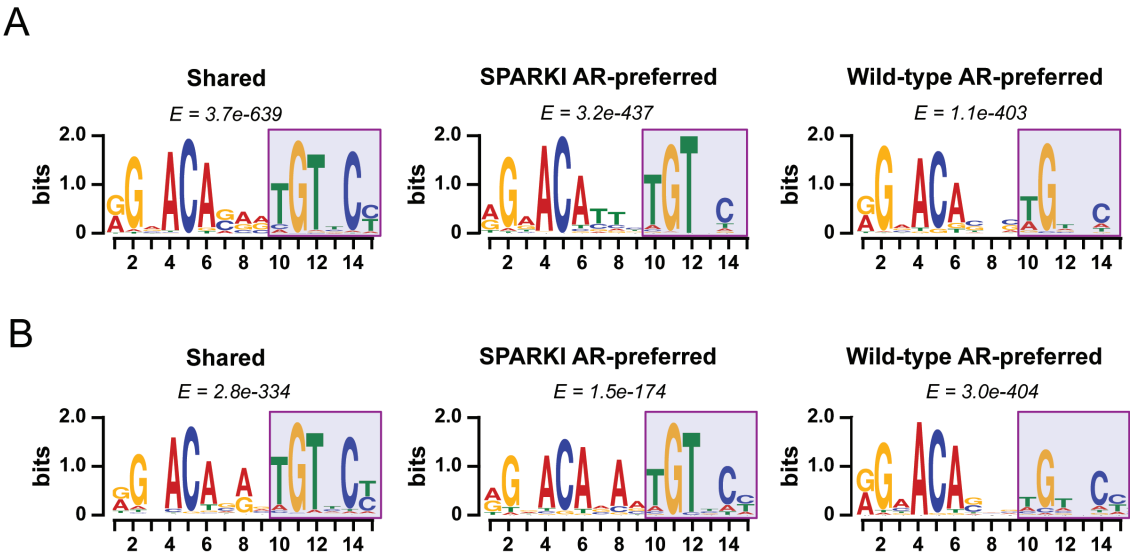

Figure 13. *Cis*-elements identified by *de novo* motif analysis within AR-binding sites shared by wild-type and SPARKI AR, and for SPARKI AR-preferred and wild-type AR-preferred sites in epididymis (A) and prostate (B).

The first zinc finger in the steroid receptor DBD is responsible for the sequence-specific DNA contact, and the second zinc finger – the part that differs between wild-type and SPARKI AR – is responsible mainly for receptor dimerization. Compared to the GR DBD, the amino acid residues in the AR DBD allow stronger affinity at the dimer interface (Luisi *et al*., 1991; Shaffer *et al*., 2004). This phenomenon potentially explains why the sequence of the second hexamer is less important for AR binding, since a stronger receptor dimerization would allow selective AR binding to AREs that GR cannot bind. Moreover, single amino acid insertion in GR dimer interface has been reported to lead to redistribution of GR binding in a genome-wide scale (Thomas-Chollier *et al*., 2013).

FUTURE PERSPECTIVES

ChIP-seq is a powerful method for studying chromatin-associated proteins and covalent modifications, as demonstrated by the wealth of data generated over the past decade using next-generation sequencing approaches. However, genome-wide mapping of receptor occupancy is just the first step towards more comprehensive understanding of transcriptional regulation. The ENCODE Project Consortium has produced detailed maps of gene regulatory elements in a wide range of cell types and commenced systematic analyses of the architecture of transcriptional networks. Nevertheless, assigning defined sets of regulatory elements to a particular transcription unit is a challenge that requires further research efforts and development of new methodology, such as the novel genome editing tools.

Few recent reports have initiated functional characterization of the distal transcription factor-binding sites. First, a polymorphic enhancer residing 335 kb upstream of the *Myc* oncogene was deleted in mice leading to a decrease in *Myc* expression and resistance to intestinal tumorigenesis (Sur *et al*., 2012). Second, novel technology combining transcription activator-like (TAL) effectors with LSD1 was used to demethylate lysines at specific enhancer region of stem cell leukemia locus leading to decreased expression of proximal genes (Mendenhall *et al*., 2013). Furthermore, interactome maps have revealed physical connections and chromosome conformation within the genome (Kieffer-Kwon *et al*., 2013), and analyses of DNase I-hypersensitivity sites recently uncovered asymmetrical chromatin opening by a subset of pioneer factors (Sherwood *et al*., 2014). Information pertaining to transcription factor binding and genetic polymorphisms combined with methodological advancements is anticipated to lead to elucidation of novel features and functional relationships between distal enhancers and transcriptional regulation.

Many of the pioneer factors identified for steroid receptors, such as FoxA1, Hnf4α, and AP-2α that were studied in this thesis work, are crucial regulators of embryonic development and organogenesis. Thus, knocking out of the genes encoding these factors

in mice results in embryonically lethal phenotypes. It would be interesting to study the effects of cell type-specific or inducible depletion of these factors on the AR pathway *in vivo*. Furthermore, despite recent reports characterizing chromatin landscape associated with transcriptional regulation, the mechanisms that determine pioneer factor binding are still elusive and intriguing questions persist. Do pioneer factors recognize a specific histone code and, if yes, how is the code established? Is there a functional hierarchy between collaborating pioneer factors? Do pioneer factors have redundant functions? What initiates and regulates specific pioneer factor function during development? And finally, what is the full spectrum of pioneer factor effects on steroid receptor signaling?

SUMMARY AND CONCLUSIONS

Genome-wide technologies for mapping transcription factor binding events, chromatin modifications, and gene expression profiles have revealed novel features of transcriptional regulation. The core of steroid receptor function – hormone-induced DNA binding of a ligand-occupied receptor – is necessary but not sufficient for physiological hormone action in a native chromatin environment. Collaborating transcription factors and the coregulatory proteins along with local chromatin features, such as histone modifications, facilitate context-specific transcriptional outcome. Distinct AR cistromes in three androgen-responsive tissues – prostate, kidney, and epididymis – established in this thesis work have contributed to our understanding of tissue-specific androgen-dependent gene regulation. Two novel pioneer factors for AR were identified, Hnf4α in kidney and AP-2α in epididymis. The role of DBD in and of itself in specifying receptor-binding events was studied using transgenic SPARKI mouse model that expresses chimeric AR, the second zinc finger of which is replaced with that of GR. SPARKI AR fails to bind or binds with attenuated affinity to wild-type AR-preferred genomic loci. Sequence analysis of these selective enhancers was used to determine characteristic features of AR selectivity *in vivo*.

Naturally occurring dietary chemicals with the ability to modulate steroid hormone signaling are promising compounds in prevention of hormone-dependent cancers. Several lines of evidence from epidemiological studies and murine models link phytoestrogen genistein to prostate cancer risk and disease progression. Our findings support the potential role of genistein in prostate cancer prevention, in that it modulates AR-mediated gene expression *in vivo* in a tissue-specific and context-dependent manner at a dose relevant to human exposure on soy-rich diet. However, overall cancer risk of an individual is determined by a combination of genetic and environmental factors, including dietary compounds.

Collectively, this thesis work provided novel insights into AR function *in vivo*. The main conclusions are as follows:

1. An androgen reporter mouse with robust androgen-responsive reporter gene expression in multiple tissues was generated. It was a useful tool for delineating the effects of chemical compounds perturbing with AR signaling *in vivo*.

2. Genistein is a tissue-specific androgen receptor modulator that exhibits both agonistic and antagonistic effects on AR signaling in prostate and brain but not in lung or skeletal muscle *in vivo*.

3. Divergent AR cistromes in three murine androgen-responsive tissues – prostate, kidney, and epididymis – drive androgen-dependent tissue-specific transcriptional programs.

4. Distinct tissue-specific pioneering/licensing factors – FoxA1 in prostate, Hnf4α in kidney, and AP-2α in epididymis – guide AR-binding events in different tissues, and the enhancers shared by AR and these factors associate with active chromatin marks.

5. The second zinc finger in the AR DBD directs AR binding to selective androgen response elements on prostatic and epididymal chromatin. Distinct cistromes of wild-type and SPARKI AR are associated with differential transcriptional outcomes *in vivo*.

6. Selectivity of AR binding to chromatin over other steroid receptors is achieved by less stringent requirement for the *cis*-element DNA sequence.

7. Overall, the results clarified several molecular mechanisms employed by AR *in vivo* and provided important mechanistic insights that are potentially important for the development of better treatments and diagnostic tools for hormone-dependent disorders.

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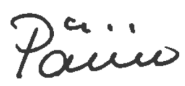

Helsinki, March 17th, 2014

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