ANDROGEN ACTIONS IN POLYCYSTIC OVARIAN SYNDROME LIVER

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ABSTRACT

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In women, polycystic ovarian syndrome (PCOS) is a common fertility disorder that is associated with metabolic dysfunction such as, non-alcoholic fatty liver disease (NAFLD), obesity, and insulin resistance. Androgen excess, the main feature of PCOS, significantly contributes to the development of NAFLD in women with PCOS. Recently, clinical reports establish that independent of insulin resistance and obesity, hyperandrogenism by itself causes NAFLD. Here, using a liver-specific androgen receptor knockout (AR-KO) mouse and well-characterized mouse PCOS model, we determine the role of androgens in NAFLD development in hyper-androgenic conditions. We show that hyperandrogenism causes fat accumulation, increases triglyceride levels and dysregulates metabolic gene expression in the liver. Interestingly, we found that KO of the AR in the liver rescues the NALFD phenotype and metabolic gene expression in PCOS conditions. Mechanistically, androgens modulate gene expression indirectly by regulating the trimethylation of lysine 27 of histone H3 (H3K27me3) repressive mark in downstream gene promotors both in mouse and human hepatocytes. This study shows that androgen actions, through the AR, are necessary for the development of NAFLD and liver dysfunction. However, in the female liver, androgens regulate gene expression indirectly rather than direct. This study demonstrates that the AR can be used as a therapeutic target to prevent NALFD in PCOS. Moreover, the novel *in vivo* and *in vitro* models created in this study, can be used to specifically differentiate the effects of nuclear vs extranuclear androgen actions both at a physiological and cellular level in the liver.

This thesis is dedicated to my mother, my life's inspiration,
and my sisters, for their endless support.
Thanks for believing in me.

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KEY TO SYMBOLS AND ABBREVIATIONS

~ Approximately

+/+ Homozygous

+/- Heterozygous

-/- Homozygous knockout

% Percent

× Multiplication sign

+ Plus sign

± Plus-minus sign

≥ Greater than or equal to

≤ Less than or equal to

* $P \leq 0.05$

** $P \leq 0.01$

*** $P \leq 0.001$

ADCY6 adenylate cyclase 6

AF transcriptional activating function

AKT serine/threonine-specific protein kinase

ANOVA analysis of variance

APOA1 apolipoprotein a1

AR androgen receptor

ARE androgen response element

ASGR1 asialoglycoprotein receptor 1

ATXN1 ataxin-1

BDM basal differentiation medium

BMAL brain and muscle arnt-like 1

BMP4 bone morphogenetic protein 4

BSA bovine serum albumin

cAMP cyclic adenosine monophosphate

ChIP chromatin immunoprecipitation

C-MYC c-proto-oncogene, BHLH transcription factor

CT computed tomography

C807 cysteine amino acid 807

DAPI 4′,6-diamidino-2-phenylindole

DBD DNA binding domain

DE definitive endoderm

DHEA dehydroepiandrosterone

DHT 5α-dihydrotestosterone

DMSO dimethyl sulfoxide

DNA deoxyribonucleic acid

DOR diminished ovarian reserve

EGF epidermal growth factor

EGFR epidermal growth factor receptor

ERK extracellular signal-regulated kinase

EZH2 polycomb group protein enhancer of zeste homolog 2

FGF2 basic fibroblast growth factor

FIB-4 fibrosis score 4

FLOX flanked by loxP site

FSH follicle-stimulating hormone

FSHR follicle-stimulating hormone receptor

F805 phenylalanine amino acid 805

GC granulosa cell

HBM hepatocyte basal medium

HE hepatic endoderm

HGF hepatocyte growth factor

HIF-1α hypoxia-inducible factor-1 alpha

HNF4α hepatocyte nuclear factor 4 alpha

HPO hypothalamus-pituitary-ovarian

HSP heat shock protein

H3 histone 3

H3K27me3 trimethylation of lysine 27 of histone H3

IMH immature hepatocyte stage

iPSC induced-pluripotent stem cell

JMJD3 lysine demethylase 6B

KDM5C lysine demethylase 5c

KLF4 kruppel-like factor 4

KO knockout

LBD ligand binding domain

LH luteinizing hormone

LL812-813 luecine amino acids 812 and 813

MAPK mitogen-activated protein kinase

MH mature hepatocyte

MMP matrix metalloproteinase

MOAR membrane-only androgen receptor

MRI magnetic resonance imaging

mRNA messenger ribonucleic acid

mTOR mammalian target of rapamycin

NAFL non-alcoholic fatty liver

NAFLD nonalcoholic fatty liver disease

NASH non-alcoholic steatohepatitis

NES nuclear export signal

NLS nuclear localization signal

NOAR nuclear-only androgen receptor

NTD N-terminal transcriptional regulation domain

OCT4 octamer-binding transcription factor

P phosphorylation

PAT palmitoyl acyltransferases

PBS phosphate-buffered saline

PCOS polycystic ovarian syndrome

PCR polymerase chain reaction

PI3K phosphatidylinositol 3-kinase

RNA ribonucleic acid

RPL19 60s ribosomal protein l19

RUNX1 runt-related transcription factor 1

SEM standard error of the mean

SERPINA1 serpin family a member 1

siRNA small interfering RNA

SOX2 sex determining region Y-box 2

SRC proto-oncogene tyrosine-protein kinase

TC theca cell-specific

TF transferrin

TFM testicular feminization

TSS transcription start site

WT wild type

αGSU alpha subunit of gonadotropins

Chapter 1: Introduction

Androgens have an well-established role in the development of the male reproductive system and the formation of secondary male characteristics during puberty [1]. Additionally, they have a crucial function in the development and maintenance of musculoskeletal, cardiovascular, immune, neural, and haemopoietic systems, as well as in metabolism [2-7]. Most of the physiological effects of androgens are mediated by the steroid hormone testosterone, and its more potent metabolite 5α-dihydrotestosterone (DHT). Biologically, DHT is about 100-fold more active than testosterone [8]. In the steroidogenic pathway, DHT is converted from its precursor testosterone by an enzyme known as 5α-reductase. This enzyme is part of the cytochrome P450 family which are essential in the steroidogenesis pathway and convert other precursor androgens such as dehydroepiandrosterone sulphate, dehydroepiandrosterone (DHEA), and androstenedione to testosterone [9]. Furthermore, androgens are primarily produced in the gonads and the adrenal gland and are regulated by the hypothalamic-pituitary-gonadal axis as well as through paracrine and autocrine mechanisms. Like other steroid hormones, androgens are mostly transported in the blood by a specialized protein known as sex hormone binding globulin and to a lesser extent by albumin [10]. However, low amounts androgens remain in its unbound form in the blood [10].

Androgen Receptor Structure and Function

The effects of androgens are mediately by binding to the androgen receptor (AR), a transcription factor and member of the steroid nuclear receptor family. The human AR gene is about 90 kb in length and codes for a 919 amino acids long AR (Fig. 1). Moreover, the rodent AR has an identical amino acid sequence in the DNA and ligand-binding domains and has 85% overall sequence

homology compared to the human AR [11]. Similar to other steroid receptors, the AR is made of three functional domains (Fig. 1): the N-terminal transcriptional regulation domain (NTD), the DNA binding domain (DBD) and the ligand binding domain (LBD) [3]. From these domains, the DBD is highly conserved and specific for each nuclear receptor family while the NTD is the most variable. The highly conserved DBD domain consist of zing fingers that recognize specific DNA sequences and enables the AR to bind to specific AR-regulated gene promoters and enhancers, thus modulating their expression [3]. Furthermore, the DBD and the LBD are linked together by a hinge region. The LBDs main function is to mediate the interplay between the AR, the chaperone and heat-shock proteins [3]. In addition, it also interacts with the NTD to stabilize bound androgens.

The AR also contains several signal sequences. For example, two transcriptional activating function (AF) signals have been identified. One of them is the ligand-independent AF-1 located in the NTD which is necessary for the maximal activity [12]. The other is the ligand-dependent AF-2 that is located in the LBD. This signal plays a role in mediating interactions between the NTD and LBD as well as forming the coregulator binding site [13]. When androgens are not bound to the AR, the AR is found in the cytoplasm and is associated with heat-shock and chaperone proteins. However, once androgens are bound to the AR, it causes a conformational change which results in the dissociation of its associated proteins and ultimately exposure of the nuclear localization signal (NLS). The NLS is located between the DBD and the hinge region of the AR and is responsible for importing the AR into the nucleus. Once the AR does it job in the nucleus, its ligand becomes unbound and a nuclear export signal (NES) located in the LBD exports the AR back into the cytoplasm, respectively [3].

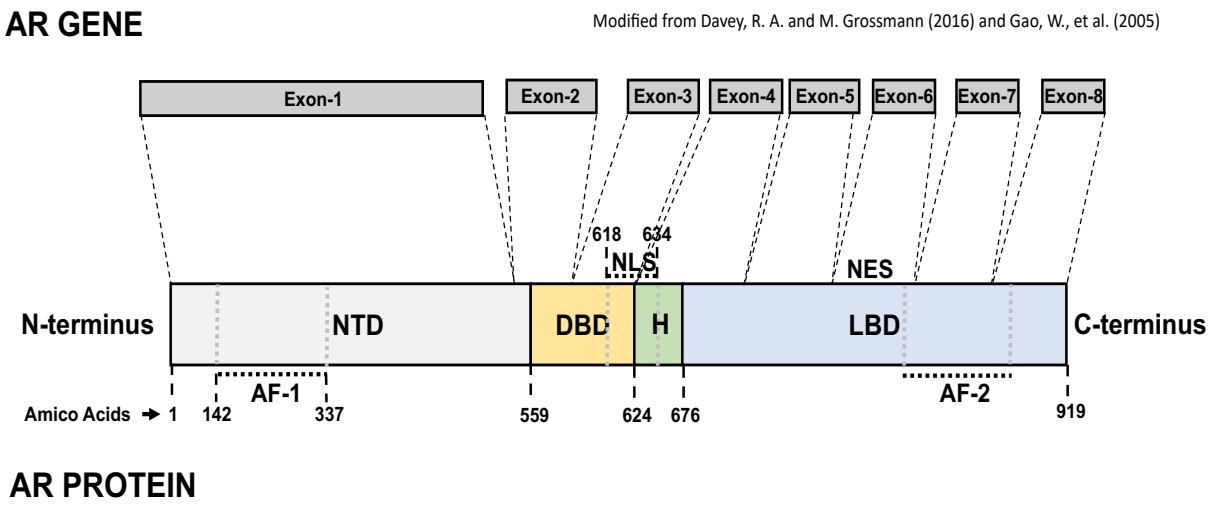


Figure 1. Androgen Receptor Gene and Protein Structure. Domains of the Androgen Receptor: N-terminal domain (NTD), DNA binding domain (DBD), hinge region (H), ligand binding domain (LBD), transcriptional activation function 1 (AF-1), transcriptional activating function 2 (AF-2), nuclear localization signal (NLS), nuclear export signal (NLS).

Proteins known as coregulators are responsible for the transcriptional activity of the AR. These proteins bind to the ligand-bound AR and serve as coactivators or corepressors to modulate gene expression by recruiting transcriptional machinery as well as regulate chromatin accessibility and histone modifications [3]. Importantly, there has been numerous AR coregulators identified and they've been extensively studied and reviewed [14-16].

Androgen Receptor Signaling

The AR is a member of the nuclear hormone receptor family and upon induction by its ligand, dimerizes, translocates to the nucleus, and regulates gene expression by binding to specific DNA sequences found in regulatory regions of genes called androgen response elements (AREs) [17].

This form of AR signaling is referred to as classical, genomic or "nuclear" androgen signaling. Moreover, it's now well established that there are ARs associated near the membrane of cells and upon activation, results in rapid membrane-initiated cytoplasmic signals such as, Erk, MAPK1/3 and PI3K/Akt signaling to mediate intracellular responses, such as proliferation and apoptosis [18]. This form of AR signaling is referred to membrane initiated or "extra-nuclear" androgen signaling (Fig. 2) [19-21]. This membrane-initiated androgen signaling can be transcription-independent involving regulation of enzymatic activity [22], translation initiation process [23-25], and protein degradation [25] or can also cause gene expression in an AR-ARE-independent fashion. Nuclear and extra-nuclear signaling has been primarily and extensively studied in male reproduction [26] and prostate cancer [27-29]. Additionally, various studies now show that these androgen-induced signaling pathways play a role in other tissues such as in, bone [30, 31], muscle [30], and the cardiovascular [5] and immune [30] systems.

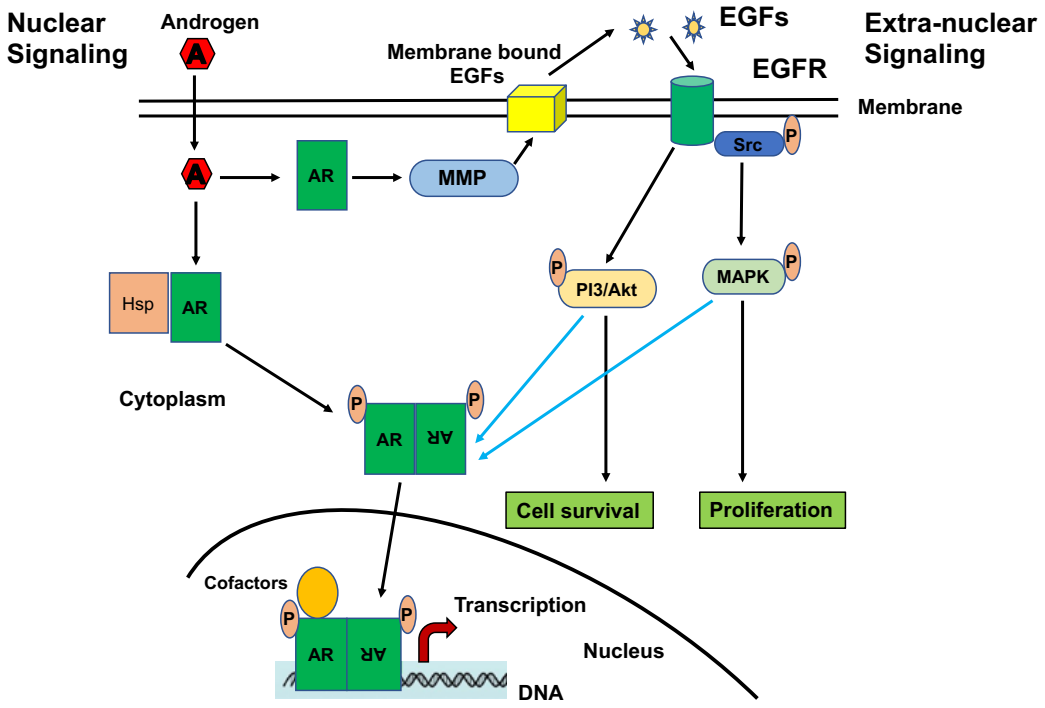


Figure 2. Nuclear and Extra-Nuclear Androgen Signaling.

Figure 2 (cont'd)

Androgens, bind to ARs localized in the cytoplasm, causing a protein conformational change which results in the dissociation of its associated proteins. Then, dimerizes, translocates to the nucleus, and regulates gene expression by binding to androgen response elements (AREs). This form of AR signaling is referred to as genomic or "nuclear" androgen. Moreover, androgens, through membrane-localized ARs, trigger matrix metalloproteinase (MMP)-mediated transactivation of the epidermal growth factor receptor (EGFR), which in turn leads to cytoplasmic PI3K/Akt and MAPK3/1 signaling. Heat shock protein (HSP), Phosphorylation (P).

Androgen Actions in the Female: Lessons from ARKO Mouse Models

Traditionally, androgens are considered male sex steroids. In females, for long androgens were considered only as a precursor of estrogen in the steroidogenic pathway. However, animals studies and clinical reports have established that androgens play important roles in female non-reproduction tissues, both in a physiological and pathophysiological condition [32-34], thereby affecting women's health, in general.

In the female, the notion that ARs could play in a role in follicular development and ovarian physiology came from studies in the 1990s showing that ARs are expressed in different cells throughout the stages of the developing follicle such as, theca cells, granulosa cells (GCs), and the oocyte [33, 35-45]. In most species [35-42], the expression levels of ARs are abundant in the preantral to antral stages of follicular development and then later decline when follicles mature to the preovulatory stage [33]. In general, this expression pattern suggested that when follicles grow from the pre-antral to the antral/preovulatory phase, the expression levels of the AR decrease, and the estrogen receptor becomes the dominant steroid receptor. Thereafter, multiple *in vivo* and *in*

vitro studies across different species such as, rodents [46-49], porcine [50], ewes [51], cattle [52, 53], and primates [54, 55] reported that androgen stimulation had a direct stimulatory effect on follicular development. However, the question remained whether the observed effects were direct effects of androgens through the AR or due to estrogenic effects involving aromatization of androgens to estrogen.

Originally, before the AR gene was identified, its gene locus located in the X chromosome was called testicular feminization (Tfm). One of the first AR knockout (ARKO) female mouse model had a homozygous mutation in the Tfm on the X chromosome (tfm/tfm) [56]. Initially, these female mice had normal ovulation, mating potential, and pregnancies. However, later in life their reproductive potential decreased [56]. Since the tfm (AR) gene is located in the X chromosome, and male mice lacking a functional AR gene are infertile, generating female mice lacking the AR was difficult. It wasn't until the development of the Cre/LoxP technology that later allowed the generation of three different global ARKO female mouse models. Two of these models were generated by targeting the deletion of exon-1 ($AR_1^{-/-}$) [57] or exon-2 ($AR_2^{-/-}$) [58] of the AR resulting in the complete loss of the AR protein. The other model targeted the deletion of exon-3 ($AR_3^{-/-}$) of the AR gene leading to a nonfunctional AR protein [59]. Nonetheless, all these three global ARKO models exhibited normal ovarian morphology, however, where sub-fertile, with $AR_3^{-/-}$ showing a mildly less severe phenotype than $AR_1^{-/-}$ and $AR_2^{-/-}$. In addition, all these models had small litter size, fewer corpus lutea, and higher rates of atretic follicles. Thus, leading to a premature depletion of their follicle pool, a common phenotype seen in women with diminished ovarian reserve (DOR). These studies established that in the absence of androgens, the reproductive potential of females isn't fully accomplished and that androgen actions are not essential for survival and reproduction in female mice.

Hypothalamic-Pituitary-Ovarian Axis

In years to come, the fact that ARs were found to be expressed throughout the hypothalamus-pituitary-ovarian (HPO) axis led to the development of cell-specific ARKO animal models. ARs have been knocked out in all three major cell-types of the ovary such as, oocyte, theca, and granulosa cells. In 2010, a study generated oocyte-specific and granulosa cell (GC)-specific ARKO female mice by crossing AR ($AR_2^{-/-}$) -floxed mice with growth differentiation factor-9 driven cre (oocyte-specific) or anti-Mullerian hormone receptor II (AMHR2)- driven cre (GC-specific) mice [60]. The GC-specific ARKO model replicated the phenotype generated by the global ARKO mice models. These animals were sub-fertile displaying lower numbers of litters, had decreased number of ovulated oocytes, increased rate of follicular atresia, and longer estrus cycle leading to early infertility issues. Moreover, their ovaries had higher numbers of pre-antral and atretic follicles, with decreased levels of corpora lutea and antral follicles [60]. In contrast, the oocyte-specific ARKO model showed no reproductive phenotype, suggesting that AR actions in oocytes are not critical for female fertility [60]. Following these studies, another GC-specific ARKO mouse model was developed by crossing AR ($AR_3^{-/-}$) -floxed with AMHR2-cre [61]. Similar to the previous mentioned GC-specific ARKO model [60], this model also displays a sub-fertile phenotype, with increase atretic follicle numbers, longer estrous cycles, and fewer litters and pups [61]. However, importantly, this model displayed a less potent ovarian phenotype, showing no reduction in corpora lutea and no depletion of antra follicles. This effect could be observed because the first GC-specific ARKO (using $AR_2^{-/-}$ floxed animals) lead to the complete loss of ARs in GCs, while the second GC-specific ARKO (using $AR_3^{-/-}$ animals) retained a nonfunctional AR protein in GCs. Future studies need to determine whether this nonfunctional AR

is able to activate androgen-induced signaling events and thus influence folliculogenesis in the ovary. The last ovarian cell-specific ARKO model that was developed is the theca cell-specific (TC) ARKO mouse model [62], which was made by crossing $AR_2^{-/-}$ floxed with Cyp17-iCre mice that express Cre recombinase under cytochrome P450 17A1 promoter [62]. This model has a normal reproductive phenotype, exhibiting normal ovaries and no signs of reduced reproduction capacity. Altogether, with regard to the ovary, androgen actions through the AR expressed in GCs are key regulators of follicular development and female fertility (Table 1).

For the past decade, the primarily focus of our laboratory has been to elucidate androgen actions in the female. Our laboratory and others have shown that androgens, regulate critical

Table 1: Androgen Receptors Expressed in GCs are Key Regulators of Female Fertility

	Global ARKO [57] (Exon 1), [58] (Exon 2), [59] (Exon 3)	**GC-ARKO** [60] (Exon-2), [61] (Exon-3)	**Oocyte-ARKO** [60] (Exon-2)	**TC-ARKO** [62] (Exon-2)	**Neuron-ARKO** [66] (Exon-3)
Defective Folliculogenesis	✓	✓	X	X	✓
High Atresia	✓	✓	X	X	✓
Decreased Fertility	✓	✓	X	X	ND
Premature Ovarian Failure	✓	✓	X	X	X

ND= Not Determined

aspects of follicular development through nuclear and extra-nuclear signaling [22, 60, 63]. The underlying mechanisms of androgen actions in regulating follicular development and female fertility have been reviewed in various reports [32, 33]. Briefly, our laboratory found that ARs in

GCs regulate pre-antral to antral follicular transition by increasing the expression of the follicle-stimulating hormone receptor (FSHR) in a translation-independent (extra-nuclear) manner [63]. Additionally, androgens, prevent follicular atresia through nuclear signaling (AREs) by inhibiting pro-apoptotic proteins via the induction of miRNA-125b [63]. Moreover, other studies indicate that androgens also promote steroidogenesis in GCs by inducing the expression of steroidogenic enzymes such as StAR, P450scc, 3ßHSD [64] and aromatase resulting in an increase of steroidogenesis.

In efforts to further understand AR actions in the HPO-axis, another group generated a gonadotroph-specific (pituitary-specific) ARKO. This model was created by crossing $AR_2^{-/-}$ floxed animals with an alpha subunit of gonadotropins (aGSU)-Cre promoter driven mice [65]. Pituitary-specific ARKO females were sub-fertile showing a reduction in their pups per litter and altered late stage-ovarian function exhibiting reduced number of corpus lutea, and increased number of unhealthly antral follicles. Importantly, follicular development was not impaired in these females. However, the sub-fertile phenotype could be established as a consequence of a lower basal and surge levels of FSH hormone levels, an important mediator of follicle maturation. Moreover, the pre-ovulatory surge levels of both luteinizing hormone (LH) and FSH hormones were lower in pituitary-specific ARKO females. This study suggested that androgen actions may be involved in the priming of gonadotrophs for the preovulatory surge, leading to the reduction of LH hormone surge levels and ultimately leading to fewer ovulation rates and reduction of pups per litter [65]. However, how androgen signaling in the gonadotrophs causes this effect still remains to be determined.

Lastly, KO of the AR in the female brain and pituitary (nuero-ARKO) impacted ovarian physiology [66]. For example, this mouse model showed reduction in large antral follicles and

increased follicular atresia [66]. However, didn't develop premature ovarian failure. In addition, relative to control, nuero-ARKO female mice had a comprised kisspeptin/gonadotropin-releasing hormone/LH cascade which lead to the ovarian phenotype. Differences between the nuero-ARKO and pituitary-specific ARKO could be could be observed because the pituitary-specific mouse models (using $AR_2^{-/-}$ floxed animals) lead to the complete loss of ARs in gonadotrophs, while the nuero-ARKO (using $AR_3^{-/-}$ animals) retain a nonfunctional AR protein in the brain and pituitary.

Pancreas

Androgen excess (hyperandrogenemia) is a well-known diagnostic marker for women with polycystic ovarian syndrome, and recent clinical reports show that these women have several degrees of pancreatic β-cell dysfunction [67, 68]. One hypothesis is that excess androgens predisposes β-cell dysfunction by inducing β-cell stresses [7] such as, insulin resistance and circulating oxidative stress, both typically seen in PCOS women [69] and mouse models [70]. However, the mechanism of how excess androgens causes β-cell dysfunction hasn't been elucidated yet. Recently, a study reported that androgen (DHT) excess coupled with a western diet (high-fat and sugar diet) causes female mice to develop hyperinsulinemia and insulin resistance which was shown to be linked to pancreatic β cell failure, thus, leading to hyperglycemia [71]. However, these comorbidities were not seen in the female β-cell specific ARKO. Moreover, this study also reported that *in vitro* treatment with DHT of mouse and human pancreatic islets, via extra-nuclear androgen signaling (cAMP and mTOR pathway), resulted in an insulin hypersecretion response to glucose [71]. Future studies need to look at the effects of only androgen excess in β-cell and pancreatic physiology. Regardless, this study showed the potential of AR actions in female β-cells.

Overall, it is now recognized that in females, androgens play a pivotal role in female reproduction and fertility displaying effects all throughout the HPO-axis [66]. Moreover, recent ARKO animals and clinical studies show that AR actions extend to other female tissues besides reproductive organs such as the pancreas. However, the mechanisms of androgen actions in the female are complex and remain frustratingly unclear appearing to have cell-specific effects. Therefore, understanding the transcription-dependent and transcription-independent effects regulated by nuclear and extra-nuclear androgen actions in specific female tissues is paramount. A major limitation towards elucidating androgen actions is the inability to differentiate *in vivo* the potential role of each of these pathways in physiological or pathophysiological conditions. In **Chapter 3** of this thesis, we transition to a more basic science scenario and try to further understand androgen actions both at a physiological and cellular level. To do so, we have developed mouse models and generated AR clones (Chapter 3) with nuclear-only and membrane-only AR signaling that we propose to use in future studies to determine the relative contribution of extra-nuclear and nuclear AR in normal physiological and/or disease conditions.

Androgens in Male and Female Disease

Androgens have been linked to the development of several pathophysiological conditions. For example, in males, a reduction in androgen signaling leads to under virilization and infertility [72], and mutations of the AR gene can lead to androgen insensitivity disorders [8]. Additionally, the AR has a well-established role in prostate cancer development and progression [73].

For decades, androgens were known to be detrimental to women by being associated with pathophysiological conditions involving ovarian physiology and female fertility. While high levels of androgens is the clinical biomarker of polycystic ovarian syndrome (PCOS) which leads to

excess follicular development [74], disrupted androgen signaling is associated with abnormal follicular development leading to a negative impact in female fertility [75, 76]. Moreover, ARKO animal models clearly established that androgen actions are necessary to maintain normal ovarian function and fertility. Furthermore, androgen priming is now being considered as a fertility treatment option in women with diminished ovarian reserve [76]. In summary, it's now recognized that a balanced level of androgens is required for normal ovarian function and adequate female fertility [33].

Polycystic Ovarian Syndrome and Non-alcoholic Fatty Liver Disease

Polycystic ovary syndrome (PCOS) affects 10-15% of women and primarily causes anovulation and fertility problems in women. However, PCOS women also develop or are more likely to develop diseases and conditions like, nonalcoholic fatty liver disease (NAFLD) [77], type 2 diabetes, hypertension, and cardiovascular problems [78, 79] thereby affecting the overall health of women. One of the clinical diagnostic markers of PCOS is high levels of androgens (hyperandrogenism). For years it was speculated that NALFD development in PCOS patients was a consequence of metabolic comorbidities present in PCOS such as obesity and insulin resistance. Intriguingly, recent clinical reports show that independent of obesity and insulin resistance, hyperandrogenism by itself is a cause for NALFD development [80-82]. However, how chronic high levels of androgens cause liver dysfunction and whether the AR in the liver can be targeted to prevent NALFD in a PCOS condition still remains to be determined. Therefore, this thesis will shed some light into how chronic high levels of androgens induce liver dysfunction and address the question of whether the AR can be a therapeutic option to prevent NALFD in PCOS.

In **Chapter 2**, using a well-established mouse PCOS model, we found a mechanism through which chronic high levels of androgens cause liver dysfunction and contribute to the development of NALFD in the mouse. Additionally, using the Cre-LoxP system, a liver specific-ARKO PCOS mouse model was developed to determine if KO of the AR in the liver will prevent the development NALFD in a PCOS condition.

Significance

- Androgen excess, the main feature of PCOS, significantly contributes to the development of NAFLD in women with PCOS [82, 83]. However, the underlying mechanism(s) of androgen effects on liver metabolism and its manifestation in the form of liver dysfunction in women with PCOS condition is unknown and is one of the focus of this thesis. Moreover, the mechanistic insights gained from this study will have a number of implications for the diagnosis and treatment of various diseases associated with PCOS or for women's health, in general.
- To date, obesity and type 2 diabetes associated with PCOS were considered the primary cause of NAFLD in PCOS. Importantly, this study demonstrates a direct effect of androgens on liver metabolism and its role in the pathophysiology of NAFLD in PCOS condition, thereby establishing AR as a potential therapeutic target for NALFD in PCOS conditions. Current AR inhibitors like Enzalutamide or Flutamide that are in clinical use or in clinical trials for prostate cancer are potential therapeutics for PCOS women.
- Over the years there have been a large number of studies on androgen actions in the male liver. This is the first comprehensive study to delineate androgen actions in the female liver. Molecular insights gained from this study will help to understand the sexual dimorphic nature of androgen actions in the liver.

- This study for the first time has developed molecular tools and mouse models to elucidate *in vivo* and *in vitro* the genomic and non-genomic effects on androgens and its role in pathophysiological conditions**.**
- Changes in epigenetic marks may be an early event in pathogenesis and progression of various disorders [84]. Our preliminary studies have identified specific epigenetic modifiers that are modulated by excess androgens. Thus, studies on mechanisms of epigenetic modifications can contribute to our understanding of long-term effects of PCOS and shed light on disease prevention/treatment.

Chapter 2: Androgen Actions in Liver Dysfunction and Non-Alcoholic Fatty Liver Disease (NALFD) Development in a PCOS Condition

NALFD is a chronic disease that is characterized by the accumulation of fat in the liver which is typically referred to as hepatic steatosis (fatty liver) and is histologically the same as alcoholic fatty liver disease. This disease is highly prevalent affecting ~20-30% of the world [85, 86] and is the most common cause of chronic liver disease. In the United States alone, the cases of NALFD is estimated to increase from 83.1 million in 2015 (~25% of the population) to 100.9 million in 2030 [87]. In the clinic, NALFD comprises of a wide spectrum that can start from a fatty liver, which is also referred to as non-alcoholic fatty liver (NAFL), and can advance to a more severe stage called non-alcoholic steatohepatitis (NASH) [88, 89]. NASH is characterized by severe steatohepatitis, lobular inflammation, hepatocyte ballooning and is typically accompanied by a variable degree of pericellular fibrosis [89] which can ultimately lead to liver cirrhosis and hepatocellular carcinoma [90]. Fatty liver is characterized by the accumulation of fat in the liver and is diagnosed when ~5% of the liver mass is fat. Commonly, fatty liver is easily diagnosed in the clinic through non-invasive imaging studies such as, ultrasound, computed tomography (CT) or magnetic resonance imaging (MRI). However, it's typically suspected when patients have elevated levels of serum hepatic enzymes such as, aminotransferases. While NAFL is fairly easy to diagnose, accurately diagnosing NASH involves histological analysis by obtaining an invasive liver biopsy. However, elastography techniques and the NALFD fibrosis score (FIB-4) have been commonly used as a non-invasive way to identify fibrosis [89].

Recently, animals studies using models of PCOS created by androgen excess, show that long term androgen treatment causes hepatic steatosis [91]. Additionally, lean hyper-androgenic mouse models show direct androgen effects on hepatocyte metabolism [92]. Similarly, clinical studies

show that rates of NAFLD are increased in women with PCOS [93, 94] both lean [95] or normal-weight [82]. In addition to body mass index and dysglycemia, androgen excess is a potential additional contributing risk factor for NAFLD development in PCOS [81, 96]. For a long time, it was suggested that common metabolic issues present in PCOS such as obesity and diabetes where causing NALFD in PCOS. However, recent clinical reports of PCOS patients show that, NALFD is highly prevalent as a result of hyperandrogenism, independent to insulin resistance and obesity. For example, a cross sectional study using 400 Chinese women with PCOS established that, independent of obesity and insulin resistance, a higher free androgen index is associated with increased accumulation of fat in the liver and NAFLD prevalence, when compared to control women [80]. Similarly, another case study using 29 PCOS patients provided evidence that hyperandrogenic PCOS patients have increased fat in their liver (hepatic steatosis) when compared with non-hyperandrogenic PCOS patients or with their healthy controls, independent of BMI, adipose tissue volume, and insulin resistance [83]. All together, these clinical studies suggest that high levels of androgens associated with PCOS by itself is a cause of NALFD. However, direct evidence of how androgens cause NALFD hasn't been elucidated.

In this study we have used a well-established DHT-pellet mouse PCOS model [91], with well characterized reproductive and metabolic phenotypes [91, 97], to determine the role of androgens in NAFLD in hyper-androgenic conditions. To elucidate how high levels of androgens cause liver dysfunction our laboratory initially did an RNA-seq study in Placebo vs DHT-pellet treated livers (unpublished data). Results show that 787 were differentially regulated genes among DHT pellet vs placebo livers out of which 361 genes were upregulated and 426 genes were downregulated. Moreover, 236 genes were metabolic genes amongst which 103 were upregulated and 133 were downregulated. Surprisingly, we found that about 240 circadian genes were also

differentially regulated in the DHT pellet livers, amongst which 127 were upregulated and 113 were downregulated. Thereafter, the overall objective of this study was to determine if the changes in the liver physiology (and gene expression) is through direct AR actions in the liver. Furthermore, our laboratory has reported that in the ovary [22], androgens regulate gene expression through epigenetic modulation. Thus, another goal of this study was to determine if this mechanism is conserved in the liver. Finally, using human hepatocytes derived from induced-pluripotent stem cells (iPSC) we have extended our observations in the mouse model to the human.

Materials and Methods

Generation of Liver-specific AR-KO Mice and PCOS model

Mouse studies were performed in accordance with the guidelines for the care and use of laboratory animals and were approved by the University Committee on Animal Resources at the Michigan State University (MSU). Mice were maintained under standard housing conditions (ad libitum access to food and water in a temperature and humidity controlled, 12-hour light cycle environment). To generate liver-specific ARKO, we used mice in which exon 2 of the WT-AR ($AR_2^{-/-}$) was floxed [98] and then mated them with existing Albumin Cre (The Jackson Laboratory, Bar Harbor, ME) as shown in Fig. 3. Briefly, AR-loxP$^{flox/flox}$ females were crossed with Albumin Cre males. Thereafter, the AR-loxP$^{flox/y}$; Albumin Cre +/- males where crossed with another AR-loxP$^{flox/flox}$ female to generate a AR-loxP$^{flox/flox}$; Albumin Cre +/- female. Genomic DNA was isolated from tail snips of 14-15 d-old animals, and PCR genotyping with appropriate primers (Table. 2) was used to identify mice with AR-WT, floxed, and cre transgene, revealing bands of 860 (AR-WT), 930 (AR floxed), 390 and 150 (Albumin Cre) bp, respectively.

PCOS mouse model was develop as previously described [91]. Briefly, at 3-4 weeks of age C57BL/6J (WT) mice (The Jackson Laboratory, Bar Harbor, ME) and liver-specific AR-KO female mice were implanted subcutaneously with a 90-day continuous-release pellets containing DHT (Innovative Research of America, Sarasota, FL). These pellets contained 2.5 mg of DHT (daily dose, 27.5 µg). Placebo pellets were implanted as control. At the end of the 90-day mark, livers and ovaries were collected.

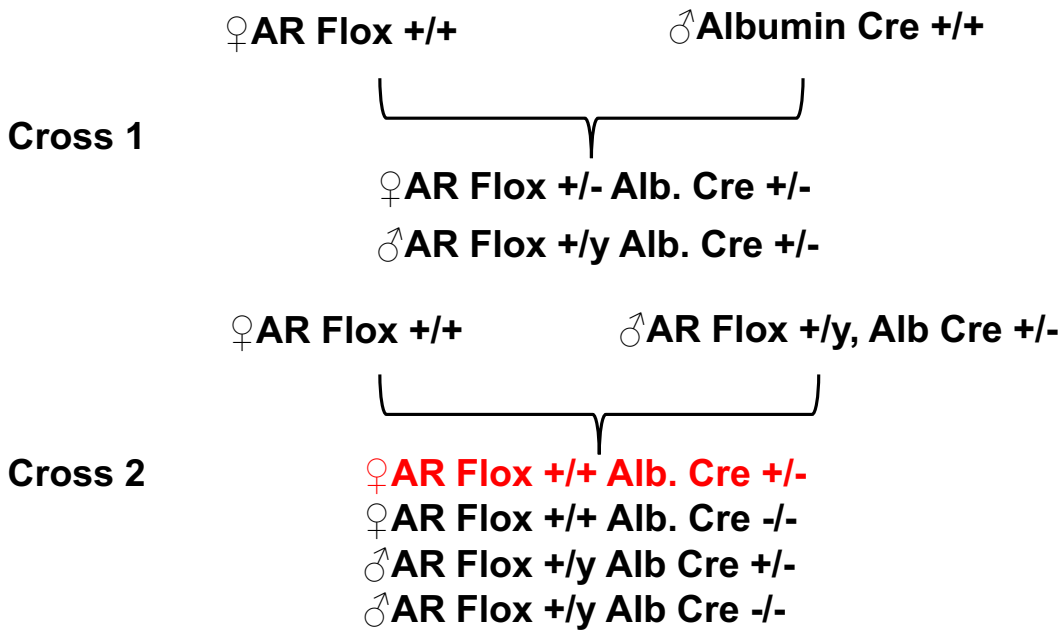


Figure 3: Schematic of Generation of Liver-specific AR-KO. AR-loxP$^{flox/flox}$ females were crossed with Albumin Cre males (Cross 1). Thereafter, the AR-loxP$^{flox/y}$; Albumin Cre +/- was crossed with another AR-loxP$^{flox/flox}$ female to generate a AR-loxP$^{flox/flox}$; Albumin Cre +/- female (Cross 2).

Table 2. Genotyping PCR Primers

PCR Primers	Sequence
WT Albumin Cre Forward	5'- TGCAAACATCACATGCACAC-3'
Albumin Cre Common Reverse	5'- TTGGCCCCTTACCATAACTG-3'
Albumin Cre Mutant Reverse	5'- GAAGCAGAAGCTTAGGAAGATGG-3'
WT and AR Flox Forward	5'- AGCCTGTATACTCAGTTGGGG-3'
WT and AR Flox Reverse	5'- AATGCATCACATTAAGTTGATACC-3'

Generation of Hepatocytes Derived from iPSCs and Cell Culture

Cell Culture

Induced-pluripotent stem cells (iPSCs) were reprogrammed to pluripotency from healthy human female fibroblasts by transiently expressing the 4 Yamanaka factors ((octamer-binding transcription factor (Oct4), sex determining region Y-box 2 (Sox2), Kruppel-like factor 4 (Klf4), and c-Proto-Oncogene, BHLH Transcription Factor (c-Myc)) with a Sendai virus. iPSCs were maintained and passaged using Essential 8 Flex (Thermo Fisher) media.

Hepatocyte Differentiating Media

Basal differentiation medium (BDM): RPMI-1640 (Corning) plus B27 supplement plus penicillin/streptomycin (100 U/ml penicillin, 100 µg/ml streptomycin, final concentration) (Thermo Scientific). Definitive endoderm (DE) medium: BDM with 100ng/ml activin A and 3µM CHIR99021. Hepatic endoderm (HE) medium: BDM with 5 ng/ml basic fibroblast growth factor (bFGF, also known as FGF2), 20 ng/ml bone morphogenetic protein 4 (BMP4) and 0.5% DMSO. Immature hepatocyte stage (IMH) medium: BDM with 20 ng/ml hepatocyte growth factor (HGF) and 0.5% DMSO. Mature hepatocyte (MH) medium: hepatocyte basal medium (HBM) (Lonza) with SingleQuots added (Lonza), as well as 20 ng/ml HGF, 20 ng/ml oncostatin M, 100 nM dexamethasone and 0.5% DMSO.

Plating and Differentiation

Generation of Hepatocytes derived from iPSCs was performed as previously described [99]. Briefly, Day 1: cultures of iPSCs were split and plated at a density of 3×10^4 cells/cm2 in mTESR (Stem Cell Technologies) with 4 μM ROCK inhibitor Thiazovivin. Days 2-4: cells were treated with DE medium. Days 5-9: cells were treated with HE medium for 5 days. Days 10-14: cells were treated with IMH medium for 5 days. Days 15-27: cells were treated with MH medium for 10-12 days. The medium was changed daily throughout differentiation. Differentiated hepatocytes were treated with 1 μM DHT for 48 hours. For all our experiments, we used DHT instead of testosterone to avoid misinterpretation of our results due to aromatization of testosterone to estradiol.

Oil-Red-O Staining and Triglycerides

Left lobe of livers where fixed for 24-48 hours in 4% buffered paraformaldehyde solution. Then, tissues were placed in a 30% sucrose buffered solution at 4C. OCT embedding and tissue sectioning (5 μm) were performed at MSU Investigative HistoPathology Lab. Thereafter, oil-red-o staining was performed using the Oil-Red-O Staining Kit (Abcam) according to manufacturer's instructions. Briefly, OCT embedded liver sections were incubated in propylene glycol for 2 minutes followed by incubation in oil-red-o solution for 6 min. Then, sections were differentiated in 85% propylene glycol for 1 min followed by a rise in water. Next, tissue sections were counterstained in hematoxylin for 1-2 min and rinsed in water 2-3 times. Tissue sections were then coversliped with an aqueous mounting medium (Sigma). Triglycerides were measured using a Triglyceride Colorimetric Assay Kit from Cayman Chemical as per manufacturer's instructions.

Western Blot Analysis

Western blot analysis was performed as described previously [22, 100, 101]. Primary antibodies used were AR (Active motif), H3 (Cell signaling), H3K27me3 (Cell signaling), RPL19 (Abcam), JMJD3 (Sigma), and EZH2 (Cell signaling) (Table 3). Each experiment consisted of n=3 liver and ovary samples from placebo, DHT-pellet (WT) and hepatocyte-specific AR-KO DHT-pellet. For *in vitro* studies, each experiment was repeated at least 3 times.

Table 3: Antibodies

Antibodies	Source	Dilution
Western Blot		
H3K27me3	Rabbit (9733S, Cell Signaling)	1:1000
H3	Rabbit (9715S, Cell Signaling)	1:1000
JMJD3	Rabbit (SAB3500956, Sigma)	
EZH2	Mouse (3147S, Cell Signaling)	1:1000
AR	Rabbit (39781, Active Motif)	1:1000
RPL19	Rabbit (ab224592, Abcam)	
Secondary antibody Anti-Rabbit	Goat (170-6515, Bio Rad)	1:5000
Secondary antibody Anti-Mouse	Goat (170-6516, Bio Rad)	1:5000
Immunofluorescence		
HNF-4-α	Rabbit (ab92378, Abcam)	1:100
Albumin	Goat (ab19194, Abcam)	1:200
Secondary antibody Anti-Rabbit	Alexa Fluor 488 Donkey (A21206, Thermo Fisher)	1:200
Secondary antibody Anti-Goat	Alexa Flour 488 Donkey (A32814, Fisher Scientific)	1:200
ChIP		
H3K27me3	Mouse (ab6002, Abcam)	5 µg/µl

RNA Extraction and Real-Time Polymerase Chain Reaction

Total RNA from livers, ovaries and hepatocytes derived from iPSCs was isolated as previously described [22] using the standard Trizol isolation method according to the manufacturer's

instructions. Levels of mRNA expression were analyzed by delta delta cycle threshold (Ct) method using One Step TaqMan gene expression assay primers (Table 4) (Applied Biosystems, Foster City, CA) and the Applied Biosystems StepOnePlus real-time polymerase chain reaction (RT-PCR) system. One microgram of RNA was used for all of the RT-PCR reactions. RPL19 was used as an endogenous control.

Table 4: Mouse Taqman (Thermofisher) RT-PCR Primer

Gene	NCBI Reference Sequence
Adcy6	Mm00475772_m1
Kdm5c	Mm00840032_m1
Cidea	Mm00432554_m1
Cidec	Mm00617672_m1
Fgf21	Mm00840165_g1
Arntl (Bmal)	Mm00500223_m1
Rpl19	Mm02601633_g1

miR-101 Isolation and Detection

Total RNA was isolated as previously described [22] using the standard Trizol isolation method according to the manufacturer's instructions. RT-PCR was performed using the TaqMan MicroRNA Reverse Transcription Kit and mouse/human miR-101 TaqMan MicroRNA Assays (Applied Biosystems). miR16 and snoRNA202 was used as an endogenous control, for human and mouse, respectively. Relative expression of miR-101 was calculated using the delta delta Ct method.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP was performed as described previously [22, 28, 63, 101] with MAGnify Chromatin Immunoprecipitation System (Invitrogen), according to the manufacturer's instructions. Briefly,

chromatin fragments were immunoprecipitated with Dynabeads coupled with mouse monoclonal anti-H3K27me3 (Abcam) and immunoglobulin-G as nonspecific control. Quantitative PCR was performed using EXPRESS SYBR GreenER^{TM} qPCR SuperMix (Invitrogen) with primers (Table 5) designed for a region of the mouse Brain and Muscle ARNT-Like 1 (*Bmal)* gene promoter. Importantly, we initially performed a ChIP assay using WT female mouse liver explants treated with DHT *in vitro* using 5 primer sets spanning up to 500 base pairs upstream of the transcription start site (TSS) of the *Bmal* promotor. Based on the results of this study, for this *in vivo* ChIP assay we specifically chose region 1 of the promoter of *Bmal* (see Fig. 8A). n=3 liver samples from placebo, DHT-pellet (WT) and liver-specific AR-KO DHT-pellet were used for ChIP studies. IgG was used as non-specific control (Fig. 8C).

Table 5: ChIP Primers

ChIP Primers	Sequence
Bmal Fwd	5'- CCAGTCAAATCCTGTGGACC -3'
Bmal Reverse	5'- GCTTGCAAGGGACTCTAACTTC -3'

Immunofluorescence

Briefly, differentiated hepatocytes were fixed in 4% paraformaldehyde for 4-8 min in microscope slides. Slides where then washed 3 times for 5 minutes in phosphate-buffered saline (PBS)-glycine (0.3M glycine) and blocked in 10% normal serum + 0.5% bovine serum albumin (BSA) + 0.5% Triton X-100 in PBS for 1 hr. Next, slides were rinsed in PBS and incubated in primary antibodies (HNF4- α and Albumin) (Table 3), diluted appropriately in 1% normal serum + 0.5% BSA + 0.05% Triton X-100 in PBS, overnight at 4 C. Further, slides were washed 3 x 5 min in PBS and incubated in secondary antibodies (Table 3) diluted in 1% normal serum + 0.5% BSA + 0.05% Triton X-100 in PBS, for a minimum of 2 hrs in dark. Slides were counterstained with DAPI and washed 3 x 5

min in PBS in dark. Lastly, slides were mounted in Prolong Gold-DAPI, coverslip and sealed with nail varnish.

Statistical Analysis

Data are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism version 7 (GraphPad Software Inc, California, USA). Statistical comparisons were made by two-tailed unpaired t-test (for comparing two groups), one-way ANOVA followed by Dunnett's multiple comparisons test (for comparing multiple groups) and results with $P \leq 0.05$ were considered significant.

Results

Selective Knockout of AR in Hepatocytes

To generate liver-specific AR KO female mice we first crossed AR-loxP$^{flox/flox}$ females with Albumin Cre males (Fig. 3). Thereafter, the AR-loxP$^{flox/y}$; Albumin Cre +/- was crossed with another AR-loxP$^{flox/flox}$ female to generate a AR-loxP$^{flox/flox}$; Albumin Cre +/- female. To confirm selective knockout of AR in hepatocytes, liver and ovarian tissues from 8- to 9-wk-old AR-loxP$^{flox/flox}$; Albumin Cre +/-, AR-loxP$^{flox/-}$; Albumin Cre +/- and WT animals were subjected to RT-PCR and Western Blot analysis for detection of AR (Fig. 4). Results show that the mRNA (Fig. 4A) (***$P \leq 0.001$) and protein levels (Fig. 4B) of the AR in the livers from AR-loxP$^{flox/flox}$; Albumin Cre +/- mice was significantly lower than livers from WT animals. Moreover, specificity of KO of the AR in hepatocytes could be established because of the equal expression of AR mRNA (Fig. 4A) and protein (Fig. 4B) in the ovaries isolated from WT, heterozygous, and AR-null mice.

In addition, a previous study also established the specificity of this cross by showing that various tissues including epididymis, heart, kidney, muscle, spleen, testis, and adipose, but not the liver, contained mRNA levels of the AR [102].

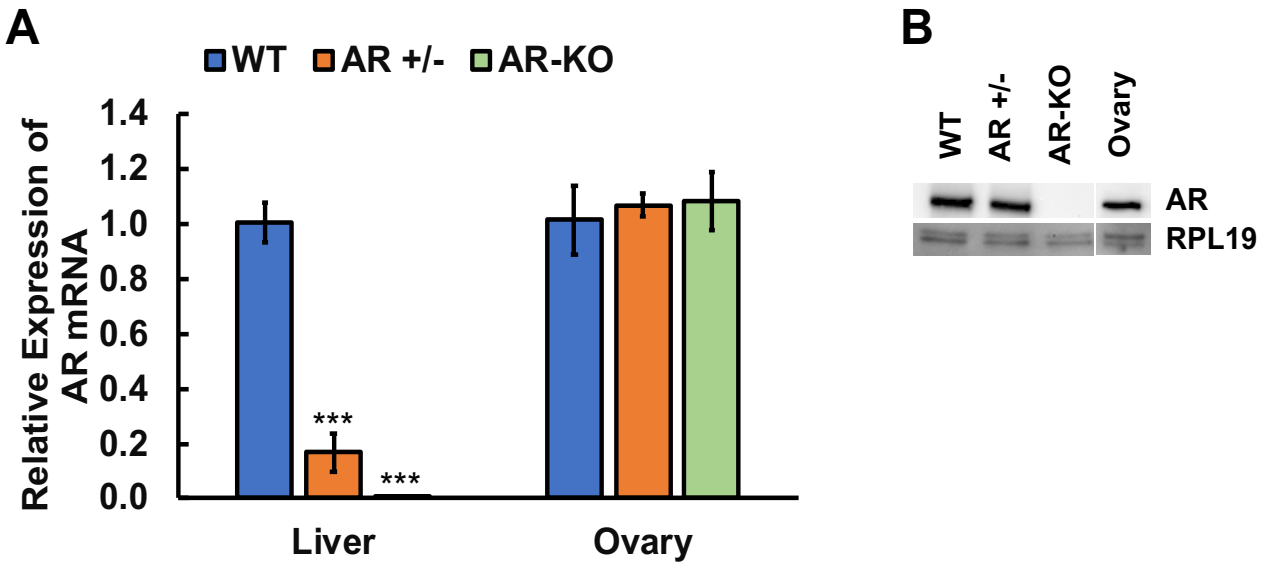


Figure 4: Conformation of AR KO in the Liver. (A) Graphic representation of AR mRNA transcripts in liver and ovary RNA extracts isolated from 8- to 9-wk-old WT, liver-specific AR +/-, and KO animals. Ovary samples were used as a positive control. The mRNA levels were measured by quantitative real-time PCR and compared with control RPL19 mRNA expression using the delta delta Ct method. Results are represented as the amount relative to WT (mean ± SE, n=3 mice per genotype). ***P ≤ 0.001 for hepatocyte-specific AR-KO and liver-specific AR +/- mice vs. WT. (B) Representative western blot demonstrating the AR expression in liver isolated from WT, liver-specific AR +/-, and KO mice (n =3 animals per genotype). The ovary was used as positive control. Similar results were observed in three separate experiments.

Knockout of the Androgen Receptor in the Liver Attenuates NALFD and Liver Dysfunction in the Mouse PCOS Model

With respect to androgen actions in women, previous studies have primarily focused on androgen actions in female reproduction and fertility [32, 33, 66]. Till date, there has been few to no studies on AR actions in the female liver with regard to NALFD development. Given that androgen excess in the context of PCOS has been associated with NAFLD and clinical reports [80, 82, 83] provide evidence that hyperandrogenism by itself causes NALFD in PCOS patients, we hypothesized that direct androgen actions, through the AR in the liver, plays a critical role in the development of NALFD in a PCOS condition. For our studies, we have evaluated the development of hepatic steatosis (fatty liver) in livers samples from WT animals treated with placebo and DHT-pellet, and liver-specific AR-KO animals treated with DHT-pellet using oil-red-o staining and by measuring triglyceride levels. Results show that WT animals treated with DHT-pellet had increased accumulation of fat (Fig. 5A) and significantly increased triglyceride levels (Fig. 5B) (***$P \leq 0.001$) in the liver compared to placebo. Moreover, DHT-pellet treated livers from the liver-specific AR-KO animals showed no accumulation of fat (Fig. 5A) and their triglyceride levels (Fig. 5B) were similar to those of placebo treated livers.

Next, we determined if KO of the AR rescue any of the differentially expressed gene levels found in the RNA seq analysis. Given that the circadian system plays a crucial role in liver physiology and disease [103, 104], we specifically chose to detect the mRNA levels of two clock-controlled metabolic genes, named *Bmal* and *Adcy6*, found to be upregulated in our RNA seq analysis. Moreover, we also detected the mRNA levels of the top gene found to be upregulated in our RNA seq data called *Kdm5c*, a histone demethylase that specifically demethylates 'Lysine 4' of histone H3 [105]. *Bmal* is a critical player in the molecular circadian clock. *ADCY6* gene codes

for a protein that is member of the adenylyl cyclase family, which are required for the synthesis of cyclic AMP (cAMP) and cAMP has a wide range of cellular functions acting as a secondary messenger system. Figure 5C shows the relative mRNA levels of *Kdm5c, Bmal* and *Adcy6.* Results show that liver lysates from WT animals treated with DHT-pellet had a significantly (***P≤ 0.001 for *Kdm5c* and *P ≤ 0.05 for *Bmal* and *Adcy6*) increased mRNA levels of all three genes, as expected, compared to placebo (Fig. 5C). Interestingly, DHT-pellet treated liver lysates from the liver-specific AR-KO animals had similar mRNA levels of all three genes as placebo treated livers (Fig. 5C).

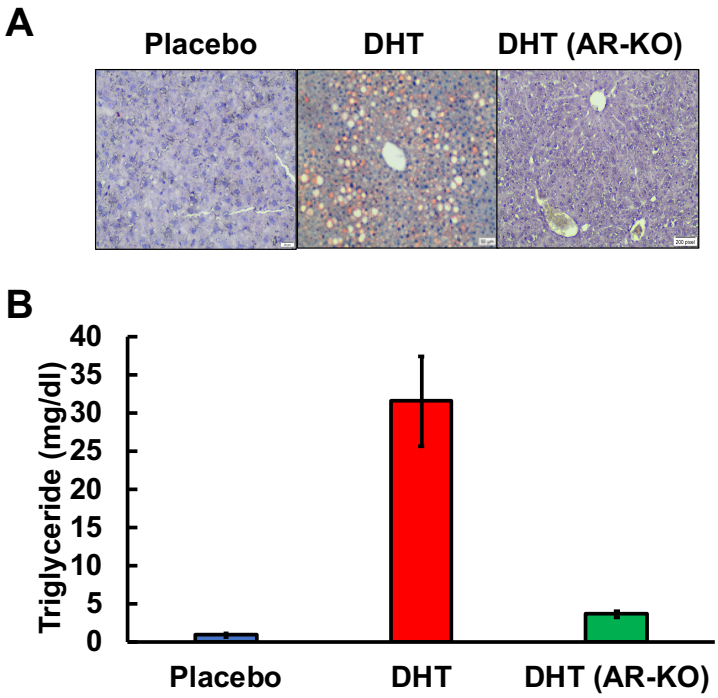
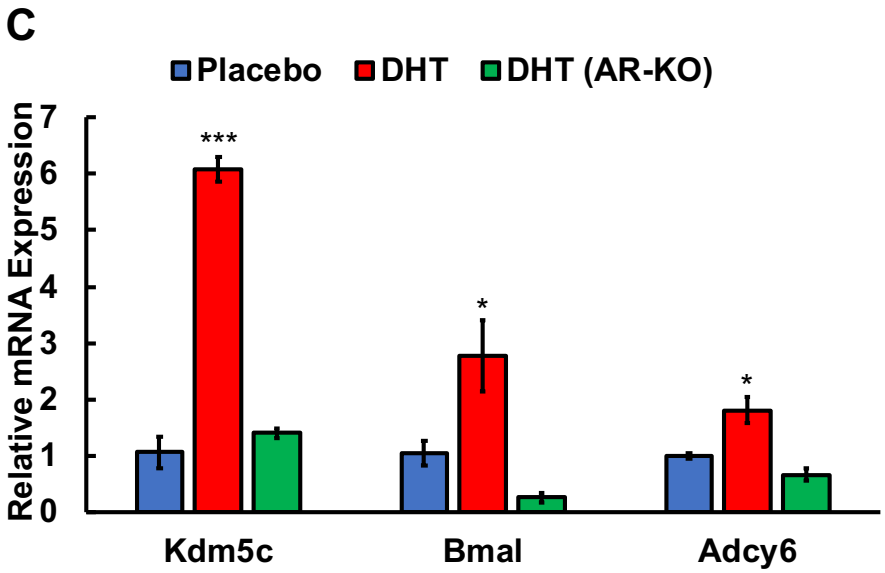


Figure 5: KO of AR in the Liver Rescues Fatty Liver Development and Metabolic Gene Expression in PCOS.

Figure 5 (cont'd)



(A) Representative images of oil-red-o stained livers from placebo, DHT-pellet, and ARKO DHT-pellet treated animals. Fatty accumulation is increased in WT DHT-pellet treated animals, but not liver-specific AR-KO treated with DHT-pellet, compared to placebo. (B) Levels of triglycerides in the liver are significantly increased in DHT-pellet, but not liver-specific AR-KO treated with DHT-pellet, compared to placebo. Data are displayed as means ± SEM (n = 3) (***P ≤ 0.001 vs. placebo). (C) Relative expression of *Kdm5c, Bmal,* and *Adcy6* is significantly increased in livers from DHT-pellet treatment, but not liver-specific AR-KO treated with DHT-pellet, compared to placebo. Data are displayed as means ± SEM (n = 3) and normalized to RPL19 levels (***P≤ 0.001 vs. placebo and *P ≤ 0.05 vs. placebo).

Androgens Modulate Gene Expression Through the Modulation of H3K27me3 Histone Mark and KO of the AR in the Liver Attenuates it

While androgens have been shown to play a major role in follicular development and ovarian physiology, very few ovarian genes, so far, have been identified as direct AR targets (containing

ARE-sequences) in the female. Recently, our lab has shown that in the ovary, androgens have a more global effect on gene expression by modulating chromatin accessibility which is primarily mediated through indirect rather than direct androgen actions [22]. Chromatin remodeling via histone modification is now considered as one of the key control points for transcription and a primary target of signal transduction. Post-translational modification [106] through kinase-dependent phosphorylation [107-109] is one of the major ways through which the activity of epigenetic programmers can be regulated. We have shown that the trimethylation of lysine 27 of histone H3 (H3K27me3), a gene silencing mark, is a major downstream target of androgens [22] (Fig. 6). Androgens, through extra-nuclear kinase signaling (PI3K/Akt pathway) rapidly phosphorylate and inhibit activity of the Polycomb group protein enhancer of zeste homolog 2 (Ezh2). Ezh2 is a histone methyltransferase that promotes H3K27me3 mark. Moreover, androgens through nuclear signaling (AREs) induce the expression of an anti-Ezh2 microRNA, *miR-101* that targets and decreases Ezh2 expression (Fig. 6). In addition, in the ovary, androgens through a transcription-independent (non-genomic) mechanism induces the expression of hypoxia-inducible factor-1 alpha (Hif-1a) which in turn increases the expression of a histone demethylase called lysine demethylase 6B (JMJD3/KDM6B) (unpublished data) (Fig. 6). JMJD3 specifically demethylates (removes) the H3K27me3 gene repressive histone mark [110]. Ultimately, this results in the overall removal of H3K27me3 mark, which in turn modulates chromatin accessibility and allow the expression of ovulatory genes such as, *RUNX1* [22].

Therefore, we next investigated whether this androgen-induced modulation of histone enzymes, and ultimately the H3K27me3 mark, is conserved in the liver. We determined the protein levels of Ezh2, JMJD3, and the global H3K27me3 mark, through western blot analysis, and the mRNA levels of *miR101* in liver tissue lysates from placebo, DHT-pellet (WT) and liver-specific

AR-KO DHT-pellet treatments (Fig. 7). Results show that liver lysates from WT animals treated with DHT-pellet had increased protein levels of JMJD3 (Fig. 7A) and mRNA levels of *miR101* (Fig. 7B). Correspondingly, protein levels of Ezh2 and H3K27me3 (Fig. 7A) were lower in liver samples from DHT-pellet compared to placebo animals. Importantly, protein levels of Ezh2, JMJD3, H3K27me3 (Fig. 7A) and mRNA levels of *miR101* (Fig. 7B) in DHT-pellet treated liver lysates from the liver-specific AR-KO animals were similar to placebo treated livers from WT-animals.

Given the importance of histone modifications in gene expression, we next determined if changes in the level of the H3K27me3 epigenetic mark in the liver from placebo, DHT-pellet (WT) and liver-specific AR-KO DHT-pellet treatments had any effect on gene expression. To correlate the H3K27me3 epigenetic mark with the upregulated genes obtained from RNA-seq, we performed ChIP analysis to study post-translational modifications occurring specifically on the gene promoter of *Bmal. Bmal* was one of the genes that was found to be upregulated in the RNA-seq analysis, and this gene is a critical player in the molecular circadian clock. As mentioned previously, the reason for choosing this particular gene is primarily because the circadian system plays a crucial role in liver physiology and disease. Figure 8B shows ChIP analysis of region one (see Fig. 8A) of the promotor of *Bmal* with anti-H3K27me3. Results show that the H3K27me3 gene repressive mark was significantly lower ($*P \leq 0.05$) in the promoter region of *Bmal* of liver lysates from WT animals treated with DHT-pellet compared to placebo treated livers (Fig. 8B). Notably, DHT-pellet treated liver lysates from the liver-specific AR-KO animals had similar levels of the H3K27me3 histone repressive mark in the *Bmal* gene promotor as placebo treated livers (Fig. 8B).

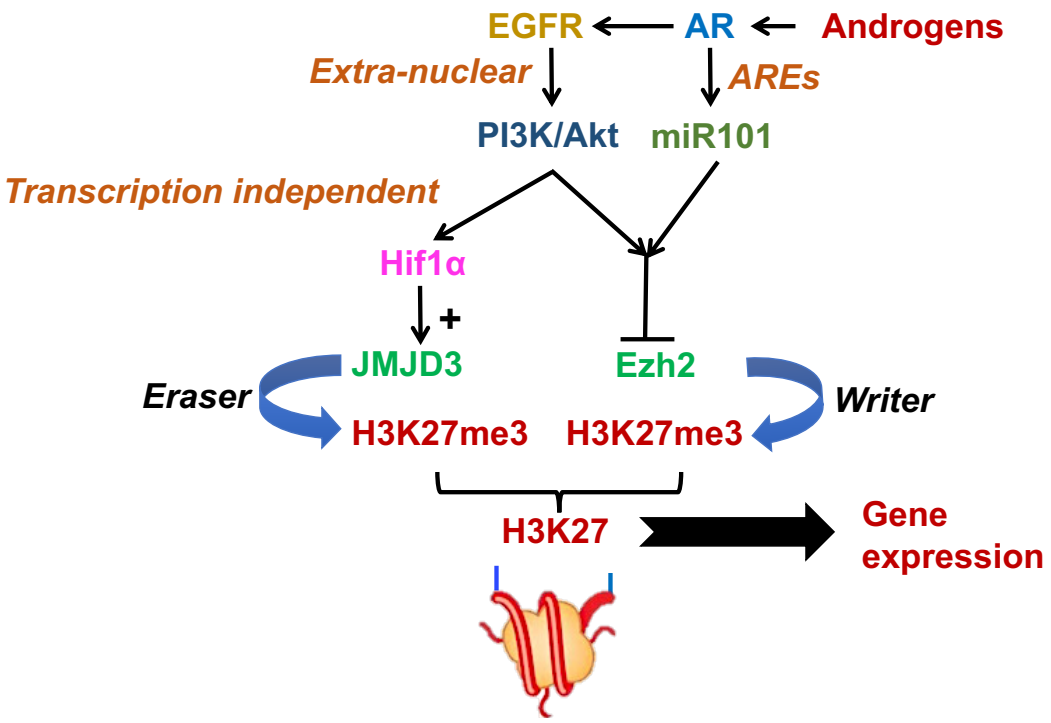


Figure 6: Proposed Model for Androgen-Induced Modulation of H3K27me3. Androgens, through extra-nuclear kinase signaling (PI3K/Akt pathway) rapidly phosphorylate and inhibit activity of the Polycomb group protein enhancer of zeste homolog 2 (Ezh2). Ezh2 is a histone methyltransferase that promotes H3K27me3 mark. Moreover, androgens through nuclear signaling (AREs) induce the expression of an anti-Ezh2 microRNA, miR101 that targets and decreases Ezh2 expression. In addition, in the ovary, androgens through a transcription-independent (non-genomic) mechanism induces the expression of hypoxia-inducible factor-1 alpha (Hif-1a) which in turn increases the expression of a histone demethylase called lysine demethylase 6B (JMJD3/KDM6B) (unpublished data). JMJD3 specifically demethylates (removes) the H3K27me3 gene repressive histone mark. Ultimately, this results in the overall removal of H3K27me3 mark, which in turn modulates chromatin accessibility and allow the expression of genes.

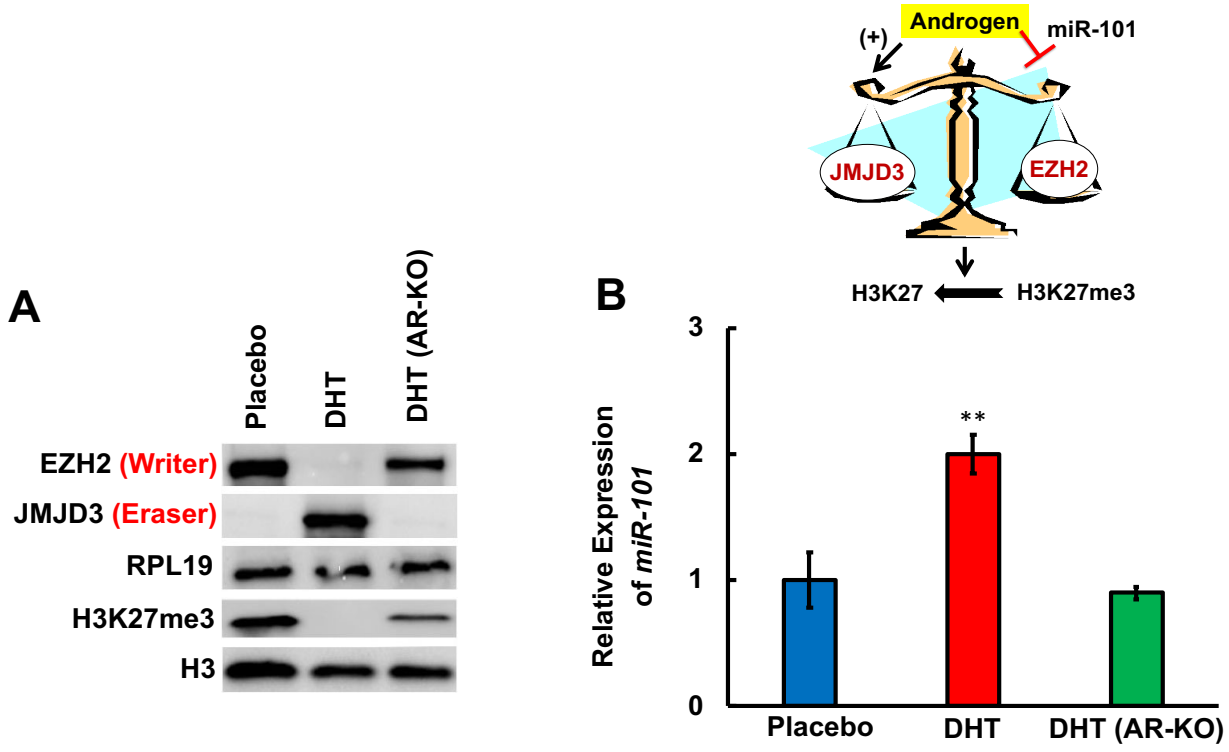


Figure 7: Androgen-induced Modulation of Histone Enzymes and H3K27me3 Mark is Conserved in the Female Liver. (A) Representative Western blot for Ezh2, JMJD3, RPL19, H3K27me3 and H3 in liver lysates from WT (placebo and DHT-pellet) and liver-specific ARKO treated with DHT-pellet. Similar blots were observed in three separate experiments. (B) Relative expression of miR-101 in the liver are significantly increased in WT animals treated with DHT-pellet, but not liver-specific AR-KO treated with DHT-pellet, compared to placebo. Data are displayed as means ± SEM (n = 3) and normalized to snoRNA202 levels (**P ≤ 0.01 vs. placebo).

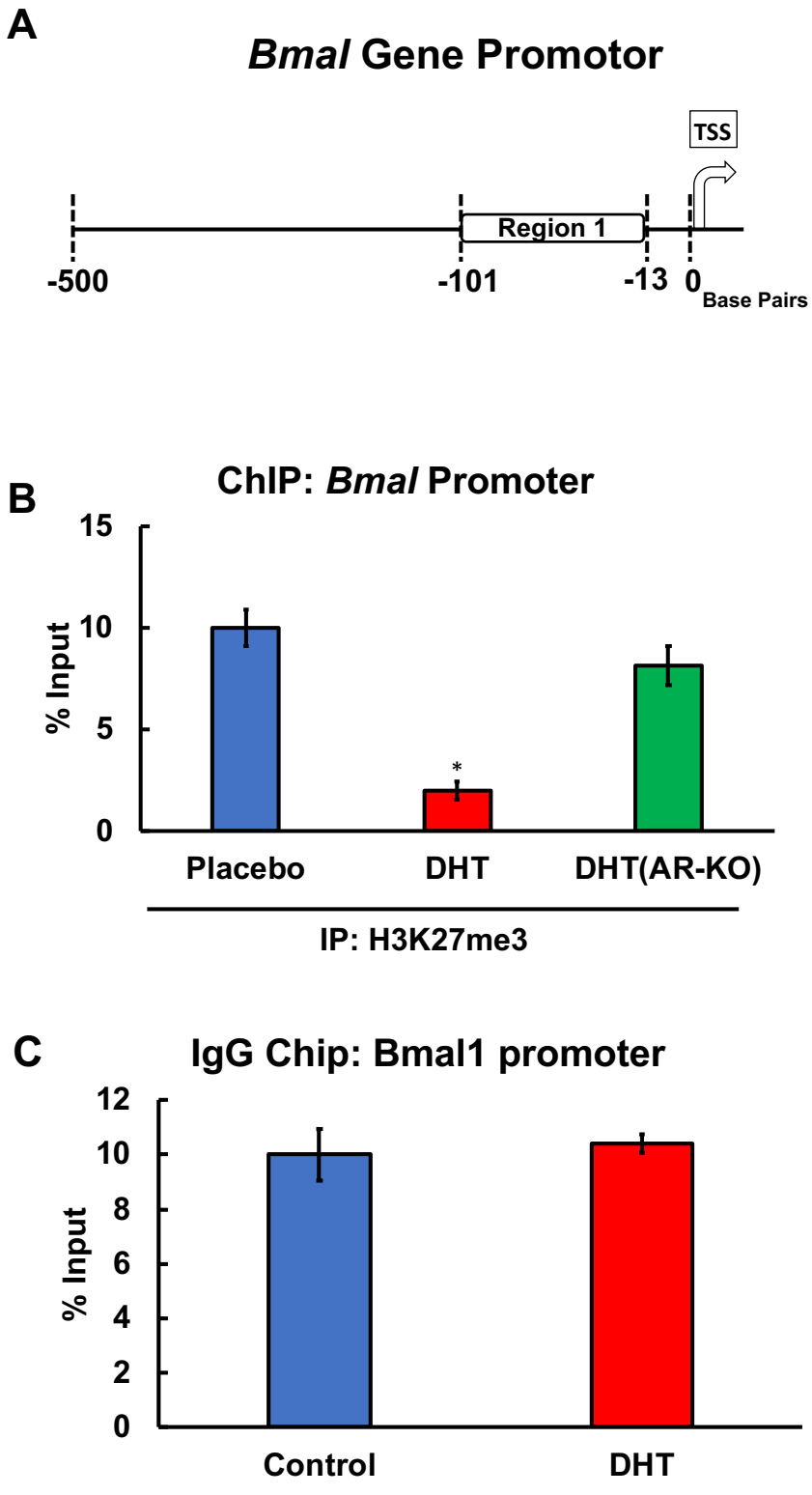


Figure 8: Levels of H3K27me3 in *Bmal* Promoter.

Figure 8 (cont'd)

(A) Schematic diagram showing the region of the *Bmal* promoter (Region 1) in which the H3K27me3 mark was measured. (B) ChIP assay revels that H3K27me3 gene repressive mark was significantly lower in the promoter region of *Bmal* of liver lysates from WT animals treated with DHT-pellet compared to placebo treated livers. DHT-pellet treated liver lysates from the liver-specific AR-KO animals had similar levels of the H3K27me3 histone repressive mark in the *Bmal* gene promotor as placebo treated livers. (C) ChIP assay using IgG as nonspecific control in liver samples from WT animals treated with placebo and DHT-pellet. Values represent percentage input (means ± SEM, n = 3; *P ≤ 0.05 vs. placebo).

Androgen-induced Regulation of Epigenetic Modulators is Conserved in Human Hepatocytes Derived from induced-Pluripotent Stem Cells (iPSCs)

Next, we wanted to determine whether the androgen-induced epigenetic modulation of the gene expression is conserved in humans. However, acquiring primary hepatocytes and/or liver samples from females, especially PCOS women typically requires an invasive liver biopsy and are rarely done. Therefore, using a well-established hepatocyte differentiation protocol (abcam) we have used human hepatocyte-like cells derived from iPSCs for our studies. Validation of the differentiated hepatocytes was established by detection of hepatocyte specific genes through immunofluorescence and RT-PCR. Staining results showed the presence of HNF4- α and Albumin positive cells (Fig. 9). Moreover, the expression of liver-specific genes such as, *Apolipoprotein A1 (APOA1)*, *Serpin Family A Member 1 (SERPINA1)*, *Asialoglycoprotein Receptor 1 (ASGR1)*, *Transferrin (TF)*, *and Albumin* are all significantly (*P ≤ 0.05) more expressed in differentiated hepatocytes compared to iPSCs (Fig. 10). All these genes have a specific hepatocyte function. For

example, HNF4- α is a nuclear receptor which regulates a hepatocyte genes involved in lipid metabolism, glucose metabolism, differentiation and morphogenesis [111]. APOA1 is a major protein component of high density lipoproteins [112] and SERPINA1 is a serine protease inhibitor and has a role in various biological functions such as coagulation, fibrinolysis, angiogenesis, inflammation and apoptosis [113]. Moreover, ASGR1 codes for a subunit of the asialoglycoprotein receptor which plays a critical role in serum glycoprotein homeostasis. TF is plasma glycoprotein that is well known for its function in iron uptake [114].

Following validation, we treated the differentiated human hepatocytes with 1μM DHT *in vitro* and using western blot analysis, we determined if androgen-induced modulation of H3K27me3 is also found in the human. Similar to the mouse studies, DHT treatment increased protein levels of JMJD3 (Fig. 11A) and mRNA levels of *miR101* (**P ≤ 0.01) (Fig. 11B), while decreased Ezh2 and H3K27me3 protein levels (Fig. 11A) compared to control in differentiated human hepatocytes. Importantly, in this experiment we used a high dose of DHT (1μM) which equals to 1000 nmol/liter. While the heterogeneity of PCOS patients is enormous, typically hyperandrogenic PCOS patients have a wide range of testosterone levels ranging from 1.8- 61.8 nmol/liter [80, 83, 96]. Thus, the DHT dose that we used is a supraphysiological dose. Previously, are lab established that in the ovary, a 25nM DHT dose was sufficient to see an androgenic effect [22, 63]. However, since this was our first time treating this cell line with androgens, we primarily used this dose to ensure we would get an effect. Future studies in our lab plan to do a concentration gradient to establish the lowest (physiologically relevant) dose needed to induce an androgenic effect in this cell line.

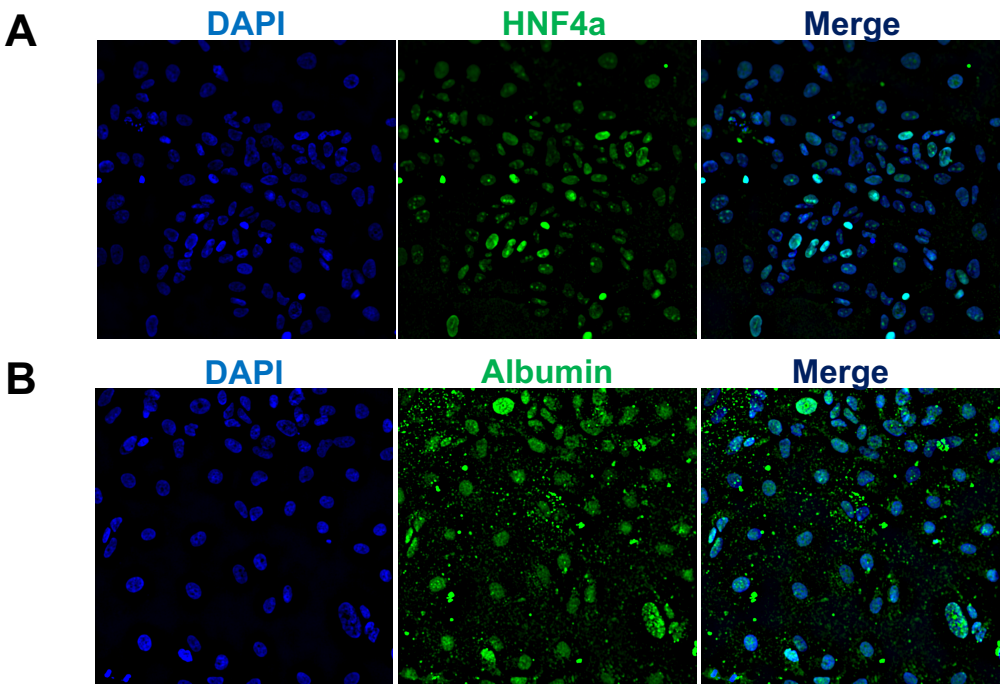


Figure 9: Albumin and HNF4a are Expressed in Hepatocytes Derived from iPSCs (A) Immunofluorescence staining images of DAPI, HNF4a, and merged (from left to right) of hepatocytes derived from iPSCs. (B) Immunofluorescence staining images of DAPI, Albumin and merged (from left to right) of hepatocytes derived from iPSCs.

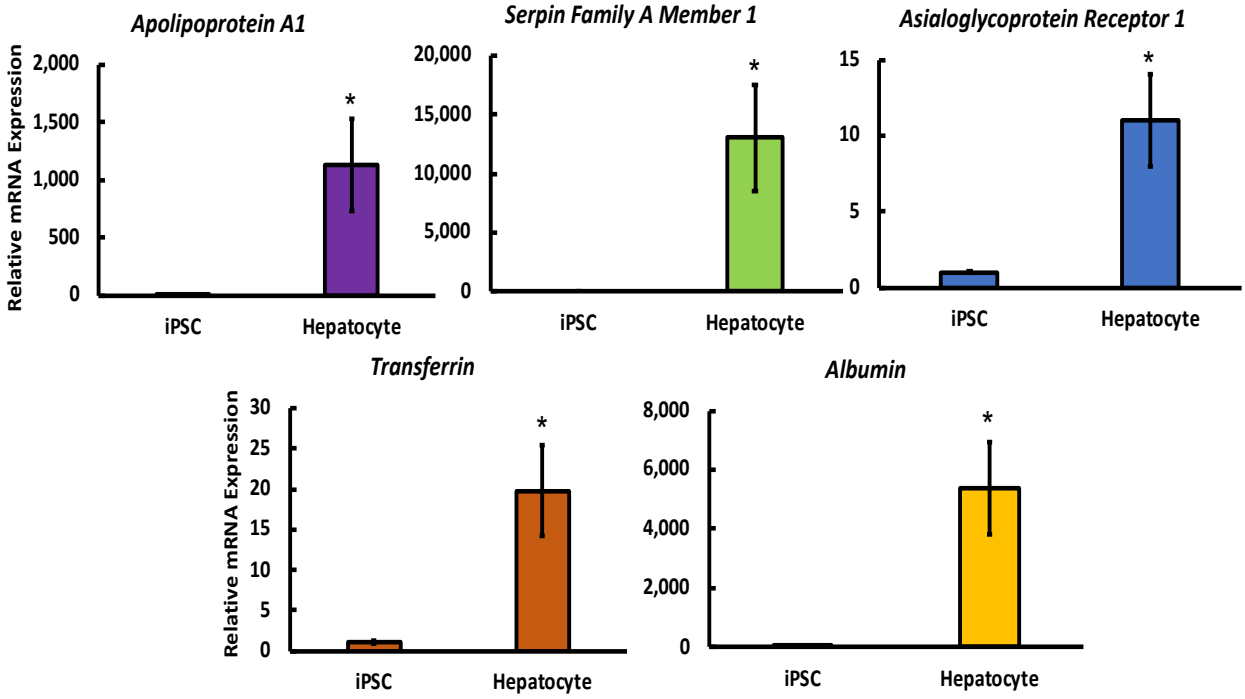


Figure 10: Differentiated Hepatocytes Have Higher Expression of Liver Specific Genes. *Apolipoprotein A1 (APOA1), Serpin Family A Member 1 (SERPINA1), Asialoglycoprotein Receptor 1 (ASGR1), Transferrin (TF)*, and *Albumin* are all significantly more expressed in differentiated hepatocytes vs iPSCs. Data are displayed as mean ± SEM (n = 3 experiments). The mRNA levels were measured by quantitative real-time PCR and compared with control RPL19 mRNA expression using the delta delta Ct method. Results are represented as the amount relative to iPSCs (mean ± SE, n=3). *P ≤ 0.05 vs. iPSC.

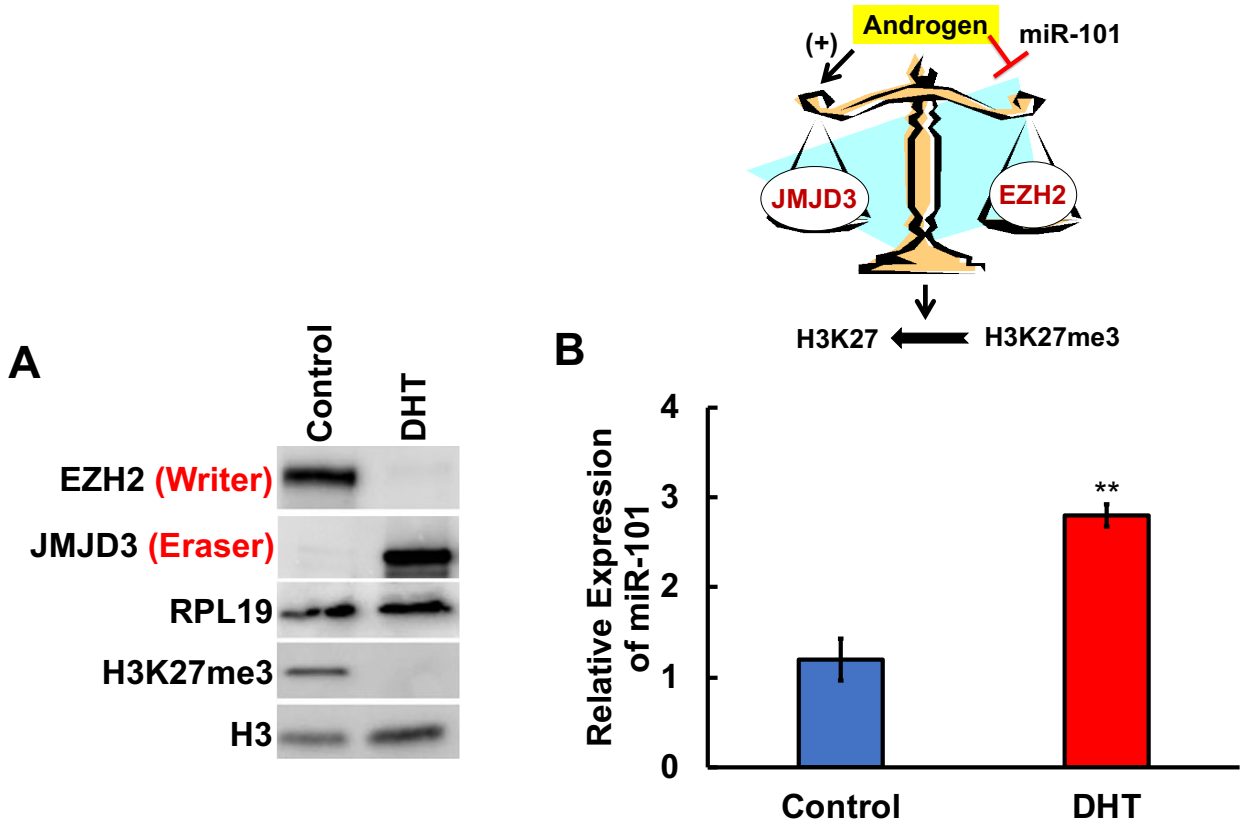


Figure 11: Androgen-induced Modulation of Histone Enzymes and H3K27me3 Mark is Conserved in Human Hepatocytes Derived from iPSCs. (A) Representative Western blots (from n = 3 plates) for Ezh2, JMJD3, RPL19, H3K27me3 and H3 in human hepatocytes derived from iPSCs treated with control and 1μM DHT. (B) Relative expression of miR-101 in human hepatocytes derived from iPSCs treated with control and 1μM DHT. Data are displayed as means ± SEM (n = 3) and normalized to miR-16 levels (**P ≤ 0.01 vs. placebo).

Discussion

This study provides an insight into how chronic high levels of androgens cause liver dysfunction in a PCOS condition. This is the first study to show that KO of the AR in the female liver rescues

the fatty liver phenotype established in the mouse PCOS model. Additionally, our studies demonstrate a direct relationship between androgen-induced changes in H3K27me3 histone repressive mark and gene expression.

Our studies show that chronic hyperandrogenism produced by our PCOS model, causes accumulation of fat and increases triglyceride levels in the liver (Fig. 5A-B). Similarly, previous studies in the rat reported that prenatal exposure to DHT during late gestation increases circulating and hepatic triglycerides levels in the adult females [115]. Moreover, prenatal exposure to androgens also causes hepatic lipid accumulation in the mouse [97], rat [115] and sheep [116] adult livers. This suggest that independent of time, whether prenatal or post-natal, hyperandrogenism can cause fatty liver. Interestingly in males, while normal androgen level and signaling prevent hepatic lipid accumulation, androgen deficiency is associated with fatty liver [117]. A previous study demonstrated that males, but not female mice lacking the AR in the liver developed diet-induced (high-fat diet) hepatic steatosis [102]. This coupled with other reports suggest that in the liver, androgens have differential effects in men and women [111]. In males, androgen deficiency is associated with fatty liver, while normal androgen levels and signaling prevent hepatic lipid accumulation [117]. In contrast, in females, abnormally high level of androgens increases fat deposition in the liver. Androgens therefore have differential effects in men and women [111]. Hence, determining the underlying mechanism of this sexual dimorphism is important. While a lot of data exist on androgen actions in the male liver, this study provides significant mechanistic insights about androgen actions in the female liver that could be used to decipher this sexual disparity with respect to androgen actions in the liver.

Previously, RNA seq analysis determined that 240 circadian genes were differentially regulated in the DHT pellet livers (unpublished data, Sen lab). Furthermore, chronic high levels of

androgens disrupt the circadian system in the liver of the mouse PCOS model (unpublished data, Sen lab). It is well established that genes involved in the molecular clock such as *BMAL*, modulate liver lipid metabolism, and disruption of the circadian rhythm in the liver induces lipid accumulation and contributes to NAFLD development [104]. Genetic modified animals have allowed us to better understand the downstream tissue-specific physiological effects of *BMAL* and circadian clocks. For example, Bmal1$^{-/-}$ knock-out mice, when fed regular chow, exhibit glucose intolerance, hypoinsulinemia, reduced fat storage, increased circulating fatty acids, increased ectopic fat formation in the liver and muscle, and hepatic steatosis [104]. Intriguingly, liver-specific Bmal1$^{-/-}$ knock-out mice exhibit metabolic discrepancies such as, high levels of hepatic triglycerides and circulating free fatty acids [118]. Additionally, they also develop hypoglycemia during fasting, and increased ability of glucose clearance despite having dyslipidemia and normal production of insulin [118].

This study also provides a mechanistic insight to the androgen-induced modulation of the hepatic circadian clock. A recent study showed that Ezh2 was downregulated in both *in vivo* and *in vitro* models of NAFLD and consequently exacerbated lipid accumulation and inflammation in the liver [119]. Moreover, Ezh2 is required for the proper function of the mammalian circadian clock [120] and loss of Ezh2 disrupts rhythmic expression of circadian clock genes in zebrafish [121]. This study highlights that androgens indirectly regulate gene expression in the female liver through regulation of Ezh2 and JMJD3 (Fig. 7), leading to the removal of the H3K27me3 gene repressive histone mark in downstream gene promoters. One such downstream gene is *Bmal*, where androgen-induced modulation of Ezh2 and JMJD3 removes the H3K27me3-repressive mark from the *Bmal* promoter (Fig. 8B) ultimately leading to its' expression. In addition, our *in vitro* studies show that this androgen-induced regulation of epigenetic enzymes is conserved in

human hepatocytes derived from iPSCs (Fig. 11). Thus, we propose that androgens, through the regulation of epigenetic enzymes, are indirectly modulating the levels of H3K27me3 of key circadian genes such as *Bmal* and consequently disrupting the circadian clock in the liver and in turn causing liver dysfunction and hepatic steatosis.

Similar to the *Bmal* promoter, it is likely that changes in H3K27me3 mark occurs in the promoter region of multiple genes. Therefore, future ChIP-seq studies are needed to correlate differentially regulated genes from in the RNA seq studies (unpublished data, Sen lab) with the levels of H3K27me3-repressive mark on their promotors. Furthermore, *in silico* analysis of 787 differentially expressed genes found in the RNA seq analysis of DHT-pellet livers show that most of these genes also contains AREs (unpublished data). Thus, direct androgen actions through nuclear signaling (AREs) is also a likely underlying mechanism of regulation of the molecular clock and liver dysfunction in NALFD. In addition to hepatic steatosis, inflammation plays a major role in the development and progression of NAFLD and NASH [89]. Further studies will look into androgen effects on inflammation as a number of inflammatory genes were found to be differentially expressed in the RNA-seq data (unpublished data, Sen lab).

Currently, there is no cure for PCOS, and current treatments are only meant to manage the metabolic and reproductive comorbidities of the syndrome. Importantly, given that KO of the AR in the liver attenuated the development of hepatic steatosis and rescued the gene expression phenotype, this study establishes AR as a potential therapeutic target for NAFLD in PCOS women. In fact, in the clinic, targeting the AR with inhibitors such as, flutamide is already being implemented as a therapeutic option for PCOS patients. For example, a clinical report showed that treatment with flutamide (12-week regimen) significantly improved the lipid profile of PCOS patients by reducing the serum levels of cholesterol, low density lipoproteins, and triglycerides

[122]. However, there was no significant effect in improving liver function dictated by measurement of liver enzymes. Furthermore, another clinical report looked at the beneficial effects of a long-term treatment (12-months) regimen of flutamide, metformin, and their combination coupled with a hypocaloric diet on the reproductive and metabolic clinical features of obese-PCOS patients [123]. This study showed that flutamide alone can counteract hyperandrogenism and even improve metabolic comorbidities such as, insulin resistance and lipid abnormalities [123]. Moreover, this study found that flutamide also had beneficial effects on the frequency of menstruation [123]. Thus, supporting the pathophysiological role of excess androgens in the syndrome. However, whether treatment with flutamide or any other AR inhibitor can aid in treating NAFLD in PCOS patients remains to be determined.

In summary, our studies provide evidence that direct androgen actions, through the AR in the female liver, are necessary for the development of fatty liver in PCOS. In addition, we show that androgen-induced modulation of the H3K27me3 repressive mark is one of the underlying causes for differential expression of genes in both the PCOS liver and human hepatocytes. Future studies are needed to further understand how this histone mark affects downstream liver physiology.

Chapter 3: Extra-Nuclear and Nuclear Androgen Signaling Models

Androgen actions are mediated by *"nuclear"* transcriptional signals or *"extra-nuclear"* kinase actions [18]. Classically androgens act through ARs that function as transcriptional regulators. However, androgens (or any other steroid hormones) evoke rapid responses that cannot be explained through transcriptional regulation. It is now well-established across tissue systems and species that there exists a pool of extra-nuclear AR associated with the plasma membrane that are involved in membrane-initiated (MAPK and PI3K/Akt) signaling [30, 56]. The androgen-induced membrane-initiated signaling involves transactivation of the epidermal growth factor receptor (EGFR) through the Src kinase [124, 125]. Additionally, in the last couple of years we have reported [22] that androgens may also influence gene expression by modulating various epigenetic marks. Alternately, the extra-nuclear AR-induced membrane initiated signaling can also have "transcription-independent" non-genomic effects mediated either through activation of proteins / enzymes or by affecting protein levels by regulating translation and/or protein degradation pathways. Unlike the estrogen receptor and the progesterone receptor, till date, no membrane-specific AR has been found [18].This emphasizes that the membrane-associated extra-nuclear AR and the nuclear AR are the same protein. This concept is further supported from small interfering RNA (siRNA) and KO studies involving AR showing that deletion of the AR completely inhibits androgen-induced MAPK signaling [28, 29, 63]. Most steroid receptors, including AR, contain a highly conserved palmitoylation site [126]. Following palmitoylation, about 5-10% of steroid receptors associate with caveolin-1, which targets palmitoylated receptors to the caveolae of the plasma membrane, where they engage in signaling [126]. This palmitoylation motif includes amino acids F805, C807, and LL812-813 which serve as recognition sites for palmitoyl acyltransferases (PATs). The cysteine amino acid (C807) gets palmitoylated by PAT [127] which

causes caveolin-1 binding and recruitment to the membrane [128] (Fig. 12). Upon ligand-binding, membrane associated AR receptors interact with Src to activate Erk, MAPK1/3, and PI3K/Akt signaling [18, 22, 63].

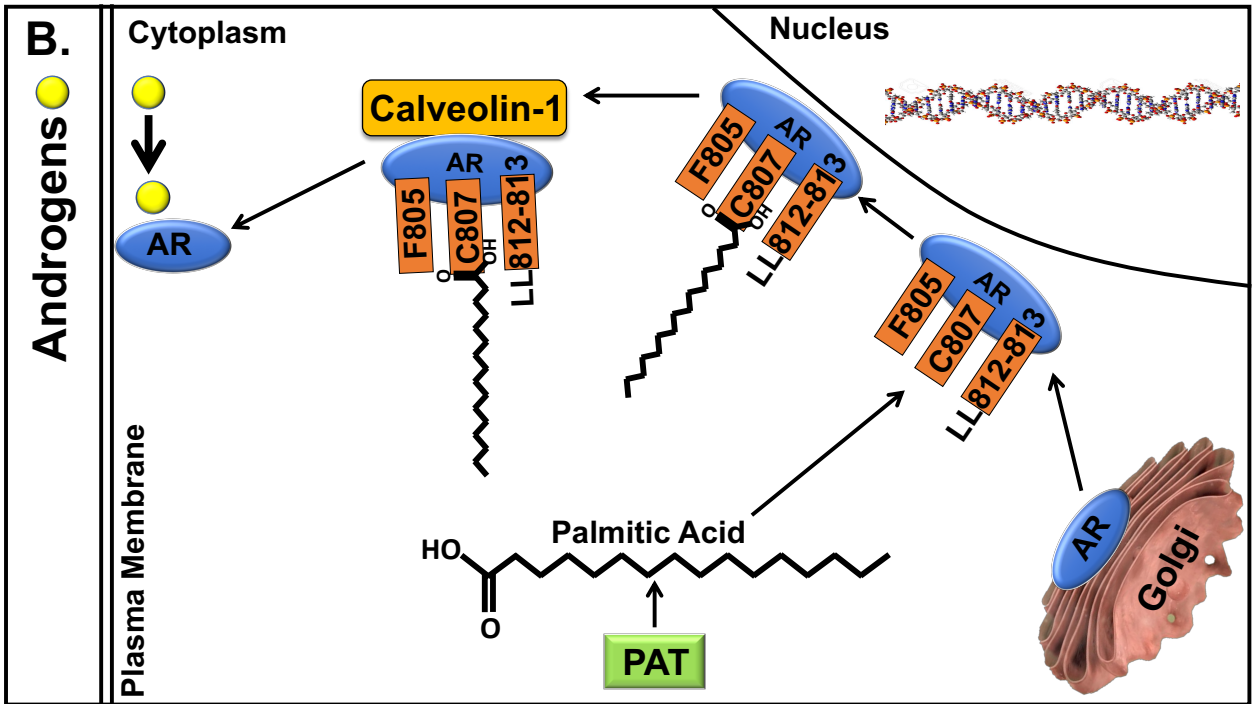


Figure 12: Schematic Diagram of AR Translocation to the Membrane. *See text above.*

Given the importance of androgen signaling in normal and various pathophysiological conditions, differentiating the genomic with the membrane-initiated androgen signaling as well as identifying the downstream targets that are either specifically or synergistically regulated by these two androgen signaling pathways is imperative towards developing biomarkers and/or therapeutics for diseases involving hyper-androgenemia like PCOS and prostate cancer. Given that the same AR protein is involved in nuclear and membrane signaling and contributing to downstream physiological processes, a major limitation towards elucidating androgen actions is the inability to differentiate *in vivo* the potential role of each of these pathways in physiological or

pathophysiological conditions. In this study, we have developed two genetic mouse models, involving Nuclear-Only Androgen Receptor signaling (NOAR) and Membrane-Only Androgen Receptor signaling (MOAR) to determine the relative contribution of extra-nuclear and nuclear AR. Furthermore, we have created AR-mutant clones using site-directed mutagenesis and mutated the palmitoylation motif and the DNA-binding site of the AR. Thereafter, these powerful and novel *in vivo* and *in vitro* models, for the first time, can be used to specifically differentiate the effects of nuclear vs extranuclear androgen actions both at a physiological and cellular level.

Materials and Methods

*Generation of in vivo NOAR/WT-*AR-loxP$^{flox/flox}$ *and MOAR/WT-AR-loxP$^{flox/flox}$ Mouse Models*

NOAR/WT-AR-loxP$^{flox/flox}$

The extra-nuclear AR-induced membrane-initiated signaling is mediated through transactivation of the EGFR involving direct interaction between AR and Src tyrosine kinase. AR interacts with Src by binding of a polyproline sequence between residues 371 and 381 of the AR NTD to Src homology domain 3 [26, 124, 125]. A number of cancer studies [129-131] have targeted this AR-Src interaction to inhibit the extra-nuclear AR signaling. To create a nuclear-only androgen receptor signaling mouse model, we created a 46 base pair deletion mutation (Exon-1) in the Src binding site of the AR (Cyagen Biosciences) and named this mouse the NOAR. We initially created a transgenic mouse model where we inserted a mutant AR containing a 45 bp deletion (15 amino acids) in exon 1 at the AR-Src interaction site and then crossed it with AR-floxed mouse to create a mouse that has the mutant AR with the WT-AR being floxed (Fig. 12). The idea is that when this mouse is crossed with tissue-specific CRE, for example albumin-CRE (liver–specific),

only the mutant AR is expressed in the targeted (liver) tissue. Given that nuclear and extra-nuclear AR are the same protein, this mutant AR will retain the nuclear activity but the extra-nuclear membrane-initiated signaling is blocked. However, the membrane-initiated signaling can be rescued *in vitro*, by treating cells isolated from the targeted tissues (liver) with EGF.

Briefly, WT-AR-loxP$^{flox/flox}$ females were crossed with NOAR males. Thereafter, the NOAR/WT-AR-loxP$^{flox/y}$ was crossed with another WT-AR-loxP$^{flox/flox}$ female to generate a NOAR/WT-AR-loxP$^{flox/flox}$ female (Fig. 13). It is important to note that the AR is located in the X chromosome, hence, it took more than one crossed to get the AR-loxP allele in both X chromosomes of the female.

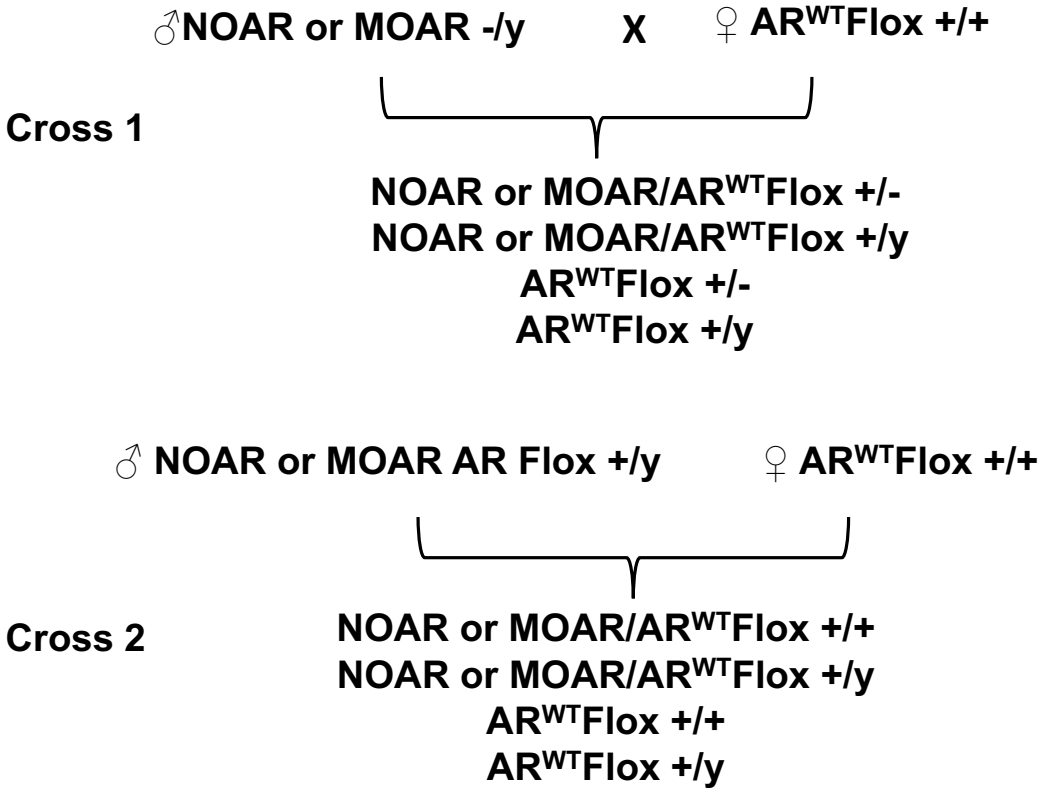


Figure 13: Schematic for the Generation of NOAR or MOAR/WT-AR-loxP$^{flox/flox}$ Females.

Figure 13 (cont'd)

Briefly, WT-AR-loxP$^{flox/flox}$ females were crossed with NOAR or MOAR males. Thereafter, the NOAR/WT-AR-loxP$^{flox/y}$ or MOAR/WT-AR-loxP$^{flox/y}$ was crossed with another WT-AR-loxP$^{flox/flox}$ female to generate a NOAR/WT-AR-loxP$^{flox/flox}$ or MOAR/WT-AR-loxP$^{flox/flox}$ females.

Genotyping:

Genomic DNA was isolated from tail snips of 14-15 d-old animals, and PCR genotyping with appropriate primers (Table 6) was used to identify mice with AR-WT, floxed, and Exon-1 mutants, revealing bands of 860 (AR-WT), 930 (AR floxed), 507 (internal control) and 280 (Exon-1 transgene mutant) bp, respectively (Fig. 14).

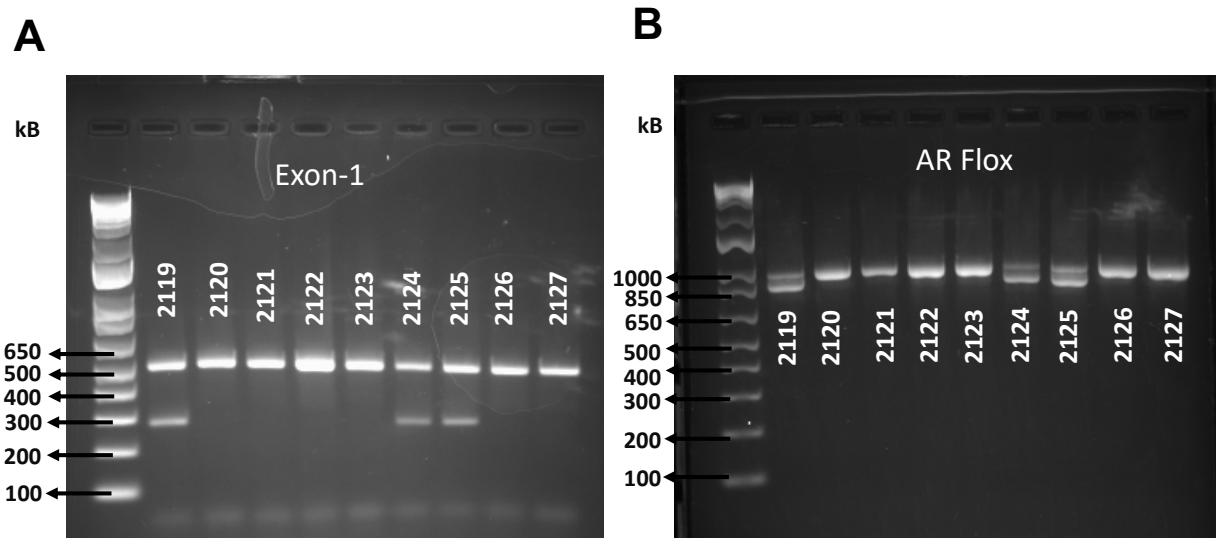


Figure 14: Genotype PCR Analysis of NOAR Mutant Female Animals.

Figure 14 (cont'd)

(A) Results of Exon-1 detection. Exon-1 positive animals are 2119, 2124 and 2125 showing bands in 280bp for Exon-1 transgene and 507bp Exon-1 Internal Control, respectively. (B) Results of AR-Flox detection. All Exon-1 positive animals (2119, 2124, and 2125) show AR-Flox heterozygous status, as expected.

Table 6. Genotyping PCR Primers

PCR Primers	Sequence
Exon-1 Mutant Forward	5'- GTGATGGCTTCCATGTCGGC -3'
Exon-1 Mutant Reverse	5'- CGCTTACGCAGGGCATCCAT -3'
Exon-1 Internal Control Forward	5'- CTATCAGGGATACTCCTCTTTGCC -3'
Exon-1 Internal Control Reverse	5'- GATACAGGAATGACAAGCTCATGGT -3'
WT and AR Flox Forward	5'- AGCCTGTATACTCAGTTGGGG-3'
WT and AR Flox Reverse	5'- AATGCATCACATTAAGTTGATACC-3'
Exon-2 Mutant Forward	5'- TGCACTTCTGGCAGGAGGAG 3'
Exon-2 Mutant Reverse	5'- CCGGACCATTCTTGCGTCTG -3'
Exon-2 Internal Control Forward	5'- GCAGAAGAGGACAGATACATTCAT -3'
Exon-2 Internal Control Reverse	5'- CCTACTGAAGAATCTATCCCACAG -3'

MOAR/WT-AR-loxP$^{flox/flox}$

Similarly, this model contains a mutant AR with a point mutation in exon 2 that corresponds to the DNA-binding domain of the AR along with the WT-AR being floxed. When crossed with CRE, in this mouse, the nuclear actions of AR will be blocked but the membrane-initiated signaling through the extra-nuclear AR is active. To create this mouse model, we inserted a two-base pair point mutation (Exon-2) in the DNA binding site of the AR (Cyagen Biosciences) and named this mouse MOAR. Briefly, WT-AR-loxP$^{flox/flox}$ females were crossed with NOAR males. Thereafter, the MOAR/WT-AR-loxP$^{flox/y}$ was crossed with another WT-AR-loxP$^{flox/flox}$ female to generate a MOAR/AR-loxP$^{flox/flox}$ female (Fig. 13).

Genotyping:

Genomic DNA was isolated from tail snips of 14-15 d-old animals, and PCR genotyping with appropriate primers (Table 6) was used to identify mice with AR-WT, floxed, and Exon-2 mutants, revealing bands of 860 (AR-WT), 930 (AR floxed), 689 (internal control) and 444 (Exon-2 transgene mutant) bp, respectively (Fig. 15).

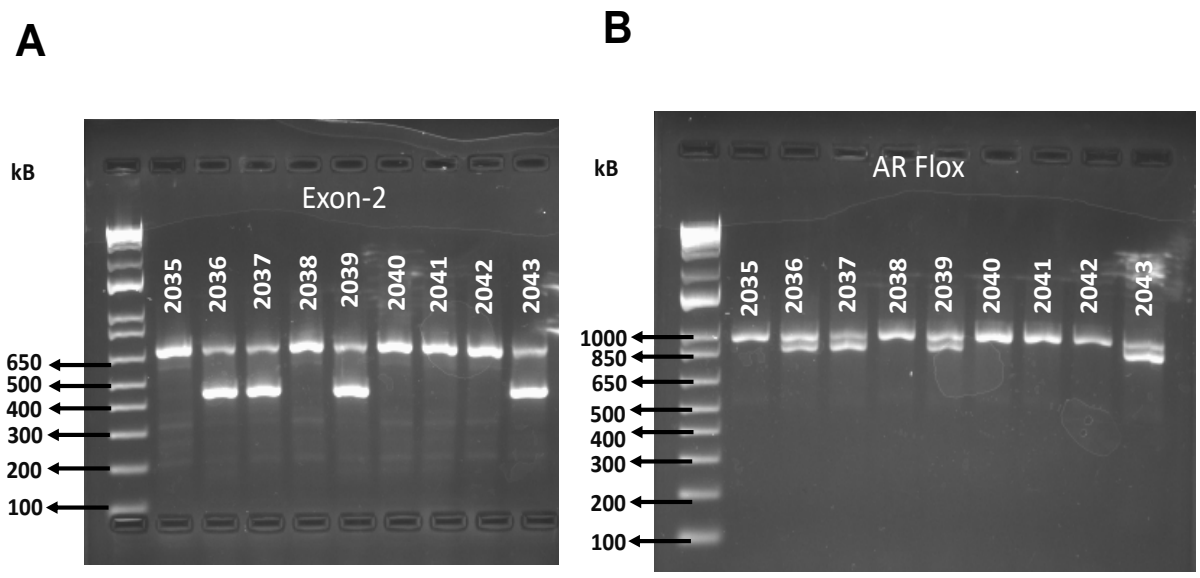


Figure 15: Genotype PCR Analysis of MOAR Mutant Female Animals. (A) Results of Exon-2 detection. Exon-2 positive animals are 2036, 2037, 2039 and 2043 showing bands in 444bp for Exon-2 transgene and 689bp Exon-2 Internal Control, respectively. (B) Results of AR-Flox detection. All Exon-2 positive animals (2036, 2037, 2039 and 2043) show AR-Flox heterozygous status, as expected.

Generation of in vitro AR Mutant Clones

Previously, it has been shown that the cysteine amino acid (C807) of the AR palmitoylation motif gets palmitoylated by PAT [127] which causes caveolin-1 binding and recruitment to the membrane [128]. However, the goal was to mutate individual as well as all of the amino acids

from the palmitoylation motif (F805, C807, and LL812-813) to alanine, to identify the importance of them for membrane translocation of the AR. To develop AR mutant clones, pCMV6-AC-GFP, mammalian vector containing human AR gene (NM_000044= Nucleotide Accession Number) that is C-terminal tGFP (Green Fluorescent Protein) tag (Origene) was used as a template to create mutant clones of the AR. Primers were designed for the mutations of palmitoylation sites (F805, C807 and LL812-813) and the DNA-binding site of the AR (two-point mutation) (Table 7). All the palmitoylation sites and DNA-binding site were changed to Alanine (A805, A807, AA812-813) through PCR (polymerase-chain reaction) based site-directed mutagenesis protocol (Agilent). All mutants were sent out to sequence (Genewiz) for mutation verification using sanger sequencing. The idea is that by mutating the palmitoylation sites in the AR, it will not get palmitoylated and thus, will not get recruited to the membrane. Therefore, these clones will only have nuclear signaling active. Additionally, by mutating the DNA-binding site of the AR, it will prevent it from binding to AREs and regulate gene expression. Therefore, this clone will only have extra-nuclear signaling active.

Table 7. Site-Directed Mutagenesis- PCR Primers

Site-Directed Mutagenesis Primers	Sequence
AR-A807 Forward	5’ CCCCAGGAATTCCTGGCCATGAAAGCACTGCTGCTACT 3’
AR-A807 Reverse	5’ AGTAGCAGTGCTTTCATGGCCAGGAATTCCTGGGG 3’
AR-AA812-813 Forward	5' CATGAAAGCACTGGCAGCCTTCAGCATTATTCCA 3'
AR-AA812-813 Reverse	5' AATAATGCTGAAGGCTGCCAGTGCTTTCATGCA 3'

Results

Mouse Genotyping

PCR analysis of DNA extracted from NOAR mice shows (Fig. 14) the presence of Exon-1 mutant band (280bp), internal control (507bp) and AR Flox allele (930bp). Furthermore, PCR analysis of DNA extracted from MOAR mice shows (Fig. 15) the presence of Exon-2 mutant band (444bp), internal control (689bp) and AR Flox allele (930bp). It is important to note that the AR-Flox primers detect not only the WT-AR (that is floxed) but also the NOAR or MOAR (without the flox). Thus, all female mice with exon 1 or exon 2 mutations show double bands for the AR-flox primers.

Plasmid Sequencing

Sanger sequencing (Genewiz) was used to verify that mutations occurred. To create AR-A807 mutant, the 807-cysteine (TGC) amino acid residue was mutated to alanine (GCC). Fig. 16 shows the alignment of the sanger sequencing results from AR-A807 mutant with the human WT-AR gene and established the correct mutation. Further, to create AR-A812-813 mutant, the double 812-813 leucine (812-CTA, 813-CTC) amino acid residues where mutated both to alanine (812-GCA, 813-GCC) (Fig.17). Fig. 17 shows the alignment of the sanger sequencing results from AR-A812-813 mutant with the human WT-AR gene and established the correct mutation. Other mutants remain to be made.

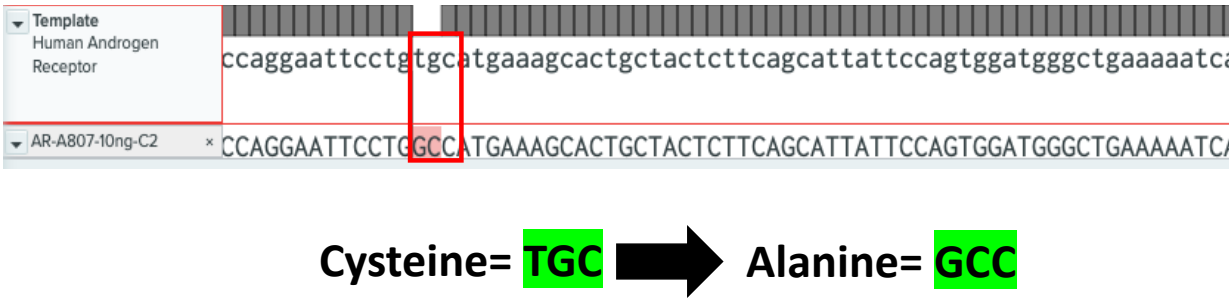


Figure 16: Sequencing Alignment of AR-A807 Mutant. Amino acid C807 (TGC codon) was mutated to A807 (GCC codon)

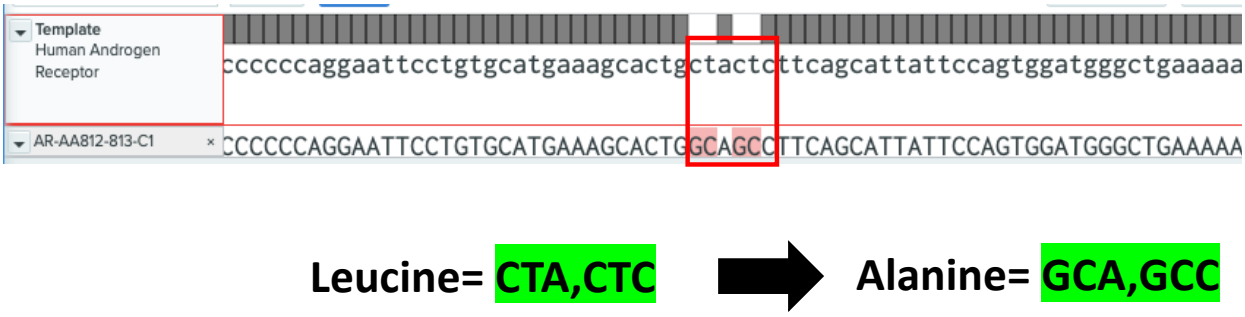


Figure 17: Sequencing Alignment of AR-AA812-813 Mutant. Amino acid L812 (CTA codon) was mutated to A812 (GCA codon) and amino acid L813 (CTC codon) was mutated to A813 (GCC codon)

Future Studies

Future studies in our lab will be validating the *in vivo* and *in vitro* models. Importantly, these *in vivo* models have already been validated and used to elucidate androgen actions in male reproductive physiology (William H. Walker- unpublished data). However, to validate our models in our lab we will cross NOAR/WT- AR-loxP$^{flox/flox}$ and MOAR/WT- AR-loxP$^{flox/flox}$ mice with

Albumin Cre mice to generate liver-specific NOAR or MOAR. Thereafter, liver explants and/or hepatocytes isolated from the liver-specific NOAR or MOAR will be stimulated with DHT, *in vitro* and MAPK and PI3K/Akt activation [22, 63] as well as expression of AR-regulated genes like miR101, *Kdm5c* (upregulated in our RNA-seq data and contains ARE-sequence on the promoter region) will be determined. Furthermore, ChIP studies will be performed with AR antibody and primers spanning ARE sequences on the promoter region of these genes will also be done for validation of these mouse models. We anticipate that DHT treatment will activate both Erk and PI3K/Akt pathways in hepatocytes isolated from WT animals but not from the NOAR mice. Moreover, ChIP assay should show AR still binds to the ARE sequence on the *Kdm5c* gene causing gene expression upon DHT treatment. In contrast, in the MOAR mice, while DHT should activate PI3K/Akt and Erk, AR will not bind to the DNA and there should be no DHT-induced expression of *Kdm5c.* Additionally, the *in vitro* models will also be validated. Thereafter, these models will be used to differentiated androgen-induced genomic and membrane-initiated signaling in liver metabolism.

Conclusion

This thesis highlights a potential role of the AR in female liver physiology and establishes the importance of the AR in the development hepatic steatosis in a PCOS condition. As shown in Fig. 5 of Chapter 2, direct androgen actions are necessary for the development of fatty liver in the mouse PCOS model. Mechanistically, this study determined that androgen modulate gene expression indirectly by regulating the H3K27me3 mark in downstream gene promotors both in mouse and human hepatocytes derived from iPSCs. However, further studies need to determine the effects that direct androgen actions (through AREs) and androgen-induced membrane-initiated signaling pathways such as Erk, MAPK1/3 and PI3K/Akt have in female liver physiology and dysfunction.

To do so, Chapter 3 of this thesis focused on developing the tools to further elucidate androgen actions in the liver. Once validated, we can create a liver-specific NOAR or MOAR PCOS mouse model and further delineate the relative contribution of nuclear or extra-nuclear androgen signaling in the development of NALFD and liver dysfunction at a physiological level. On a cellular level, our *in vitro* models can help us further understand in androgen actions in cultured hepatocytes. These models will help us answer questions regarding androgen actions in the liver such as, what are the pathways regulated by membrane-initiated signals? What are the pathways regulated by nuclear androgen signaling? Moreover, this study and previous studies in our lab (unpublished data, Sen lab) determined that hyperandrogenism significantly disrupts the circadian system. Thus, these models will further help us elucidate further whether androgens do this in a direct or indirect manner. Recently, our lab has also performed metabolomics studies (unpublished data, Sen lab) on livers of WT animals treated with DHT-pellet and further bioinformatic studies are needed to correlate the results with our RNA-seq analysis to further

determine candidate pathways affected by androgens. In future, ChIP-seq studies, coupled with our RNA-seq data, can be used to determine the global effect of the H3K27me3 repressive mark and identify the genes regulated though epigenetic modulation in DHT-treated livers.

In conclusion, this thesis demonstrates that the AR may be a potential therapeutic target to prevent NALFD in PCOS. Further, we conclude that androgen-induced modulation of H3K27me3 is one of the underlying causes for liver dysfunction in the PCOS liver. Targeting H3K27me3 at target genes may possibly serve as a second therapeutic target for treating NALFD in PCOS.

REFERENCES

REFERENCES

1. Nef, S. and L.F. Parada, *Hormones in male sexual development.* Genes Dev, 2000. **14**(24): p. 3075-86.

2. O'Connell, M.D. and F.C. Wu, *Androgen effects on skeletal muscle: implications for the development and management of frailty.* Asian J Androl, 2014. **16**(2): p. 203-12.

3. Davey, R.A. and M. Grossmann, *Androgen Receptor Structure, Function and Biology: From Bench to Bedside.* Clin Biochem Rev, 2016. **37**(1): p. 3-15.

4. Rana, K., R.A. Davey, and J.D. Zajac, *Human androgen deficiency: insights gained from androgen receptor knockout mouse models.* Asian J Androl, 2014. **16**(2): p. 169-77.

5. Lucas-Herald, A.K., et al., *Genomic and non-genomic effects of androgens in the cardiovascular system: clinical implications.* Clin Sci (Lond), 2017. **131**(13): p. 1405-1418.

6. Ma, W.L., et al., *Androgen receptor roles in hepatocellular carcinoma, fatty liver, cirrhosis and hepatitis.* Endocr Relat Cancer, 2014. **21**(3): p. R165-82.

7. Navarro, G., et al., *The role of androgens in metabolism, obesity, and diabetes in males and females.* Obesity (Silver Spring), 2015. **23**(4): p. 713-9.

8. Matsumoto, T., et al., *The androgen receptor in health and disease.* Annu Rev Physiol, 2013. **75**: p. 201-24.

9. Bon-chu, C. and H. Meng-Chun, *Androgen Biosynthesis And Degradation*, in *Androgens and Androgen Receptor: Mechanisms, Functions, and Clinical Applications*, C. Chang, Editor. 2002, Springer US: Boston, MA. p. 1-15.

10. Lobl, T.J., *Androgen transport proteins: physical properties, hormonal regulation, and possible mechanism of TeBG and ABP action.* Arch Androl, 1981. **7**(2): p. 133-51.

11. Gao, W., C.E. Bohl, and J.T. Dalton, *Chemistry and structural biology of androgen receptor.* Chem Rev, 2005. **105**(9): p. 3352-70.

12. Callewaert, L., N. Van Tilborgh, and F. Claessens, *Interplay between two hormone-independent activation domains in the androgen receptor.* Cancer Res, 2006. **66**(1): p. 543-53.

13. Slagsvold, T., et al., *Mutational analysis of the androgen receptor AF-2 (activation function 2) core domain reveals functional and mechanistic differences of conserved*

residues compared with other nuclear receptors. Mol Endocrinol, 2000. **14**(10): p. 1603-17.

14. Heemers, H.V. and D.J. Tindall, *Androgen receptor (AR) coregulators: a diversity of functions converging on and regulating the AR transcriptional complex.* Endocr Rev, 2007. **28**(7): p. 778-808.

15. Thakur, M.K. and V. Paramanik, *Role of steroid hormone coregulators in health and disease.* Horm Res, 2009. **71**(4): p. 194-200.

16. Heinlein, C.A. and C. Chang, *Androgen receptor (AR) coregulators: an overview.* Endocr Rev, 2002. **23**(2): p. 175-200.

17. Mangelsdorf, D.J., et al., *The nuclear receptor superfamily: the second decade.* Cell, 1995. **83**(6): p. 835-9.

18. Levin, E.R. and S.R. Hammes, *Nuclear receptors outside the nucleus: extranuclear signalling by steroid receptors.* Nat Rev Mol Cell Biol, 2016. **17**(12): p. 783-797.

19. Evaul, K., et al., *Testosterone and progesterone rapidly attenuate plasma membrane Gbetagamma-mediated signaling in Xenopus laevis oocytes by signaling through classical steroid receptors.* Mol Endocrinol, 2007. **21**(1): p. 186-96.

20. Lutz, L.B., et al., *Evidence that androgens are the primary steroids produced by Xenopus laevis ovaries and may signal through the classical androgen receptor to promote oocyte maturation.* Proc Natl Acad Sci U S A, 2001. **98**(24): p. 13728-33.

21. Lutz, L.B., et al., *G protein beta gamma subunits inhibit nongenomic progesterone-induced signaling and maturation in Xenopus laevis oocytes. Evidence for a release of inhibition mechanism for cell cycle progression.* J Biol Chem, 2000. **275**(52): p. 41512-20.

22. Ma, X., et al., *Androgens Regulate Ovarian Gene Expression Through Modulation of Ezh2 Expression and Activity.* Endocrinology, 2017. **158**(9): p. 2944-2954.

23. Liang, T. and S. Liao, *A very rapid effect of androgen on initiation of protein synthesis in prostate.* Proc Natl Acad Sci U S A, 1975. **72**(2): p. 706-9.

24. Liang, T. and S. Liao, *Dihydrotestosterone and the initiation of protein synthesis by prostate ribosomes.* J Steroid Biochem, 1975. **6**(5): p. 549-50.

25. Rossetti, M.L., J.L. Steiner, and B.S. Gordon, *Androgen-mediated regulation of skeletal muscle protein balance.* Mol Cell Endocrinol, 2017. **447**: p. 35-44.

26. Walker, W.H., *Testosterone signaling and the regulation of spermatogenesis.* Spermatogenesis, 2011. **1**(2): p. 116-120.

27. Lonergan, P.E. and D.J. Tindall, *Androgen receptor signaling in prostate cancer development and progression.* J Carcinog, 2011. **10**: p. 20.

28. Sen, A., et al., *Paxillin mediates extranuclear and intranuclear signaling in prostate cancer proliferation.* J Clin Invest, 2012. **122**(7): p. 2469-81.

29. Sen, A., et al., *Paxillin regulates androgen- and epidermal growth factor-induced MAPK signaling and cell proliferation in prostate cancer cells.* J Biol Chem, 2010. **285**(37): p. 28787-95.

30. Sen, A., H. Prizant, and S.R. Hammes, *Understanding extranuclear (nongenomic) androgen signaling: what a frog oocyte can tell us about human biology.* Steroids, 2011. **76**(9): p. 822-8.

31. Vanderschueren, D., et al., *Androgens and bone.* Endocr Rev, 2004. **25**(3): p. 389-425.

32. Astapova, O., B.M.N. Minor, and S.R. Hammes, *Physiological and Pathological Androgen Actions in the Ovary.* Endocrinology, 2019. **160**(5): p. 1166-1174.

33. Prizant, H., N. Gleicher, and A. Sen, *Androgen actions in the ovary: balance is key.* J Endocrinol, 2014. **222**(3): p. R141-51.

34. Walters, K.A. and D.J. Handelsman, *Role of androgens in the ovary.* Mol Cell Endocrinol, 2018. **465**: p. 36-47.

35. Chadha, S., et al., *Androgen receptor expression in human ovarian and uterine tissue of long-term androgen-treated transsexual women.* Hum Pathol, 1994. **25**(11): p. 1198-204.

36. Suzuki, T., et al., *Immunohistochemical distribution of progesterone, androgen and oestrogen receptors in the human ovary during the menstrual cycle: relationship to expression of steroidogenic enzymes.* Hum Reprod, 1994. **9**(9): p. 1589-95.

37. Hillier, S.G., M. Tetsuka, and H.M. Fraser, *Location and developmental regulation of androgen receptor in primate ovary.* Hum Reprod, 1997. **12**(1): p. 107-11.

38. Weil, S.J., et al., *Androgen receptor gene expression in the primate ovary: cellular localization, regulation, and functional correlations.* J Clin Endocrinol Metab, 1998. **83**(7): p. 2479-85.

39. Duffy, D.M., et al., *Androgen receptor mRNA expression in the rhesus monkey ovary.* Endocrine, 1999. **11**(1): p. 23-30.

40. Slomczynska, M. and Z. Tabarowski, *Localization of androgen receptor and cytochrome P450 aromatase in the follicle and corpus luteum of the porcine ovary.* Anim Reprod Sci, 2001. **65**(1-2): p. 127-34.

41. Cardenas, H. and W.F. Pope, *Androgen receptor and follicle-stimulating hormone receptor in the pig ovary during the follicular phase of the estrous cycle.* Mol Reprod Dev, 2002. **62**(1): p. 92-8.

42. Juengel, J.L., et al., *Oestrogen receptor alpha and beta, androgen receptor and progesterone receptor mRNA and protein localisation within the developing ovary and in small growing follicles of sheep.* Reproduction, 2006. **131**(1): p. 81-92.

43. Tetsuka, M. and S.G. Hillier, *Androgen receptor gene expression in rat granulosa cells: the role of follicle-stimulating hormone and steroid hormones.* Endocrinology, 1996. **137**(10): p. 4392-7.

44. Szoltys, M. and M. Slomczynska, *Changes in distribution of androgen receptor during maturation of rat ovarian follicles.* Exp Clin Endocrinol Diabetes, 2000. **108**(3): p. 228-34.

45. Gill, A., M. Jamnongjit, and S.R. Hammes, *Androgens promote maturation and signaling in mouse oocytes independent of transcription: a release of inhibition model for mammalian oocyte meiosis.* Mol Endocrinol, 2004. **18**(1): p. 97-104.

46. Mori, T., et al., *Evidence for androgen participation in induced ovulation in immature rats.* Endocrinology, 1977. **101**(2): p. 623-6.

47. Ware, V.C., *The role of androgens in follicular development in the ovary. I. A quantitative analysis of oocyte ovulation.* J Exp Zool, 1982. **222**(2): p. 155-67.

48. Wang, H., et al., *Effect of adrenal and ovarian androgens on type 4 follicles unresponsive to FSH in immature mice.* Endocrinology, 2001. **142**(11): p. 4930-6.

49. Murray, A.A., et al., *Effect of androgens on the development of mouse follicles growing in vitro.* J Reprod Fertil, 1998. **113**(1): p. 27-33.

50. Cardenas, H. and W.F. Pope, *Administration of testosterone during the follicular phase increased the number of corpora lutea in gilts.* J Anim Sci, 1994. **72**(11): p. 2930-5.

51. Smith, P., et al., *Developmental programming: differential effects of prenatal testosterone and dihydrotestosterone on follicular recruitment, depletion of follicular reserve, and ovarian morphology in sheep.* Biol Reprod, 2009. **80**(4): p. 726-36.

52. Hampton, J.H., et al., *Androgen receptor mRNA expression in the bovine ovary.* Domest Anim Endocrinol, 2004. **27**(1): p. 81-8.

53. Yang, M.Y. and J.E. Fortune, *Testosterone stimulates the primary to secondary follicle transition in bovine follicles in vitro.* Biol Reprod, 2006. **75**(6): p. 924-32.

54. Abbott, D.H., et al., *Insights into the development of polycystic ovary syndrome (PCOS) from studies of prenatally androgenized female rhesus monkeys.* Trends Endocrinol Metab, 1998. **9**(2): p. 62-7.

55. Vendola, K.A., et al., *Androgens stimulate early stages of follicular growth in the primate ovary.* J Clin Invest, 1998. **101**(12): p. 2622-9.

56. Lyon, M.F. and P.H. Glenister, *Reduced reproductive performance in androgen-resistant Tfm/Tfm female mice.* Proc R Soc Lond B Biol Sci, 1980. **208**(1170): p. 1-12.

57. Shiina, H., et al., *Premature ovarian failure in androgen receptor-deficient mice.* Proc Natl Acad Sci U S A, 2006. **103**(1): p. 224-9.

58. Hu, Y.C., et al., *Subfertility and defective folliculogenesis in female mice lacking androgen receptor.* Proc Natl Acad Sci U S A, 2004. **101**(31): p. 11209-14.

59. Walters, K.A., et al., *Female mice haploinsufficient for an inactivated androgen receptor (AR) exhibit age-dependent defects that resemble the AR null phenotype of dysfunctional late follicle development, ovulation, and fertility.* Endocrinology, 2007. **148**(8): p. 3674-84.

60. Sen, A. and S.R. Hammes, *Granulosa cell-specific androgen receptors are critical regulators of ovarian development and function.* Mol Endocrinol, 2010. **24**(7): p. 1393-403.

61. Walters, K.A., et al., *Targeted loss of androgen receptor signaling in murine granulosa cells of preantral and antral follicles causes female subfertility.* Biol Reprod, 2012. **87**(6): p. 151.

62. Ma, Y., et al., *Androgen Receptor in the Ovary Theca Cells Plays a Critical Role in Androgen-Induced Reproductive Dysfunction.* Endocrinology, 2017. **158**(1): p. 98-108.

63. Sen, A., et al., *Androgens regulate ovarian follicular development by increasing follicle stimulating hormone receptor and microRNA-125b expression.* Proc Natl Acad Sci U S A, 2014. **111**(8): p. 3008-13.

64. Hasegawa, T., et al., *A regulatory role of androgen in ovarian steroidogenesis by rat granulosa cells.* J Steroid Biochem Mol Biol, 2017. **172**: p. 160-165.

65. Wu, S., et al., *Conditional knockout of the androgen receptor in gonadotropes reveals crucial roles for androgen in gonadotropin synthesis and surge in female mice.* Mol Endocrinol, 2014. **28**(10): p. 1670-81.

66. Walters, K.A., et al., *The Role of Central Androgen Receptor Actions in Regulating the Hypothalamic-Pituitary-Ovarian Axis.* Neuroendocrinology, 2018. **106**(4): p. 389-400.

67. Dunaif, A. and D.T. Finegood, *Beta-cell dysfunction independent of obesity and glucose intolerance in the polycystic ovary syndrome.* J Clin Endocrinol Metab, 1996. **81**(3): p. 942-7.

68. Goodarzi, M.O., et al., *beta-Cell function: a key pathological determinant in polycystic ovary syndrome.* J Clin Endocrinol Metab, 2005. **90**(1): p. 310-5.

69. Gonzalez, F., et al., *Increased activation of nuclear factor kappaB triggers inflammation and insulin resistance in polycystic ovary syndrome.* J Clin Endocrinol Metab, 2006. **91**(4): p. 1508-12.

70. Liu, S., G. Navarro, and F. Mauvais-Jarvis, *Androgen excess produces systemic oxidative stress and predisposes to beta-cell failure in female mice.* PLoS One, 2010. **5**(6): p. e11302.

71. Navarro, G., et al., *Androgen excess in pancreatic beta cells and neurons predisposes female mice to type 2 diabetes.* JCI Insight, 2018. **3**(12).

72. Feldman, S.R., *Androgen insensitivity syndrome (testicular feminization): a model for understanding steroid hormone receptors.* J Am Acad Dermatol, 1992. **27**(4): p. 615-9.

73. Fujita, K. and N. Nonomura, *Role of Androgen Receptor in Prostate Cancer: A Review.* World J Mens Health, 2019. **37**(3): p. 288-295.

74. Azziz, R., *Androgen excess is the key element in polycystic ovary syndrome.* Fertil Steril, 2003. **80**(2): p. 252-4.

75. Gleicher, N., et al., *Hypoandrogenism in association with diminished functional ovarian reserve.* Hum Reprod, 2013. **28**(4): p. 1084-91.

76. Gleicher, N., A. Weghofer, and D.H. Barad, *The role of androgens in follicle maturation and ovulation induction: friend or foe of infertility treatment?* Reprod Biol Endocrinol, 2011. **9**: p. 116.

77. Vassilatou, E., *Nonalcoholic fatty liver disease and polycystic ovary syndrome.* World J Gastroenterol, 2014. **20**(26): p. 8351-63.

78. Sirmans, S.M., et al., *Epidemiology and comorbidities of polycystic ovary syndrome in an indigent population.* J Investig Med, 2014. **62**(6): p. 868-74.

79. Gilbert, E.W., et al., *Comorbidities and complications of polycystic ovary syndrome: An overview of systematic reviews.* Clin Endocrinol (Oxf), 2018. **89**(6): p. 683-699.

80. Cai, J., et al., *High-free androgen index is associated with increased risk of non-alcoholic fatty liver disease in women with polycystic ovary syndrome, independent of obesity and insulin resistance.* Int J Obes (Lond), 2017. **41**(9): p. 1341-1347.

81. Rocha, A.L.L., et al., *Non-alcoholic fatty liver disease in women with polycystic ovary syndrome: systematic review and meta-analysis.* J Endocrinol Invest, 2017. **40**(12): p. 1279-1288.

82. Kim, J.J., et al., *Polycystic ovary syndrome with hyperandrogenism as a risk factor for non-obese non-alcoholic fatty liver disease.* Aliment Pharmacol Ther, 2017. **45**(11): p. 1403-1412.

83. Jones, H., et al., *Polycystic ovary syndrome with hyperandrogenism is characterized by an increased risk of hepatic steatosis compared to nonhyperandrogenic PCOS phenotypes and healthy controls, independent of obesity and insulin resistance.* J Clin Endocrinol Metab, 2012. **97**(10): p. 3709-16.

84. Padmanabhan, V., R.C. Cardoso, and M. Puttabyatappa, *Developmental Programming, a Pathway to Disease.* Endocrinology, 2016. **157**(4): p. 1328-40.

85. Younossi, Z.M., et al., *Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes.* Hepatology, 2016. **64**(1): p. 73-84.

86. Clark, J.M., *The epidemiology of nonalcoholic fatty liver disease in adults.* J Clin Gastroenterol, 2006. **40 Suppl 1**: p. S5-10.

87. Estes, C., et al., *Modeling the epidemic of nonalcoholic fatty liver disease demonstrates an exponential increase in burden of disease.* Hepatology, 2018. **67**(1): p. 123-133.

88. Matteoni, C.A., et al., *Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity.* Gastroenterology, 1999. **116**(6): p. 1413-9.

89. Friedman, S.L., et al., *Mechanisms of NAFLD development and therapeutic strategies.* Nat Med, 2018. **24**(7): p. 908-922.

90. Bugianesi, E., et al., *Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma.* Gastroenterology, 2002. **123**(1): p. 134-40.

91. van Houten, E.L., et al., *Reproductive and metabolic phenotype of a mouse model of PCOS.* Endocrinology, 2012. **153**(6): p. 2861-9.

92. Andrisse, S., et al., *Low-Dose Dihydrotestosterone Drives Metabolic Dysfunction via Cytosolic and Nuclear Hepatic Androgen Receptor Mechanisms.* Endocrinology, 2017. **158**(3): p. 531-544.

93. Karoli, R., et al., *Prevalence of hepatic steatosis in women with polycystic ovary syndrome.* J Hum Reprod Sci, 2013. **6**(1): p. 9-14.

94. Kumarendran, B., et al., *Polycystic ovary syndrome, androgen excess, and the risk of nonalcoholic fatty liver disease in women: A longitudinal study based on a United Kingdom primary care database.* PLoS Med, 2018. **15**(3): p. e1002542.

95. Gambarin-Gelwan, M., et al., *Prevalence of nonalcoholic fatty liver disease in women with polycystic ovary syndrome.* Clin Gastroenterol Hepatol, 2007. **5**(4): p. 496-501.

96. Vassilatou, E., et al., *Increased androgen bioavailability is associated with non-alcoholic fatty liver disease in women with polycystic ovary syndrome.* Hum Reprod, 2010. **25**(1): p. 212-20.

97. Caldwell, A.S., et al., *Characterization of reproductive, metabolic, and endocrine features of polycystic ovary syndrome in female hyperandrogenic mouse models.* Endocrinology, 2014. **155**(8): p. 3146-59.

98. De Gendt, K., et al., *A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis.* Proc Natl Acad Sci U S A, 2004. **101**(5): p. 1327-32.

99. Peters, D.T., et al., *Asialoglycoprotein receptor 1 is a specific cell-surface marker for isolating hepatocytes derived from human pluripotent stem cells.* Development, 2016. **143**(9): p. 1475-81.

100. Ma, X., et al., *Leptin-Induced CART (Cocaine- and Amphetamine-Regulated Transcript) Is a Novel Intraovarian Mediator of Obesity-Related Infertility in Females.* Endocrinology, 2016. **157**(3): p. 1248-57.

101. Roy, S., et al., *Oocyte-Derived Factors (GDF9 and BMP15) and FSH Regulate AMH Expression Via Modulation of H3K27AC in Granulosa Cells.* Endocrinology, 2018. **159**(9): p. 3433-3445.

102. Lin, H.Y., et al., *Increased hepatic steatosis and insulin resistance in mice lacking hepatic androgen receptor.* Hepatology, 2008. **47**(6): p. 1924-35.

103. Tahara, Y. and S. Shibata, *Circadian rhythms of liver physiology and disease: experimental and clinical evidence.* Nat Rev Gastroenterol Hepatol, 2016. **13**(4): p. 217-26.

104. Shi, D., et al., *Circadian Clock Genes in the Metabolism of Non-alcoholic Fatty Liver Disease.* Front Physiol, 2019. **10**: p. 423.

105. Tumber, A., et al., *Potent and Selective KDM5 Inhibitor Stops Cellular Demethylation of H3K4me3 at Transcription Start Sites and Proliferation of MM1S Myeloma Cells.* Cell Chem Biol, 2017. **24**(3): p. 371-380.

106. Badeaux, A.I. and Y. Shi, *Emerging roles for chromatin as a signal integration and storage platform.* Nat Rev Mol Cell Biol, 2013. **14**(4): p. 211-24.

107. Cha, T.L., et al., *Akt-mediated phosphorylation of EZH2 suppresses methylation of lysine 27 in histone H3.* Science, 2005. **310**(5746): p. 306-10.

108. Wei, Y., et al., *CDK1-dependent phosphorylation of EZH2 suppresses methylation of H3K27 and promotes osteogenic differentiation of human mesenchymal stem cells.* Nat Cell Biol, 2011. **13**(1): p. 87-94.

109. Yan, J., et al., *EZH2 phosphorylation by JAK3 mediates a switch to noncanonical function in natural killer/T-cell lymphoma.* Blood, 2016. **128**(7): p. 948-58.

110. Xiang, Y., et al., *JMJD3 is a histone H3K27 demethylase.* Cell Res, 2007. **17**(10): p. 850-7.

111. Schiffer, L., et al., *MECHANISMS IN ENDOCRINOLOGY: The sexually dimorphic role of androgens in human metabolic disease.* Eur J Endocrinol, 2017. **177**(3): p. R125-R143.

112. Mangaraj, M., R. Nanda, and S. Panda, *Apolipoprotein A-I: A Molecule of Diverse Function.* Indian J Clin Biochem, 2016. **31**(3): p. 253-9.

113. Marques, P.I., et al., *SERPINA2 is a novel gene with a divergent function from SERPINA1.* PLoS One, 2013. **8**(6): p. e66889.

114. Ponka, P., C. Beaumont, and D.R. Richardson, *Function and regulation of transferrin and ferritin.* Semin Hematol, 1998. **35**(1): p. 35-54.

115. Demissie, M., et al., *Transient prenatal androgen exposure produces metabolic syndrome in adult female rats.* Am J Physiol Endocrinol Metab, 2008. **295**(2): p. E262-8.

116. Hogg, K., et al., *The in utero programming effect of increased maternal androgens and a direct fetal intervention on liver and metabolic function in adult sheep.* PLoS One, 2011. **6**(9): p. e24877.

117. Mody, A., et al., *Relevance of low testosterone to non-alcoholic fatty liver disease.* Cardiovasc Endocrinol, 2015. **4**(3): p. 83-89.

118. Pan, X., C.A. Bradfield, and M.M. Hussain, *Global and hepatocyte-specific ablation of Bmal1 induces hyperlipidaemia and enhances atherosclerosis.* Nat Commun, 2016. **7**: p. 13011.

119. Vella, S., et al., *EZH2 down-regulation exacerbates lipid accumulation and inflammation in in vitro and in vivo NAFLD.* Int J Mol Sci, 2013. **14**(12): p. 24154-68.

120. Etchegaray, J.P., et al., *The polycomb group protein EZH2 is required for mammalian circadian clock function.* J Biol Chem, 2006. **281**(30): p. 21209-15.

121. Zhong, Y., et al., *Ezh2 promotes clock function and hematopoiesis independent of histone methyltransferase activity in zebrafish.* Nucleic Acids Res, 2018. **46**(7): p. 3382-3399.

122. Diamanti-Kandarakis, E., et al., *The effect of a pure antiandrogen receptor blocker, flutamide, on the lipid profile in the polycystic ovary syndrome.* J Clin Endocrinol Metab, 1998. **83**(8): p. 2699-705.

123. Gambineri, A., et al., *Treatment with flutamide, metformin, and their combination added to a hypocaloric diet in overweight-obese women with polycystic ovary syndrome: a randomized, 12-month, placebo-controlled study.* J Clin Endocrinol Metab, 2006. **91**(10): p. 3970-80.

124. Leung, J.K. and M.D. Sadar, *Non-Genomic Actions of the Androgen Receptor in Prostate Cancer.* Front Endocrinol (Lausanne), 2017. **8**: p. 2.

125. Giovannelli, P., et al., *Androgens Induce Invasiveness of Triple Negative Breast Cancer Cells Through AR/Src/PI3-K Complex Assembly.* Sci Rep, 2019. **9**(1): p. 4490.

126. Pedram, A., et al., *A conserved mechanism for steroid receptor translocation to the plasma membrane.* J Biol Chem, 2007. **282**(31): p. 22278-88.

127. BASU, J., *Protein palmitoylation and dynamic modulation of protein function.* Current science 2004. **87**(2): p. 212-217.

128. Razandi, M., et al., *ERs associate with and regulate the production of caveolin: implications for signaling and cellular actions.* Mol Endocrinol, 2002. **16**(1): p. 100-15.

129. Castoria, G., et al., *Targeting androgen receptor/Src complex impairs the aggressive phenotype of human fibrosarcoma cells.* PLoS One, 2013. **8**(10): p. e76899.

130. Migliaccio, A., et al., *Steroid-induced androgen receptor-oestradiol receptor beta-Src complex triggers prostate cancer cell proliferation.* EMBO J, 2000. **19**(20): p. 5406-17.

131. Migliaccio, A., et al., *Inhibition of the SH3 domain-mediated binding of Src to the androgen receptor and its effect on tumor growth.* Oncogene, 2007. **26**(46): p. 6619-29.