REVIEW

Androgen-induced epigenetic modulations in the ovary

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Abstract

In recent years, androgens have emerged as critical regulators of female reproduction and women's health in general. While high levels of androgens in women are associated with polycystic ovary syndrome (PCOS), recent evidence suggests that a certain amount of direct androgen action through androgen receptor is also essential for normal ovarian function. Moreover, prenatal androgen exposure has been reported to cause developmental reprogramming of the fetus that manifests into adult pathologies, supporting the Developmental Origins of Health and Disease (DOHaD) hypothesis. Therefore, it has become imperative to understand the underlying mechanism of androgen actions and its downstream effects under normal and pathophysiological conditions. Over the years, there has been a lot of studies on androgen receptor function as a transcriptional regulator in the nucleus as well as androgen-induced rapid extra-nuclear signaling. Conversely, new evidence suggests that androgen actions may also be mediated through epigenetic modulation involving both the nuclear and extra-nuclear androgen signaling. This review focuses on androgen-induced epigenetic modifications in female reproduction, specifically in the ovary, and discusses emerging concepts, latest perceptions, and highlight the areas that need further investigation.

Key Words

- ▶ androgens
- ▶ epigenetics
- ▶ female reproduction
- ▶ developmental programming
- ▶ histone modification
- ▶ DNA methylation

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Introduction

Steroid hormones participate in almost every biological process operative in the mammalian system. However, the basic mechanisms involved in steroid hormone action are highly complicated and remain frustratingly unclear. To date, the physiological actions of steroids were thought to be mediated by the classical 'nuclear' and non-classical 'membrane-initiated' steroid receptor signaling (Hammes & Levin 2011, Levin & Hammes 2016). It is now well-established that this crosstalk between steroid-induced extra-nuclear kinase signaling and intra-nuclear transcription is critical for many biological processes

(DeFranco 2002, Edwards 2005, Levin 2015, Levin & Hammes 2016, Klinge 2018, Wilkenfeld *et al.* 2018). Interestingly, emerging evidence suggests that steroid hormones may also influence or alter gene expression through epigenetic modifications (Matsumoto *et al.* 2013). Control of gene expression occurs at different levels, one of which is the accessibility of genes and their controlling elements to the transcription machinery. Accessibility is regulated largely by the degree of chromatin compaction, which is modulated by several epigenetic markers (Kim & Kaang 2017). This represents

an independent avenue for steroids to coordinate gene regulation apart from the established pathways. Steroid receptors recruit several epigenetic modulators as an integral part of its transcription machinery and this has been extensively studied across species and cells/tissues (Heemers & Tindall 2007, Thakur & Paramanik 2009). However, the underlying mechanism(s) of how steroid-induced signaling causes epigenetic modifications and regulate downstream physiological functions are poorly understood. This review specifically focuses on androgen-induced epigenetic modifications with respect to ovarian function and overall female health. Here we discuss emerging concepts, latest perceptions, and highlight the areas that need further investigation.

Moreover, steroid-mediated events occurring during critical fetal developmental phases can alter the anatomy and physiology of steroid-targeted developing organs and systems (Solano & Arck 2019). It is now widely accepted that an adverse peri-conceptional and/or intrauterine environment is associated with fetal epigenetic malprogramming causing a predisposition to chronic disorders later in life (Puttabyatappa & Padmanabhan 2018). Epigenetics is thought to be by far the most likely mechanism by which intrauterine or postnatal steroid exposure affects the health and disease of the offspring. Therefore, we also discuss how prenatal and postnatal exposure to androgen results in epigenetic modulation and its manifestation of adult pathologies later in life.

Androgen actions and female fertility

In the last few years, there has been much focus on understanding androgen actions in women and androgens have emerged as a critical regulator of follicular development and female fertility (Prizant *et al.* 2014, Walters 2015) in both normal and pathophysiological conditions. In women, androgens are produced in the ovaries (Franks & Hardy 2018), adrenal glands (Burger 2002), and fat cells (Cadagan *et al.* 2014) which exhibit its actions directly through androgen receptors (AR). In female reproduction, high levels of androgens have been considered detrimental to women's health leading to polycystic ovary syndrome (PCOS), hirsutism, and metabolic dysfunctions such as abdominal visceral adiposity, insulin resistance, and metabolic disturbances (London 1987, Escobar-Morreale *et al.* 2014). In contrast, global (Hu *et al.* 2004, Shiina *et al.* 2006, Walters *et al.* 2007) and granulosa cell-specific (Sen & Hammes 2010, Walters *et al.* 2012) AR knockout female mouse models

as well as clinical studies (Barad & Gleicher 2005, Barad & Gleicher 2006, Gleicher *et al.* 2013a,b) have established that direct androgen actions through AR in the ovary are critical for female fertility (Sen & Hammes 2010, Sen *et al.* 2014). Disruption of androgen signaling negatively impacts female fertility by being associated with follicular dysfunction, premature ovarian failure (Hu *et al.* 2004, Shiina *et al.* 2006, Sen & Hammes 2010, Wu *et al.* 2014, Ma *et al.* 2017b, Walters *et al.* 2018), and diminished ovarian reserve (Gleicher *et al.* 2013a). It is now believed that there exists a balance between the essentiality of androgen actions and its detrimental effects associated with high androgen levels. Understanding the mechanisms of androgen actions in regulating female reproduction has thus been the primary focus in the last couple of years (Prizant *et al.* 2014, Walters 2015, Walters *et al.* 2016, 2019, Franks & Hardy 2018, Walters & Handelsman 2018).

Moreover, in the last decade, several studies across species have shown that prenatal or perinatal androgen exposure has profound effects throughout an individual's lifespan (Abbott *et al.* 2002, Poston 2010, Padmanabhan *et al.* 2016). This concept of the fetal origin of adult diseases is now an area of intense research and has evolved into the field of Developmental Origins of Health and Disease (Barker 2007). Epigenetic programming appears to play a central role in prenatal or postnatal exposure to different steroid hormones and is one of the most common conditions for the 'fetal origins of adult disease'. It has been hypothesized that changes in the histone/DNA methylation/acetylation during critical periods of gestation is one of the mechanisms through which prenatal and postnatal exposure to androgens cause developmental programming (Padmanabhan *et al.* 2015). It is well-established across species that the neuroendocrine-reproductive axis, as well as metabolism, is significantly impacted by steroidal signaling during prenatal and postnatal periods (Gore 2008, Padmanabhan & Veiga-Lopez 2013, Zambrano *et al.* 2014). Excess androgens disrupt gonadotropin-releasing hormone secretion, facilitating loss of the positive feedback response to estrogen by epigenetically reprogramming the refractoriness of the brain (Foecking *et al.* 2008). The epigenetic modulation by androgens on ovarian function can either be due to postnatal or prenatal exposure (Abbott *et al.* 2002, Nelson-DeGrave *et al.* 2004, Zhang *et al.* 2014, Walters 2015, Guo *et al.* 2019, Sinha *et al.* 2020). Misinterpretation of the *de novo* DNA methylation and active histone replacement during the oogenesis-specific developmental program has been reported in PCOS patients (Xu *et al.* 2010, Yu *et al.* 2017).

Children born to mothers with PCOS or congenital adrenal hyperplasia, diseases characterized by excess androgens, are more susceptible to developing PCOS-like phenotypes, increased risk of metabolic dysfunctions, cardiovascular, and neoplastic diseases later in life (Abbott *et al.* 2002, 2005, 2008a,b, Padmanabhan *et al.* 2016). Given the importance of prenatal or perinatal androgen exposure in the fetal origin of adult diseases, here we discuss studies focused on determining the developmentally reprogrammed genes in the ovary and its downstream physiological effects caused by prenatal or perinatal androgen exposure.

Androgen-induced epigenetic modulation in the ovary

Androgen receptors transcriptionally regulate the expression of many genes either directly (Eder *et al.* 2001, Heemers & Tindall 2007) through binding to ARE sites (androgen response element) on the promoter regions of genes or indirectly (Heinlein & Chang 2002, Kang *et al.* 2004, Foradori *et al.* 2008) by modulating other transcriptional regulators via activation of different signaling pathways. Intriguingly, while androgens have been shown to play a major role in the hypothalamus–pituitary–gonadal axis in females (Sen & Hammes 2010, Walters *et al.* 2018), very few genes, so far, have been identified as direct AR targets. In the ovary, studies show that androgens may have a more global effect on gene expression which is primarily mediated through indirect rather than direct androgen actions (Ma *et al.* 2017a). Remodeling of the chromatin into an accessible conformation allowing recruitment of transcriptional elements is a key regulatory step of gene expression. Chromatin remodeling via histone modification is now considered a critical control step for transcription and a primary target of signal transduction. Post-translational modification (Badeaux & Shi 2013) through kinase-dependent phosphorylation (Cha *et al.* 2005, Wei *et al.* 2011, Yan *et al.* 2016) is one of the major mechanisms through which the activity of epigenetic enzymes can be regulated. Apart from histone modifications, androgen may regulate gene transcription through DNA methylation by interfering with the ability of transcriptional regulators to bind to the DNA. Conversely, the underlying mechanisms of how androgens regulate DNA methylation or histone modifications in the ovary and its effect on ovarian gene expression and downstream physiological processes, are poorly understood.

Histone modifications

Epigenetic modulation of gene expression is dependent partly on the dynamic balance of histone marks (methyl/demethyl and/or acetyl/deacetyl marks) on the enhancers and promoters. Enhancers are non-coding regions that are widely spread across the genome and have binding sites for transcription factors which in turn recruit co-activators. These enhancers are located either proximally or distally from the promoter region of genes in the genome and activate transcription by interacting with the promoter either by direct interaction between the enhancer-associated proteins and the promoter through looping of the DNA (Fig. 1A) or via tracking factors (like Pol II) down the DNA to connect to the promoter (Bulger & Groudine 2011). Various histone marks in the enhancer regions determine if an enhancer region is active, primed, or poised. The dynamics between histone marks in the enhancer and/or promoter regions is mediated by regulating the expression and activity of various epigenetic enzymes (methylases/demethylases and/or acetylases/deacetylases) (Blum 2015, Hyun *et al.* 2017). Androgens through both the extra-nuclear and nuclear pathways can repress/activate the expression and activity of epigenetic enzymes resulting in an increase or decrease of the histone marks affecting the expression of

Figure 1

(A) Androgen-induced histone modifications occur in the distal enhancer and/or promoter regions of genes that in turn regulate gene expression (Stelloo *et al.* 2019). (B) Mechanism of androgen-induced histone modifications: androgens through activation PI3K/Akt pathway and induction of *miR-101* expression inhibit the enzymatic activity and expression of Ezh2, a histone methyltransferase responsible for trimethylation of lysine 27 on histone 3 (H3K27me3), a gene repressive mark. (C) Androgen receptors also recruit LSD1, a lysine-specific histone demethylase 1A that demethylates mono- or di-methyl lysine 4 and 9 on histone 3 (H3K4/9me1/2).

different genes that are crucial for ovarian function (Ma *et al.* 2017a) (discussed in detail in the section 'Postnatal/ in vitro androgen exposure'). However, this effect may vary based on the timing of androgen exposure. For example, prenatal androgen exposure may regulate a specific set of epigenetic modulators controlling precise epigenetic marks on the histone and/or DNA that affect the expression of a certain set of genes. In contrast, postnatal androgens may regulate a different set of genes by modulating other epigenetic marks. Also, the androgen-induced epigenetic modulation may be cell-specific within the ovary (Guo *et al.* 2019).

Prenatal androgen exposure

It has long been known (Abbott *et al.* 2006, Nugent *et al.* 2012, Padmanabhan & Veiga-Lopez 2013, Walters 2015, Padmanabhan *et al.* 2016) that prenatal androgen exposure has a significant effect on ovarian function. However, only recently two studies (Guo *et al.* 2019, Sinha *et al.* 2020) investigated the histone modifications induced by prenatal androgen exposure in the androgenized sheep model. In one of the studies (Sinha *et al.* 2020), changes in different histone marks were determined in prenatally (from days 30 to 90 of gestation)-testosterone (T)-treated fetal (day 90) and adult (year 2) whole sheep ovaries. This study reported no epigenetic changes in D90 fetal ovaries, but prenatal testosterone treatment causes a significant increase in both gene repressive (H3K9me3-trimethylation of lysine 9 on histone 3) and gene activating (H3K27ac and H3K9ac – acetylation of lysine 27 and lysine 9 on histone 3) marks in adult (year 2) ovaries. This emphasizes that it is not imperative that the effect of prenatal androgen exposure on the epigenome occurs instantly in the fetus but can manifest even later in life. Other epigenetic marks determined in this study with no reported change were the gene repressive mark, H3K27me3 (trimethylation of lysine 9 on histone 3), and the gene activating mark, H3K4me3 (trimethylation of lysine 4 on histone 3). Intriguingly, RNA-sequencing data of ovarian gene expression revealed no change in the expression levels of any histone-modifying enzymes in adult (year 2) ovaries isolated from control and prenatal testosterone-treated sheep (Sinha *et al.* 2020). However, changes in mRNA or protein levels may not be enough to explain the downstream effects of these enzymes as the activity of these enzymes may be regulated at a post-translational level (Table 1).

In a second study (Guo *et al.* 2019), laser capture microscopy was used to compare the expression of various epigenetic enzymes and the level of histone marks

in granulosa, theca and stromal cells isolated from adult (year 2) prenatally androgenized sheep. This study (Guo *et al.* 2019) reported that prenatal testosterone exposure (from days 30 to 90 of gestation) shows a trend or causes a significant increase/decrease in various epigenetic enzymes in a cell-specific manner within the ovary. For example (Guo *et al.* 2019), in granulosa cells, prenatal testosterone exposure increases histone methyltransferases; *SUV39H1* that methylates Lys-9 of histone 3 (H3K9me3 – gene activating mark) and *EZH2* that methylates Lys-27 of histone 3 (H3K27me3 – gene repressive mark); conversely, prenatal testosterone exposure decreases *HDAC3* that deacetylates lysine residues on the N-terminal part of the core histones (H2A, H2B, H3, and H4) resulting in epigenetic repression, and *KDM1A* that demethylates both Lys-4 (H3K4me – gene activating mark) and Lys-9 (H3K9me – gene repressive mark) of histone H3, thereby acting as a coactivator or a corepressor. In theca cells (Guo *et al.* 2019), it has been reported that prenatal testosterone exposure increases *SMYD3* that specifically methylates Lys-4 of histone H3 inducing di- and tri-methylation (H3K4me2/me3 – gene activating mark) while decreasing *HDAC3* (causes epigenetic repression) and *KDM1A* (can act as both coactivator/corepressor). Similarly, in stromal cells (Guo *et al.* 2019), prenatal testosterone exposure causes an increase in *EZH2* (H3K27me3 – gene repressive mark) and a decrease in *KDM1A*. Moreover, in both granulosa and theca cells (Guo *et al.* 2019), there is an increase in *HDAC1* that catalyzes deacetylation on their substrates having lysine residues on the N-terminal part of the core histones (H2A, H2B, H3, and H4). Notably, when the change in expression of the epigenetic enzymes was correlated with epigenetic marks, it was found that there was an increase in H3K9me3 (gene repressive mark) levels in both granulosa and theca cells and an increase in H3K4me2 (gene activating mark) in theca and stromal cells (Table 1). These data (Guo *et al.* 2019) highlight two important insights: first, prenatal androgen exposure has differential effects on the epigenome in a cell-specific manner within the ovary. Second, changes in the expression of epigenetic enzymes do not always correlate with its activity and modulation of downstream epigenetic marks. This emphasizes that future studies are required to focus on correlating the expression and activity of an epigenetic enzyme with its specific downstream epigenetic marks with respect to prenatal testosterone treatment in the ovary. Importantly, further studies in other prenatal androgen exposure models like rodents and non-human primates are required to comprehend the effect of prenatal androgen exposure in the ovary across species.

Table 1 Androgen-induced site-specific histone modifications in the ovary.

Species	Site of impact	Treatment	Epigenetic enzymes		Epigenetic marks	Genes affected	References
			Up	Down			
Human	Theca cells	<i>In vitro</i>	–	–	Histone acetylation	<i>CYP11A</i> and <i>CYP17</i>	Nelson-DeGrave <i>et al.</i> 2004
Sheep	Whole ovaries	Prenatal	–	–	H3K9me3, H3K27me3, H3K9ac, H3K27ac and H3K4me3	<i>SERPINA5</i> , <i>HSD3B1</i> , <i>CTLA4</i> , <i>HSD17B14</i> , <i>SPP1</i> , <i>OXTR</i> , <i>TNK1</i> , <i>LIPG</i> , <i>HEY2</i> , <i>NDRG4</i>	Sinha <i>et al.</i> 2020
Sheep	Stromal cells	Prenatal	EZH2	KDM1A	H3K4me2, H3K9me3	–	Guo <i>et al.</i> 2019
Sheep	Theca cells	Prenatal	HDAC1, SMYD3,	HDAC3, KDM1A	H3K4me2, H3K9me3	<i>CYP17</i> , <i>AR</i> , <i>ESR1</i> , <i>ESRβ2</i> , <i>LHCGR</i> , <i>FSHR</i> , <i>INSR</i> , <i>ADIPOQ</i> , <i>PPARG</i>	Guo <i>et al.</i> 2019
Sheep	Granulosa cells	Prenatal	HDAC1, SUV39H, EZH2	HDAC3, KDM1A	H3K4me2, H3K9me3	<i>CYP19</i> , <i>CYP17</i> , <i>AR</i> , <i>ESR1</i> , <i>ESR2</i> , <i>LHCGR</i> , <i>FSHR</i> , <i>INSR</i> , <i>ADIPOQ</i> , <i>PPARG</i> , <i>INH1A</i> , <i>INH1B</i> , <i>INH1C</i> , <i>INH1D</i> , <i>INH1E</i> , <i>INH1F</i> , <i>INH1G</i> , <i>INH1H</i> , <i>INH1I</i> , <i>INH1J</i> , <i>INH1K</i> , <i>INH1L</i> , <i>INH1M</i> , <i>INH1N</i> , <i>INH1O</i> , <i>INH1P</i> , <i>INH1Q</i> , <i>INH1R</i> , <i>INH1S</i> , <i>INH1T</i> , <i>INH1U</i> , <i>INH1V</i> , <i>INH1W</i> , <i>INH1X</i> , <i>INH1Y</i> , <i>INH1Z</i>	Guo <i>et al.</i> 2019
Mouse	Granulosa cells	<i>In vitro</i>	–	Ezh2	H3K27me3	<i>Runx1</i>	Ma <i>et al.</i> 2017a

Postnatal/in vitro androgen exposure

A recent study (Ma *et al.* 2017a) shows that androgens, in both human and mouse ovarian granulosa cells, through extra-nuclear signals can rapidly activate the PI3K/Akt pathway causing phosphorylation of the Polycomb group protein enhancer of zeste homolog 2 (EZH2), that is known to block the methyltransferase activity of EZH2 (Cha *et al.* 2005, Bredfeldt *et al.* 2010, Rojanasakul 2013). EZH2 is a histone methyltransferase (HMT) that promotes H3K27me3, a gene repressive mark. Furthermore, androgens through nuclear signaling cause the long-term inhibition of *Ezh2* by increasing the expression of an anti-*Ezh2* microRNA, *miR-101* (Ma *et al.* 2017a). Figure 1A demonstrates the proposed mechanism of androgen-induced regulation of EZH2 and H3K27me3 mark. In granulosa cells, this androgen-induced inhibition of EZH2 leading to modulation of H3K27me3 (Ma *et al.* 2017a) has been shown to play a critical role in the expression of luteinizing hormone-induced *Runx1*, a transcription factor critical for ovulation (Jo & Curry 2006). Blocking of androgen-induced inhibition of *Ezh2*, *in vivo*, adversely affects *Runx1* expression and subsequent ovulation in mice (Ma *et al.* 2017a). Based on these observations, it has been proposed that androgens may have a global effect on ovarian gene expression through modulation of EZH2 and H3K27me3 mark (Table 1). Further studies are needed to identify all the genes regulated by the androgen-induced decrease of the H3K27me3 mark and its effect on ovarian physiology. However, given the importance of EZH2 in epigenetic modulation of gene expression, it can be speculated that androgen-induced inhibition of EZH2 expression and activity has far-reaching effects on female reproduction and women's health. Interestingly,

an EZH2 inhibitor, tazemetostat is currently in clinical trials for cancer patients and it has been reported that it can dramatically affect epigenetic programming in the fetal and adult oocyte (Prokopuk *et al.* 2018). Given that androgens inhibit EZH2 function and expression, it is highly possible that androgens may influence stem cell differentiation and other physiological processes through modulation of EZH2. Overall, these studies (Ma *et al.* 2017a, Prokopuk *et al.* 2018) show that EZH2 is a downstream target of androgen actions and androgens, through both extra-nuclear and nuclear signaling can regulate epigenetic modulators such as EZH2 in female reproductive tissues.

Another histone-modifying enzyme that is known (Metzger *et al.* 2005) to be involved in androgen-induced gene expression is lysine-specific histone demethylase 1A (LSD1, also referred to as KDM1A), a histone demethylase. LSD1 is an important component of the androgen receptor transcription complex. LSD1 interacts with androgen receptor and demethylates monomethylated and dimethylated H3K4/9 repressive marks which in turn stimulates AR gene transcription. While the role of LSD1 with respect to androgen actions in the ovary and/or female fertility has not yet been studied, LSD1 has been reported to be a potential mediator of ovarian cancer (Chen *et al.* 2015).

In humans, there have been very limited studies involving androgen-induced histone modifications. Nelson-DeGrave *et al.* (2004) showed an increase in androgen production in valproate (a drug used in patients with epilepsy) treated cultured human theca cells. Valproate-induced ovarian androgen biosynthesis is caused by histone acetylation of *CYP11A* and *CYP17*

that increases the transcription of these steroidogenic genes (Table 1). Further studies are needed not only to understand postnatal androgen actions in the ovary or its role in women's health in general but also to identify/study the other epigenetic histone modulators regulated by androgens and to elucidate the underlying mechanism of their regulation.

DNA modifications

In the DNA, there are a number of sites that contain cytosine nucleotides followed by guanine nucleotides arranged in a linear sequence along the 5'–3' direction called the CpG sites. The cytosines in the CpG dinucleotides can be methylated/demethylated by enzymes called methyltransferases/demethylases. Methylation of CpG sites (hyper-methylation) within a gene causes gene silencing while demethylation or a lack of methyl mark (hypo-methylation) on these sites influences gene activation (Costello & Plass 2001) (Fig. 2).

PCOS patients

A large number of studies have used DNA methylome profiling to assess if epigenetic dysregulation of critical ovarian genes may contribute to ovarian defects in PCOS (Wang *et al.* 2014, Sagvekar *et al.* 2017, Desmawati *et al.* 2018). Given that hyper-androgenism is the primary feature of PCOS (Pasquali *et al.* 2016), it is now being

hypothesized that elevated androgen levels may directly affect the methylation pattern of many of these genes. However, the underlying mechanism(s) by which androgens may regulate CpG methylation pattern of ovarian genes under PCOS condition remains to be elucidated. Interestingly, studies in prostate cancer and in the testis, show that androgens through AR can regulate the expression of TET (ten-eleven translocation) proteins that catalyzes the hydroxylation of DNA 5-methylcytosine, 5mC to 5-hydroxymethylcytosine, 5hmC (Takayama *et al.* 2015) as well as the expression and activity of DNMTs (DNA methyltransferase) (Anway & Skinner 2008, Anway *et al.* 2008, Cowin *et al.* 2010, Chu *et al.* 2014). Notably, *in silico* analysis of the 5'-promoter regions of the human DNMT1 and DNMT3A gene shows the presence of androgen response elements (AREs), thereby suggesting that the expression of these DNA methyltransferases can be directly regulated by androgens (unpublished data from authors laboratory). Furthermore, in normal prostate cells androgens through AR-mediate activity dynamically regulate the DNA methylation patterns of many of its target genes (Dhiman *et al.* 2015). The understanding of androgen-induced regulation of DNA methylation pattern in PCOS condition is still in the early stages and whether androgens regulate the expression and/or activity of TET proteins and DNMTs in the ovary needs to be established. However, the first step toward directly linking androgen actions to dynamic changes in the DNA methylation pattern is to identify the genes or the CpG sites that are hypo- or hyper-methylated in hyper-androgenic PCOS patients, so that these genes/sites can be used as downstream endpoints. Here we provide a list of genes/CpG sites that have been reported to be hypo- or hyper-methylated in PCOS ovary (has elevated androgen levels) and discuss their ovarian (dys)function with respect to PCOS pathophysiology.

Figure 2

Androgen-induced regulation of DNA methylation: It is being proposed that androgens may indirectly regulate gene expression through regulating the expression and/or enzymatic activity of DNA methyltransferases and/or DNA demethylases that in turn mediates the methylation pattern of CpG islands. The changes in the methylation pattern are tissue-/cell-type-specific and causes developmental reprogramming.

Hypo-methylation It has been proposed that aberrant DNA methylation patterns could serve as epigenetic biomarkers for the detection of PCOS. For example, it is well-established that the luteinizing hormone/choriogonadotropin receptor (LHCGR) and its ligand, LH, play a critical role in folliculogenesis/ovulation (Filicori 1999). In PCOS, LHCGR is overexpressed in granulosa cells and is thought to be an underlying cause for the anovulation phenotype associated with PCOS (Kanamarlapudi *et al.* 2016). Intriguingly, in granulosa cells and peripheral blood cells of PCOS patients the LHCGR gene has been reported to be hypo-methylated (Wang *et al.* 2014) which provides

a mechanistic understanding of how the expression of LHCGR is upregulated in a hyper-androgenic condition like PCOS. Moreover, studies (Sagvekar *et al.* 2019) in cumulus granulosa cells isolated from PCOS women show 2977 CpGs representing 2063 genes to be hypo-methylated while 2509 CpGs within 1777 genes to be hyper-methylated. Interestingly, the promoter region of the androgen receptor has also been reported to be hypo-methylated in granulosa cells of women with PCOS as compared to controls, resulting in increased AR levels in PCOS women (Desmawati *et al.* 2018). Global methylation studies provide further insights about the role of epigenetic modulation in the pathogenesis of PCOS. Granulosa cells isolated from PCOS patients show a 25% reduction in the level of 5-methylcytosine (5-mC) in comparison to the control group (Pan *et al.* 2018). Moreover, the promoter regions of several genes related to lipid metabolism and steroid synthesis (*CD9*, *NR4A1*, *EDN2*, *BNIP3*, and *LIF*) have been reported to be hypo-methylated in granulosa cells of PCOS patients (Pan *et al.* 2018) (Table 2).

Hyper-methylation Hyper-methylation of CpG islands on gene promoter led to transcriptional repression. A study (Qu *et al.* 2012) in primary granulosa cells isolated from PCOS patients has reported two hyper-methylated CpG sites in the promoter of peroxisome proliferator-activated receptor 1 (*PPARG1*) gene and five hypo-methylated CpG sites in the promoter of nuclear corepressor (*NCOR1*) gene. Correlating with this, granulosa cells from these PCOS patients have lower mRNA expression of *PPARG1* compared to non-PCOS patients (Qu *et al.* 2012) (Table 2). *PPARG1* is a nonsteroidal nuclear hormone receptor that plays an important role in ovarian function (Froment *et al.* 2003) and PPAR agonists have been used as a therapeutic option in PCOS patients to improve ovulation rates (Ehrmann *et al.* 1997, Azziz *et al.* 2001). Furthermore, granulosa cells from hyper-androgenic PCOS patients show higher levels of *NCOR1* and *HDAC3* mRNA levels (Qu *et al.* 2012). Mechanistically, inhibition of the PI3K pathway by LY294002 in human primary granulosa cells alters androgen-induced inhibition of *PPARG1* and increased expression of *HDAC3* mRNA (Qu *et al.* 2012). This shows that extra-nuclear androgen signaling is also involved in regulating *PPARG1* and *HDAC3* expression. However, whether this androgen-induced extra-nuclear signaling (PI3K pathway) also mediates androgen-induced CpG methylation thereby modulating gene expression needs further elucidation. Moreover, *NCOR1* and *HDAC3* have

been shown to epigenetically regulate circadian metabolic physiology (Alenghat *et al.* 2008) and recently, it has been proposed that disruption of the circadian system can impact female fertility and health (Sen & Sellix 2016). Therefore, it can be speculated that androgen-induced modification of the CpG methylation pattern may lead to altered expression of *PPARG1*, *NCOR1*, and *HDAC3* in granulosa cells of PCOS patients (Table 2), which may be one of the contributing factors to ovarian and metabolic dysfunction associated with PCOS.

In addition to directly affecting the CpG sites on gene promoters, androgens also indirectly regulate the expression of genes through epigenetic modulation. For example, miRNA have been shown to play a major role in PCOS pathogenesis (Mao *et al.* 2021). Combinatorial analyses of RNA-seq, miRNA-seq, and methylated DNA-binding domain sequencing in granulosa cells from PCOS patients have shown hyper-methylation of *miR-429*, *miR-141-3p*, and *miR-126-3p* promoters result in lower expression of these miRNAs that is correlated with upregulation of the genes targeted by these miRNAs, namely, *XIAP* (X-linked inhibitor of apoptosis protein), *BRD3* (Bromodomain Containing 3), *MAPK14* (Mitogen-activated protein kinase 14), and *SLC7A5* (Solute Carrier Family 7) (Mao *et al.* 2021).

The above-cited studies show mostly correlative effects and the molecular mechanisms by which androgen may regulate DNA methylation pattern in the ovary are still poorly understood and therefore needs further investigation.

Hyper-androgenic PCOS animal models

Prenatal androgen treatment Studies (Xia *et al.* 2015) in prenatally androgenized rat (3mg testosterone from days 16 to 19 of gestation) models show five CpG sites in the *Ar* gene (CpG p87, p91, p93, p98, p150) and one CpG site in *Cyp11a1* (CpG p953) that are significantly hypo-methylated (Table 2). Additionally, genome-wide CpG methylation analysis in the prenatally androgenized rat (T propionate 2.5 mg/mL/day from days 16 to 18 of gestation) ovaries revealed 2497 differentially methylated genes, 528 of which were hyper-methylated (Zhang *et al.* 2014). In this study, gene ontology analysis of these 528 hyper-methylated genes showed that these genes are mainly involved in reproductive development, embryonic development, and lipid and glucose metabolic processes (Table 2). This emphasizes that changes in the DNA methylation status of genes induced by prenatal androgenization may be an underlying mechanism for some of the phenotypes reported in the prenatally

Table 2 Androgen-induced site-specific DNA methylation in the ovary.

Species	Site of impact	Treatment	DNA methylation status	Related genes	References
Human	Peripheral blood cells and granulosa cells	Prenatal	Hypomethylation	<i>LHCGR</i>	Wang <i>et al.</i> 2014
Human	Cumulus granulosa cells	Prenatal	Hypomethylation (2977 CpGs) and hypermethylation (2509 CpGs)	2063 genes 1777 genes	Sagvekar <i>et al.</i> 2019
Human	Granulosa cells	Prenatal	Hypomethylation	<i>AR</i>	Desmawati <i>et al.</i> 2018
Human	Granulosa cells	Prenatal	Global methylation	<i>GSTA1, CD74, ANGPTL4, CD9, NR4A1, EDN2, PTX3, LIF, SLC12A8, PEX3, CYP17A1, SERPINE1, DIRAS3, BNIP3, and SPP1</i>	Pan <i>et al.</i> 2018
Human	Granulosa cells	Prenatal	CpG methylation	<i>PPARG1, NCOR1 and HDAC3</i>	Qu <i>et al.</i> 2012
Human	Granulosa cells	Prenatal	Hypermethylation (miR-429, miR-141-3p, and miR-126-3p')	<i>XIAP, BRD3, MAPK14 and SLC7A5</i>	Mao <i>et al.</i> 2021
Sheep	Stromal cells	Prenatal	Hypermethylation	<i>DNMT3A and DNMT3B</i>	Guo <i>et al.</i> 2019
Sheep	Granulosa cells	Prenatal	Hypermethylation	<i>DNMT1</i>	Guo <i>et al.</i> 2019
Zebrafish	Ovaries	Prenatal	Global DNA methylation	Genes related to glucose metabolism	Xu <i>et al.</i> 2014
Wistar rats	Theca cells	Prenatal	Hypomethylation	<i>GATA6 and StAR</i>	Salehi Jahromi <i>et al.</i> 2018
Rat	Ovaries	Prenatal	Hypomethylation	<i>AR and Cyp11a1</i>	Xia <i>et al.</i> 2015
Rat	Ovaries	Prenatal	Differential methylation	Genes involved in reproductive and embryonic development, lipid and glucose metabolic processes	Zhang <i>et al.</i> 2014
Mouse	Ovaries	Postnatal	Hypomethylation	<i>Lhr</i>	Zhu <i>et al.</i> 2010
Mouse	Oocytes	Postnatal	Hypomethylation	<i>Dnmt1</i>	Eini <i>et al.</i> 2017

androgenized animals. For example, genes involved in ovarian follicle, female genitalia, and germ cell development, as well as mammary gland development and mammary gland duct morphogenesis were significantly hyper-methylated (Zhang *et al.* 2014). Repression of gene expression by hyper-methylation in prenatally androgenized murine models leads to an increase in anogenital distance, agenesis of the lower vagina, absence of nipples, and reduced papillary numbers (McCoy & Shirley 1992, Rhees *et al.* 1997, Wolf *et al.* 2002). Offspring from prenatally androgenized (5 mg free testosterone in 500 mL solvent on day 20 of gestation) Wistar rats also show (Salehi Jahromi *et al.* 2018) hypo-methylation at –520 and –822 positions in the Gata 6 and the StAR promoter respectively (Table 2). Interestingly, in PCOS patients, expression of both Gata 6 (Ho *et al.* 2005) and StAR (Jakimiuk *et al.* 2001) genes are increased in theca cells. Similarly, the *Lhr* gene is hypo-methylated in the ovaries of the DHEA-induced mouse PCOS model (Zhu *et al.* 2010) that correlates with higher LHR levels in PCOS patients, as mentioned above.

Changes in DNA methylation are caused by DNA methyltransferases (DNMTs) and studies (Gravina *et al.* 2011, Zhu *et al.* 2011, Chu *et al.* 2014) in cancer cells have shown that androgen can increase the expression

and activity of DNMTs. Therefore, it is highly possible that in the ovary too, androgen-induced modulation of DNA methylation can be mediated through regulation of expression/activity of DNMTs (Fig. 2). For example, a recent study (Guo *et al.* 2019) comparing granulosa, theca, and stroma cells isolated from ovaries of prenatally androgenized (100 mg T propionate from days 30 to 90 of gestation) adult sheep reported a significant increase in the expression of *DNMT1* and a decrease in *DNMT3B* in the granulosa cells. Additionally, stromal cells from the same ovaries had higher expression of *DNMT3A* and *DNMT3B* genes (Table 2). *DNMT1* is the primary methyltransferase that catalyzes the addition of methyl groups to specific CpG structures in the DNA while *DNMT3A* and *DNMT3B* serve as *de novo* methyltransferases (Hervouet *et al.* 2018). Therefore, similar to histone modifications, these studies also show that in the ovary, androgens can have differential effects on DNA methylation in a cell-specific manner.

Post-natal androgen treatment There are limited studies on the effect of post-natal androgen treatment on the epigenetic modification of the DNA in the ovary. In zebrafish, exposure to testosterone, and its metabolite dihydrotestosterone (DHT), during development, show that early-life exposure can induce changes in global DNA

methylation status in the ovary (Xu *et al.* 2014) (Table 2). Furthermore, testosterone has a stronger effect on global DNA methylation in the ovary compared to DHT, thus, suggesting that the aromatization of testosterone to estrogen might play a role in the epigenetic programming effect. This study (Xu *et al.* 2014) also reported that these epigenetic effects can be trans-generationally transmitted from the exposed F1 to the unexposed F2 generation. In mammals, prepubertal female mice treated with DHEA (6 mg per 100 g body weight) show a decline in DNA methylation and reduced expression of *Dnmt1* in oocytes (Eini *et al.* 2017) (Table 2). Further studies are needed to understand how androgens regulate the expression/activity of the DNMTs.

Concluding remarks and future perspectives

Collectively, while there is now some evidence of androgen-induced epigenetic modifications, we are just beginning to understand the effect of androgen-induced epigenetic modulation in female reproduction and health. Recent studies have mainly been focused on pinpointing the changes induced by androgens in the DNA methylation status of genes, post-translational modifications of histones, and how these epigenetic modifications change gene expression. These studies now provide some mechanistic understanding of androgen actions in the ovary, both under normal and pathophysiological conditions like PCOS. However, there are very few studies and they provide a limited understanding of androgen-induced epigenetic modulation in the ovary. Moreover, how androgens regulate specific epigenetic enzymes to modulate the epigenome is poorly understood. Notably, post-translational modifications of histone-modifying enzymes, in particular phosphorylation, contribute to developmental programming (Trevino *et al.* 2015). For example, signaling pathways known to be activated by extra-nuclear androgen signaling such as PI3K/Akt, MAPK, and PKA pathways, have been shown to modify and regulate the activity of various epigenetic enzymes (Trevino *et al.* 2015, Ma *et al.* 2017a). Therefore, elucidating how androgen-induced nuclear and/or extra-nuclear signals regulate epigenetic changes will provide novel mechanistic insights about androgen actions in both normal and pathophysiological conditions as well as in androgen-induced developmental programming. Given the physiological importance of androgens in women's reproductive health, this knowledge is imperative to develop novel strategies to improve female fertility and women's health.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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