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REVIEW

NR5A1 and cell population heterogeneity: Insights into developmental and functional disparities and regulatory mechanisms

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Abstract

Background: NR5A1 plays essential roles in the development of various tissues, including the ventromedial hypothalamus, pituitary gonadotrope, adrenal cortex, spleen, testis, and ovary. Additionally, NR5A1-positive cells in these tissues exhibit developmental and functional heterogeneity.

Methods: This review summarizes recent knowledge on the relationships between physiological functions and gene cascades regulated by NR5A1 in each tissue. In addition, we also present several intriguing examples of disparities in *Nr5a1* gene regulation within the same tissues, which are relevant to developmentally and functionally heterogeneous cell populations.

Main Findings: The adrenal cortex and testicular Leydig cells exhibit clear biphasic developmental processes, resulting in functionally distinct fetal and adult cell populations in which *Nr5a1* is regulated by distinct enhancers. Similar heterogeneity of cell populations has been suggested in other tissues. However, functional differences in each cell population remain unclear, and *Nr5a1* gene regulation disparities have not been reported.

Conclusion: Some steroidogenic tissues demonstrate biphasic development, with fetal and adult cell populations playing distinct and crucial physiological roles. *Nr5a1* regulation varies across cell populations, and analyses of gene cascades centered on NR5A1 will aid in understanding the mechanisms underlying the development and maturation of reproductive capabilities.

KEYWORDS
adrenal cortex, enhancer, NR5A1, ovary, testis

1 | INTRODUCTION

Nuclear receptor subfamily 5 group A member 1 (NR5A1, previously known as steroidogenic factor-1 [SF-1] or Ad4-binding protein [Ad4BP]) was initially identified as a transcriptional regulator of steroidogenic genes in adrenocortical cells.[1,2] Subsequent studies revealed that NR5A1 is also expressed in ventromedial hypothalamic neurons, pituitary gonadotropes, splenic vascular endothelium, gonadal somatic cells, including testicular Sertoli and Leydig cells, as well as ovarian granulosa and theca cells.[3] Early investigations into NR5A1 functions through systemic gene disruption demonstrated gonadal and adrenal agenesis, highlighting the essential role of

NR5A1 in the organogenesis of the gonads and adrenal glands.[4] Since systemic *Nr5a1* gene-disrupted mice die within a few days after birth due to adrenal insufficiency, conditional *Nr5a1* knockout mice were generated to elucidate the tissue-specific functions of NR5A1. Observations of these conditional knockout mouse lines have revealed that NR5A1 plays pivotal roles in each tissue. Given the distinctive physiological functions of these tissues, it is assumed that NR5A1 regulates different sets of downstream genes in each tissue. To explore this hypothesis, numerous studies have been conducted to identify the genes whose expression is regulated by NR5A1. Furthermore, several studies have identified tissue-specific enhancers of the *Nr5a1* gene (Figure 1), suggesting that NR5A1 expression is induced by different mechanisms across tissues. Among these tissues, the adrenal gland and testicular Leydig cells exhibit biphasic development with fetal cell populations emerging before adult populations. Fetal populations not only give rise to adult populations but also play unique and indispensable physiological roles in the development of fetuses. Interestingly, *Nr5a1* gene regulation differs between fetal and adult populations, indicating that the gene cascades centered on NR5A1 are crucial for the sequential development of these distinct cell populations. In this review, we summarize previous findings on the downstream genes regulated by NR5A1, upstream factors that control *Nr5a1* gene expression, and tissue-specific regulatory regions. Additionally, this review discusses the developmental and functional heterogeneity of NR5A1-expressing cells in various tissues, including the adrenal cortex and testicular Leydig cell.

2 | VENTROMEDIAL HYPOTHALAMIC NUCLEUS

Ventromedial Hypothalamic Nucleus (VMH) is known as a classical satiety center and center for female reproductive behavior, and NR5A1 expression in the central nervous system (CNS) is restricted to the VMH neurons.

2.1 | Topology of NR5A1-positive neurons in the VMH

VMH is the largest nucleus in the hypothalamus and is traditionally divided into three subnuclei: ventromedial part (VMHvl), central part (VMHc), and dorsomedial part (VMHdm). Histological analysis of adult mouse brain revealed that NR5A1-positive neurons are predominantly distributed in the VMHdm and VMHc (Figure 2).[5]

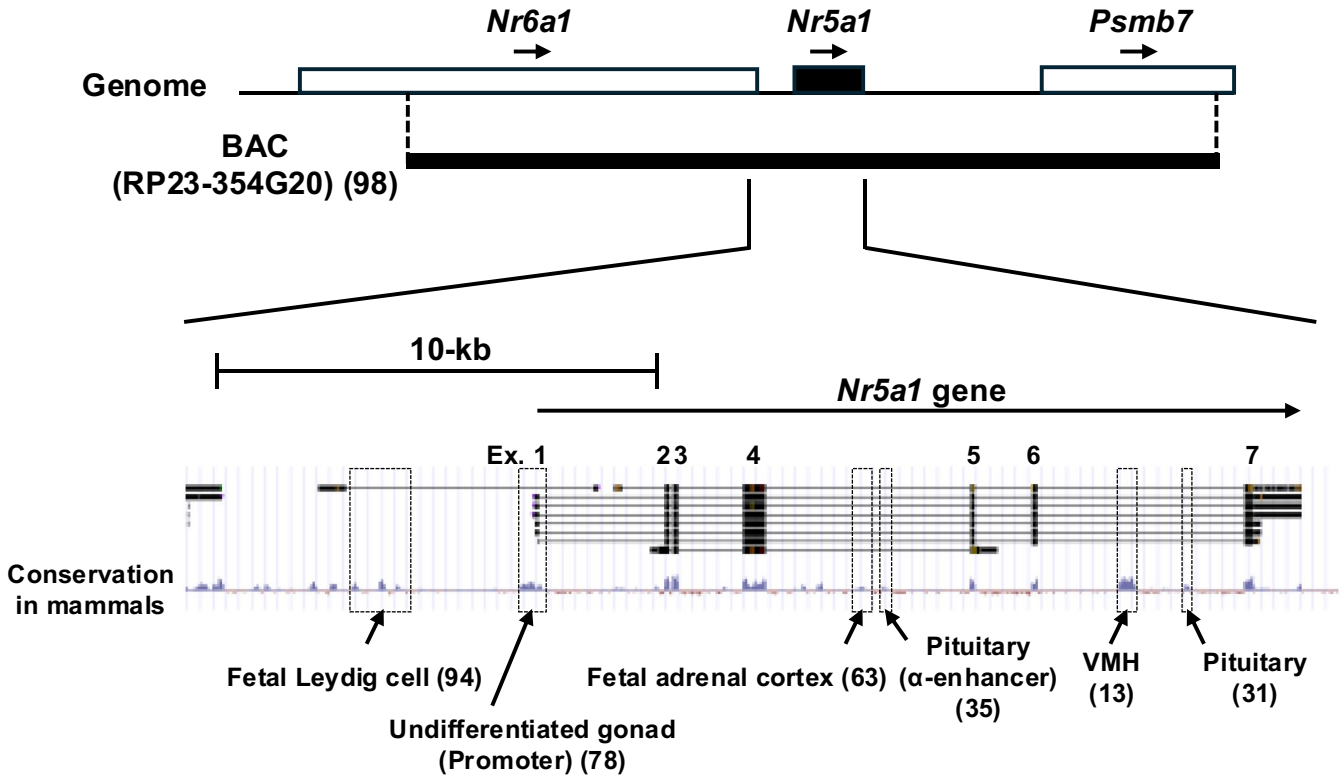

FIGURE 1 Nuclear receptor subfamily 5 group A member 1 (Nr5a1) gene structure and regulatory regions for each tissue. The positional relationship of *Nr5a1* with adjacent genes in the genome. The genomic region included in the bacterial artificial chromosome (BAC) clone (RP23-354G20), which completely recapitulates endogenous NR5A1 expression, is also shown. The *Nr5a1* gene consists of seven exons, and several tissue-specific regulatory regions have been identified in its intronic regions and upstream regions. The positions of these regulatory regions (enhancers), the references that identify each enhancer, and the conservation of enhancer sequences in mammals are shown. This figure was partially created using the University of California Santa Cruz (UCSC) Genome Browser (http://genome.ucsc.edu/).

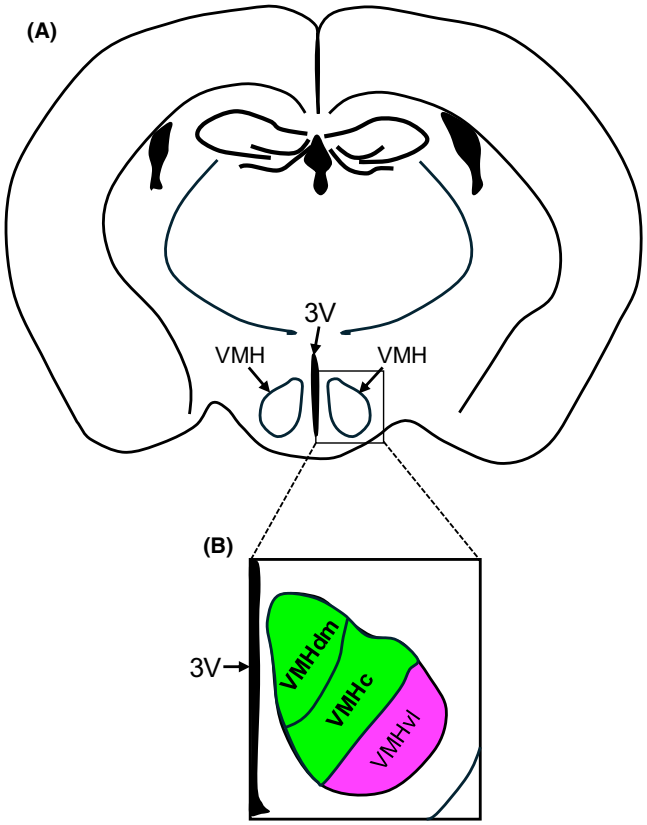

FIGURE 2 NR5A1 expression in the ventromedial hypothalamic nucleus (VMH). (A) Schematic diagram of a frontal section of the mouse brain. The VMH is located in the mediobasal region of the hypothalamus, adjacent to the third ventricle (3V). (B) Enlarged view of the VMH. NR5A1 is predominantly expressed in the dorsomedial (VMHdm) and central (VMHc) regions of the VMH (magenta). Lineage tracing analyses reveal that NR5A1 is transiently expressed in the ventrolateral region (VMHvl) of the VMH (green), but its expression diminishes at later developmental stages.

Although neurons in each subnucleus exhibit distinct aggregation patterns, neuronal morphology, and connectivity, recent transcriptomic analyses have shown that each subnucleus comprises a heterogenous neuronal population. Consequently, VMH neurons are now categorized into a greater number of subpopulations based on their gene expression patterns.[6]

2.2 | Development of NR5A1-positive neurons in the VMH

A recent study identified homeobox transcription factor NKX2.2 as one of the most broadly expressed factors in VMH neurons.[7] Detailed observations of NR5A1-positive neurons in the mouse brain from E13.5 to E16.5 have revealed that NR5A1-positive neurons originate from different locations than other NKX2.2-positive VMH neurons and migrate dorsoventrally.[7] This finding suggests that the topology of NR5A1-positive neurons in the VMH is related to their developmental origin.

Lineage tracing of NR5A1-positive cells has revealed that NR5A1 is expressed throughout VMH during fetal development but becomes restricted to VMHdm and VMHc neurons in adult mice, indicating that NR5A1 is transiently expressed in VMHvl neurons, but its expression diminishes at later stages (Figure 2).[8] NR5A1 is also expressed in the dorsal telencephalon during embryonic development, but its expression diminished in the adult brain.[9] These studies highlight the complexity of NR5A1 expression and suggest potential unknown functions of NR5A1 in developing VMH neurons and other regions of the CNS.

2.3 | Roles of NR5A1 in VMH development

In neonatal mice with a systemic disruption of *Nr5a1*, distribution of neurons in the mediobasal part of the hypothalamus is perturbed, suggesting that NR5A1 plays crucial roles in the migration and aggregation of VMH neurons.[10,11] Furthermore, mice with CNS-specific knockout of *Nr5a1*, generated using *Nestin-Cre*, also exhibited abnormal aggregation of neurons in the mediobasal hypothalamus. This finding rules out the effects of adrenal or gonadal hormones and underscores the importance of CNS-specific NR5A1 function in VMH formation.[12] Additionally, in *Nr5a1* knockout mice, the number of dendrites was reduced and exhibited variable orientations between VMH neurons and the preoptic area (POA), indicating that NR5A1 also contributes to the normal connectivity of VMH neurons.[13]

2.4 | Downstream genes of NR5A1 in the VMH

NR5A1 is involved in various physiological functions related to whole-body homeostasis. The most important and extensively studied function of NR5A1-positive neurons is their role in energy homeostasis and obesity. Systemic *Nr5a1* knockout mice, rescued by corticosteroid injection, exhibit late-onset obesity due to decreased activity, suggesting that NR5A1 regulates energy expenditure rather than food intake.[14] More recent studies have reported that NR5A1 is also essential for the beneficial effects of exercise on metabolism.[15] In NR5A1-positive neurons, leptin and insulin signaling are integrated via phosphoinositide 3-kinase signaling, which in turn regulates energy homeostasis.[16,17] Other studies have shown that Rap1, a small GTPase belonging to the Ras family, plays an important role in glucose metabolism in NR5A1-positive neurons,[18] and that growth hormone-releasing hormone (GHRH) released from these neurons also contributes to glucose metabolism.[19] A recent study reported that NR5A1-positive neurons regulate skeletal muscle functions by increasing the sympathoadrenal activity and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) expression in the skeletal muscle.[20] NR5A1-positive neurons have also been implicated in various physiological functions, including female reproductive behavior,[21] diet-induced thermogenesis,[22] maternal behavior,[23] anxiety-like behavior,[24] and the persistent defensive state.[25]

Although NR5A1 regulates multiple homeostatic parameters, its direct targets are not yet fully understood. Brain-derived

neurotrophic factor (BDNF) is one known downstream factor of NR5A1 in VMH neurons,[26] but the relationship between BDNF and various physiological functions remains controversial. Another downstream gene of NR5A1 is *Cnr1*, whose gene product, Cannabinoid receptor 1, is implicated in the regulation of energy homeostasis and anxiety-like behavior.[27] Transcriptomic analyses of the neonatal mouse VMH, followed by promoter assays, identified several potential targets of NR5A1, including *Nmdr1* encoding N-methyl-D-aspartate receptor, transcription factors such as *Fezf1* and *Nkx2.2*, as well as molecules involved in cell adhesion and guidance, such as *A2bp1*, *Amigo2*, *Cdh4*, *Nptx2*, *Sema3a*, *Slit3*, and *Netrin3*.[28] However, further studies are necessary to elucidate the molecular functions of these factors.

2.5 | Functional heterogeneity in NR5A1-expressing neurons in the VMH

NR5A1-expressing neurons in the VMH have traditionally been considered homogeneous. However, given their involvement in various physiological functions (see Section 2.4), these neurons may consist of functionally heterogeneous subpopulations. Recent multimodal analyses of VMH neurons at the single-cell level have revealed functional heterogeneity and diverse projections among the entire population of VMH neurons. This study also identified heterogeneous gene expression patterns within NR5A1-positive neurons in the VMH, although most NR5A1-positive neurons project to the dorsal periaqueductal gray (dPAG).[29]

2.6 | Regulation of NR5A1 expression in the VMH

Shima et al. reported that an approximately 0.5-kb genomic region within the sixth intron of the *Nr5a1* gene has potential for VMH-specific expression (Figure 1).[30] Although the transcription factors that bind to this enhancer have not yet been identified, several small regions within this sequence are conserved among animal species, suggesting that homeobox factors may bind to these conserved sequences and activate *Nr5a1* transcription. This enhancer is capable of fully replicating the endogenous expression of NR5A1 in CNS, including its transient expression in the dorsal telencephalon. However, the function of this enhancer within each subpopulation of NR5A1-positive neurons in the VMH remains unclear. Further detailed analyses of *Nr5a1* regulation in the VMH at the single-cell level will provide insights into the developmentally and functionally distinct subpopulations within NR5A1-positive VMH neurons.

3 | PITUITARY GONADOTROPES

NR5A1 is expressed in pituitary gonadotropes, which produce luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These two hormones consist of a common α subunit encoded by *glycoprotein hormones, alpha subunit (Cga)* gene, and distinct β-subunits such as LHβ and FSHβ. Pituitary-specific conditional knockout of the *Nr5a1* gene was achieved using *Cga-Cre*, and these mice exhibited symptoms of human hypogonadotropic hypogonadism, including markedly decreased LH and FSH expression, severely impaired gonad development, and the absence of secondary sexual characteristics.[31] These results suggest that NR5A1 plays an important role in the functional differentiation of pituitary gonadotropes.

3.1 | Regulation of NR5A1 in the pituitary Gonadotropes

The first report on the regulation of the *Nr5a1* gene was published by Harris et al. in 1998. In this study, they found that upstream stimulatory factor (USF), a basic helix–loop–helix transcription factor, binds to the E-box in the upstream region of *Nr5a1* and regulates its expression. However, transcriptional activation of *Nr5a1* by USF was observed not only in the gonadotrope-derived cell line αT3-1 but also in the adrenal cortex-derived Y-1 cell line, which did not explain the mechanisms underlying pituitary-specific *Nr5a1* expression.[32] Shima et al. later identified a pituitary-specific enhancer of the *Nr5a1* gene (Pituitary Enhancer, PE) in the sixth intron (Figure 1). They also discovered that the PITX2 binding sequence in the PE is essential for *Nr5a1* expression in the pituitary, suggesting that PITX2 directly regulates *Nr5a1* expression.[33]

Deletion of the PE of the *Nr5a1* gene from the mouse genome resulted in a complete loss of NR5A1 in the pituitary, indicating the functional importance of the PE *in vivo*.[34] Pacini et al. identified another pituitary enhancer of *Nr5a1* (designated α enhancer) in the fifth intron through assays for transposase-accessible chromatin-sequencing (ATAC-seq) analyses of pituitary gonadotrope-derived cell lines (Figure 1). This study demonstrated that the α enhancer plays a crucial role in the early differentiation process of pituitary gonadotropes. Moreover, they showed that the estrogen receptor binds to the estrogen-responsive element (RER) of the α enhancer, inducing chromatin remodeling for NR5A1 expression and gonadotrope differentiation.[35] Although disparities in *Nr5a1* gene regulation in pituitary gonadotropes have not been reported, the α enhancer may be involved in *Nr5a1* expression at early stages, and its functionality should be further examined through in vivo genome editing experiments in future studies. Most recently, RNA-binding protein Musashi1 binds to the 3′-untranslated region of key pituitary lineage specification genes, such as *Paired-like homeodomain factor 1 (Prop1)*, *Gata-binding protein 2 (Gata2)*, and *Nr5a1*, and activates their translation.[36]

3.2 | Downstream genes of NR5A1 in the pituitary gonadotropes

Pituitary gonadotrope-derived cell lines, such as αT3 and LβT2, were established almost 30 years ago.[37] The transcriptional activity of

NR5A1 has been extensively investigated by using these cell lines. For example, NR5A1 directly binds to the promoter region of the *Cga* gene and regulates its expression.[38] Similarly, promoter analyses of *Lhb*, *Fshb*, and *gonadotropin-releasing hormone receptor (Gnrhr)* suggest that NR5A1 directly regulates the expression of the gonadotropin subunits LHβ[39,40] and FSHβ,[41,42] as well as the GnRH receptor.[43] However, in vivo experiments have yielded contradictory results; although LH and FSH levels are significantly reduced, they are not completely absent in pituitary-specific *Nr5a1* knockout mice.[31] More recently, pituitary-specific *Nr5a1* deletion was achieved by deleting the PE of the *Nr5a1* gene, and transcriptome analyses of the pituitary, as well as isolated pituitary gonadotropes, confirmed that *Lhb* and *Fshb* transcripts were markedly reduced but not entirely diminished. This transcriptomic analysis also identified genes regulated by NR5A1, such as *secreted phosphoprotein 1 (Spp1)*, *gremlin1 (Grem1)*, and *transforming growth factor beta receptor 3-like (Tgfbr3l)*, whose gene products are related to interaction between gonadotropes and extracellular matrices, BMP antagonism, and TGFβ signaling, respectively. However, it is unclear whether these genes are directly regulated by NR5A1.[34]

3.3 | Functional heterogeneity in the pituitary gonadotropes

There are only a few studies on the heterogeneity of the gonadotrope cell population. In 1993, Torronteras et al. classified porcine pituitary gonadotrope cells into three subpopulations based on their density, as revealed by Percoll density gradient centrifugation. This classification correlates with cellular morphology and hormone synthetic activity,[44] reflecting the differences in cell activity rather than the presence of functionally distinct cell populations. More recently, single-cell analyses of the chicken anterior pituitary revealed heterogeneity in pituitary gonadotropes.[45] However, functional differences among these cell populations have not been clearly defined. Additionally, since the types and secretion patterns of pituitary gonadotropins vary among animal species, it is necessary to consider interspecies differences when interpreting these results. In summary, it seems reasonable to consider that pituitary gonadotropes are a functionally homogeneous cell population in mice.

4 | SPLEEN

The spleen is one of the secondary lymphoid tissues, and its physiological functions are closely related to the structure of blood vessels. It is divided into two functional regions: the red pulp and the white pulp. The red pulp serves as a filter for capturing abnormal erythrocytes, while the white pulp provides a microenvironment for immune cells responding to blood-borne antigens. The venous sinuses in the red pulp, known as splenic sinuses, have gaps between rod-like endothelial cells that function as a filter for erythrocytes. Conversely, the white pulp is surrounded by a different type of venous sinus, called the marginal sinus, which creates a microenvironment for immune cells to capture antigens from the blood flow. Interestingly, NR5A1 is expressed in the endothelial cells of both the splenic sinus and the marginal sinus (Figure 3),[46] suggesting distinct functions for NR5A1 in the red pulp and white pulp.

4.1 | NR5A1 function in the spleen

In mice with disrupted *Nr5a1* gene, spleens exhibit several morphological and functional abnormalities. The size of the spleen is markedly reduced in knockout mice. Additionally, the large gaps normally observed between venous sinus endothelial cells are absent in these mice. Consequently, there is a significant increase in abnormal blood cells bearing Howell-Jolly bodies in the peripheral blood, likely due to the decreased blood-filtering function of the red pulp.[46] On the other hand, the function of NR5A1 in the marginal sinus remains unclear.

Fatchiyah et al. attempted to rescue systemic *Nr5a1* knockout mice by inducing NR5A1 expression from a bacterial artificial

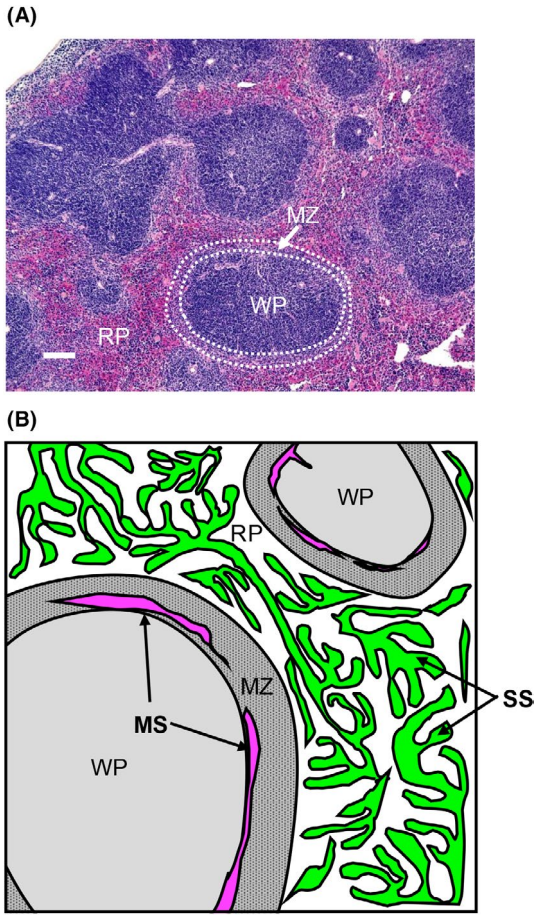

FIGURE 3 NR5A1 expression in the spleen. (A) HE-stained image of the murine spleen. The spleen is divided into three distinct regions: The red pulp (RP), the white pulp (WP), and the marginal zone (MZ), which surrounds the WP. Bar = 100 μm (B) Schematic diagram of the vascular architecture in the spleen. NR5A1 is expressed in two distinct venous sinuses: The splenic sinus (SS) (green) in the red pulp (RP) and the marginal sinus (MS) (magenta) in the marginal zone (MZ).

chromosome (BAC) transgene harboring the *Nr5a1* gene locus. The phenotypes of these mice suggest that the spleen is one of the most sensitive tissues to the dosage of the *Nr5a1* gene.[47] It has also been reported that NR5A1 mutations in humans cause abnormal splenic development, although the molecular mechanisms linking NR5A1 and spleen development are not yet understood.[48,49]

4.2 | Regulation of NR5A1 in the spleen

The mechanisms of *Nr5a1* gene regulation in the spleen are not well understood. Fukui et al. reported that disruption of the *chromobox 2 (Cbx2)* gene, which encodes the polycomb group member M33, caused a significant reduction in the size of both the adrenal gland and spleen, mimicking the phenotypes observed in *Nr5a1* heterozygous mice. This finding indicates that M33 is an upstream regulatory factor for *Nr5a1* expression in both the spleen and the adrenal gland.[50]

5 | ADRENAL CORTEX

The adrenal gland is the organ where NR5A1 shows the strongest expression in the entire body. NR5A1 was initially identified as a transcriptional activator of steroidogenic P450 enzymes, which are required for corticosterone synthesis.[2] Due to this significant background, NR5A1 functions in the adrenal gland have been extensively studied. The anatomical and functional zonation of the adrenal cortex differs between mice and humans. Most studies on the adrenal cortex have been conducted using mouse models; therefore, this review primarily discusses mouse studies, unless otherwise noted.

5.1 | Development of the fetal and definitive cortex

The NR5A1-positive cell population emerges from the intermediate mesoderm at embryonic day 10.5 (E10.5). This structure is called the adrenogonadal primordium (AGP) because it subsequently gives rise to the adrenal gland and gonad.[51] However, in humans, the adrenal gland and gonad arise from distinct anlagen.[52] The AGP splits into the gonadal primordium (GP) and the adrenal primordium (AP), with the AP subsequently giving rise to the fetal cortex. Contrary to this conventional theory, recent single-cell analyses of murine AGP from E9.0 to E12.5 suggest that the AGP arises mainly from the lateral plate mesoderm rather than the intermediate mesoderm.[53] Neural crest cells migrating from the neural tube colonize within the fetal cortex to form the adrenal medulla. Subsequently, the definitive cortex emerges, surrounds the fetal cortex, and eventually forms two or three functionally distinct layers (zones) of the adrenal cortex in mice and humans, respectively.

The fetal cortex persists into postnatal stages, forming an eosinophilic cell layer between the definitive cortex and medulla. This layer, called the X-zone, regresses in a sex-dependent manner. In female mice, X-zone starts to degenerate as early as postnatal day 32 (P32). Interestingly, the X-zone completely disappears 5–15 days after the first pregnancy, and in females that do not experience pregnancy, it disappears between 3 and 7 months after birth. In males, the X-zone regresses before P40 (Figure 4).[54–56] The molecular mechanisms underlying X-zone degeneration are not fully understood. However, since the X-zone in male mice disappears earlier than in female mice, it is assumed that androgen signaling is important for X-zone regression. Indeed, disruption of the *Ar* gene in the adrenal cortex results in an expanded X-zone in males.[57] On the other hand, *Ar* gene ablation does not affect X-zone regression during pregnancy in females,[58] suggesting that the mechanisms of X-zone degeneration are sexually dimorphic.

5.2 | Physiological functions of fetal and definitive cortex

The enzyme 20α-hydroxysteroid dehydrogenase (20α-HSD) is expressed in both the ovary and adrenal gland. In the ovary, 20α-HSD plays an important role in parturition by catabolizing progesterone.[59] In the adrenal gland, 20α-HSD expression is restricted to the X-zone. Although 20α-HSD is not required for X-zone formation, its expression pattern is closely correlated with the expansion and regression of the X-zone. Therefore, 20α-HSD is now commonly used as a marker for the X-zone in mice.[60,61] Observations from *Akr1c18* gene-disrupted mice (encoding 20α-HSD) suggested

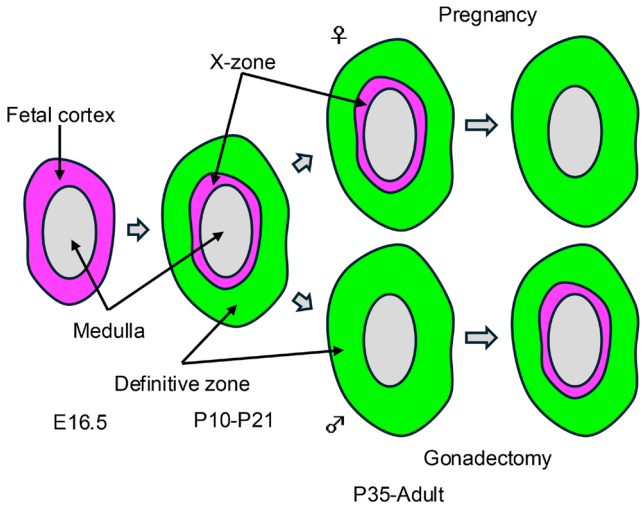

FIGURE 4 Development of two distinct layers in the adrenal cortex. During adrenal gland development, the fetal cortex (magenta) is initially formed, and neural crest cells migrate into the fetal cortex to form the medulla (gray). The definitive cortex (green) subsequently develops, surrounding the fetal cortex, which then gives rise to the X-zone. In males, the X-zone regresses before postnatal day 40 (P40) but reappears after gonadectomy. In females, the X-zone disappears during the first pregnancy or regresses 3–7 months after birth in the absence of pregnancy. E16.5: Embryonic day 16.5.

that 20α-HSD functions not only as a progesterone-catabolizing enzyme but also as an 11-deoxycorticosterone-catabolizing enzyme.[60]

In humans, the fetal adrenal cortex plays an important role in fetal development by producing steroid hormones in cooperation with the placenta. The primary function of the fetal adrenal cortex is believed to be the synthesis of dehydroepiandrosterone sulfate (DHEA-S) from cholesterol. DHEA-S is transported to the placenta, where it is desulfated and converted to androstenedione (A4), estrone (E1), and estradiol (E2), which are then released into the maternal circulation.[62] Recent studies have also revealed that the fetal adrenal cortex produces 11-oxygenated androgens, such as 11-keto-androstenedione and 11-keto-testosterone. These androgens are further metabolized in the placenta and released into the maternal circulation.[63,64] These 11-oxygenated androgens are reported to be involved in various conditions, such as congenital adrenal hyperplasia, premature adrenarche, and polycystic ovary syndrome.[65–70]

The definitive cortex eventually forms functionally mature zones outside of the fetal cortex, which serve as major sources of steroid hormones in mature organisms. Rodents have zona glomerulosa (zG) and zona fasciculata (zF), which primarily produce mineralocorticoid and glucocorticoid, respectively. In contrast, the human adrenal cortex consists of three zones, zG, zF, and zona reticularis (zR), which produces adrenal androgens and their precursors.

5.3 | *Nr5a1* gene regulation in the fetal and adult adrenal cortex

Zubair et al. identified a fetal adrenal cortex-specific enhancer (FAdE) in the fourth intron of the *Nr5a1* gene (Figure 1). This enhancer contains binding sequences for the Hox-Pbx-Prep transcription factor complex, and mutations in these sequences abolished the enhancer activity.[71] Histological analyses of mouse embryos have revealed that several transcription factors, such as GATA binding protein 4 (GATA4), Wilms' tumor 1 (WT1), and Cbp/p300-interacting transactivator 2 (CITED2), are expressed in the AGP before NR5A1 expression is initially detected. Genetic experiments in mice have shown that these factors induce the initial NR5A1 expression in the AGP.[72] However, these factors have not been shown to act directly on FAdE. FAdE activity is retained in the postnatal 20αHSD-positive X-zone, and NR5A1 SUMOylation and Dax1 are thought to repress FAdE-mediated NR5A1 expression and induce regression of the X-zone in the postnatal adrenal cortex.[73]

Importantly, FAdE induces LacZ reporter expression exclusively in the fetal cortex, while the entire cortex was labeled by FAdE-Cre, suggesting that definitive cortex originates from the fetal cortex.[74] Simon et al. have reported that adrenal capsular cells serve as stem cells for the definitive cortex and that differentiated cells migrate centripetally.[75] These two hypotheses have eventually merged, suggesting that a part of fetal adrenal cells contribute to capsular cells, which then serve as progenitors for the definitive cortex.[76]

5.4 | Downstream genes regulated by NR5A1 in the adrenal cortex

It is well known that NR5A1 regulates genes encoding steroidogenic enzymes in the adrenal cortex, such as *Star*, *Cyp11a1*, *Hsd3b1*, *Cyp17a1*, *Cyp21a1*, *Cyp11b1*, and *Cyp11b2*.[77] Additionally, NR5A1 regulates genes related to steroid hormone synthesis, including *Akr1b*,[78] *Scarb1*,[79,80] *Mc2r*,[81,82] *Nr0b1* (encoding DAX-1),[83,84] *Sult2a1*,[85] and *Adcy4*.[86] In vitro analyses of the human adrenocortical cell line NCI-H295R, using overexpression or knockdown of NR5A1, have suggested novel candidates for NR5A1 downstream genes, such as *VSNL1*, *ZIM2*, *PEG3*, *SOAT1*, and *MTSS1*. Among these genes, *SOAT1*, which encodes sterol O-acyltransferase 1, is expressed in the developing fetal cortex, suggesting an important role in maintaining cholesterol ester reserves during fetal development.[87] Another study found that *KCNJ5*, which encodes G protein-sensitive inwardly rectifying potassium channels involved in human adrenal aldosterone-producing adenomas, is directly regulated by NR5A1.[88]

Recent technological advances have facilitated the comprehensive analysis of NR5A1 downstream genes. Baba et al. performed chromatin immunoprecipitation analyses using adrenal cortex-derived Y-1 cells and revealed that NR5A1 regulates a set of genes implicated in glycolysis.[89] Moreover, their results suggested that NR5A1 also regulates NADPH production[90] and cholesterol synthesis[91] in the adrenal cortex.

It is noteworthy that recent studies have focused on the sexual dimorphism of the adrenal cortex in terms of histological structures, gene expression,[92] and cell renewal.[93] Recent transcriptomic analyses identified sexually dimorphic genes related to thyroid hormone signaling, such as *Cyp2f2* and *Dhcr24*. Intriguingly, these genes showed predominant expression in the innermost area of the zF and a part of the X-zone, which they referred to as "sub-zone".[94] The regulation of *Nr5a1* expression by distinct mechanisms in the fetal and definitive cortex suggests that different gene cascades centered on NR5A1 are deeply involved in the formation of distinct cortical zones and the physiological roles of each zone. Future studies at the single-cell level are expected to clarify the detailed functions of NR5A1 in adrenocortical cell differentiation, homeostasis, aging, and pathogenesis of various diseases.

Finally, a recent study by Takahashi et al. suggested a novel role for NR5A1 in the adrenal cortex. Their results indicated that NR5A1 expression is reduced by AR signaling in males, and suppressed global gene expression in the zF triggers decreased corticosterone production and induces sexual differences, particularly in skeletal muscles.[95]

6 | TESTIS

NR5A1 is expressed in the testicular somatic cells, such as Sertoli cells and interstitial Leydig cells. While Sertoli cells are believed to be homogeneous, interstitial Leydig cells show a two-step differentiation pattern, resulting in at least two functionally distinct cell populations in the adult testis.

6.1 | *Nr5a1* gene regulation in Sertoli cells

Nr5a1 expression in the undifferentiated gonad at early fetal stages is induced by the proximal promoter region of the *Nr5a1* gene.[96] This promoter region contains binding sequences for WT-1 and LIM homeobox 9 (LHX9), which induces *Nr5a1* expression in pre-Sertoli cells. Once a subset of cells in the undifferentiated gonad is destined to become Sertoli cells, these pre-Sertoli cells proliferate until the early postnatal period and then transition from the proliferation phase to the differentiation phase. Wood et al. reported that USF1 and USF2 bind to the *Nr5a1* gene promoter and activate *Nr5a1* transcription in Sertoli cells during the initial differentiation stages.[97]

6.2 | NR5A1 function in Sertoli cells

NR5A1 appears to play various roles in Sertoli cell development. At the beginning of testis development, the *Sry* gene is expressed in pre-Sertoli cells, and its temporal expression triggers *Sox9* expression, thereby inducing Sertoli cell differentiation. This *Sry-Sox9* gene cascade is essential for determining the fate of the undifferentiated gonad to become the testis. An initial study dissecting the involvement of NR5A1 in the *Sry-Sox9* gene cascade was conducted by Sekido et al. They identified a testis-specific enhancer of Sox9 (designated TES) and found that SRY and NR5A1 bind to the core region of TES (TESCO), synergistically activating *Sox9* gene expression.[98] Recently, Tao et al. reported that NR5A1 activates the microRNA-34b/mitogen-activated protein kinase/extracellular regulated protein kinase pathway to promote differentiation of bovine pre-Sertoli cells.[99] Another recent study identified an NR5A1-responsive enhancer approximately 5 kb upstream of the *SRY* gene in humans. This enhancer is conserved in mammalian species, and in vitro analyses have suggested its functional importance in *SRY* expression and testis development.[100] Based on these findings, NR5A1 has been used to induce the artificial differentiation of Sertoli cells. Liang et al. successfully reprogrammed human fibroblasts into Sertoli-like cells via forced expression of GATA4 and NR5A1.[101]

NR5A1 also plays an important role in maintaining Sertoli cell identity at later stages. *Nr5a1* gene disruption in differentiated Sertoli cells has been achieved using *Amh-Cre* or *Sox9-Cre*, and the phenotypes of these mice suggested that NR5A1 is essential for fetal Sertoli cell survival.[102,103] A more recent study reported that loss of NR5A1 induces anoikis of Sertoli cells and disorganization of testis cords in mice.[104]

6.3 | Developmentally distinct Leydig cell populations in mammals

It is well known that Leydig cells in the fetal testis (fetal Leydig cells, [FLCs]) demonstrate distinct morphological and functional features compared to Leydig cells in the adult testis (adult Leydig cells, [ALCs]). For example, FLCs lack 17β-HSD activity and synthesize androstenedione but not testosterone. Instead, fetal Sertoli cells express 17β-HSD enzyme and convert androstenedione to testosterone in the fetal testis.[105,106] Additionally, ALC development depends on androgen receptor (AR) signaling, while FLCs develop independently of AR signaling.[107,108] Moreover, FLC differentiation is independent of pituitary gonadotropins, whereas functional differentiation of ALCs is largely dependent on the pituitary in rodents.[109]

Mechanisms of FLC differentiation have been investigated using several knockout mice. These studies indicated that FLC differentiation is induced by several growth factors, such as desert hedgehog (DHH)[110] and platelet-derived growth factor (PDGF).[111] Inoue et al. isolated FLC progenitor cells from the fetal testis[112] and performed single-cell analyses. This study dissected molecular mechanisms downstream of DHH and PDGF, revealing that thymosin β 10 (TMSB10) plays an important role by suppressing the RAS/extracellular regulated protein kinase pathway.[113] NR5A1 is also essential for the differentiation of FLCs. In fact, when NR5A1 is specifically knocked out in FLCs, their differentiation is significantly impaired and ultimately abolished.[114] However, the functional relationship between NR5A1 and DHH, PDGF, and TMSB10 has not yet been clarified.

After playing pivotal roles in the masculinization of fetuses, FLCs persist in the postnatal testis and eventually intermingle with ALCs to form a heterogeneous Leydig cell population (Figure 5).[108] The developmental link between FLCs and ALCs has long been a subject of debate. Shima et al. suggested the possibility that some FLCs dedifferentiate and then give rise to ALCs after birth.[114] This hypothetical model is quite similar to the developmental link between the fetal cortex and definitive cortex in the adrenal gland in two aspects. Firstly, fetal steroidogenic cells enter an undifferentiated state and start to differentiate again at later stages. Secondly, fetal steroidogenic cells have the capacity to contribute to the adult steroidogenic cell population within a limited developmental time window. The most recent study by Shen et al. suggested that TCF21-positive cells retain the potential to differentiate into ALCs in the adult testis.[115] Further studies are expected to reveal the lineage relationships among FLCs, TCF21-positive mesenchymal cells, and ALCs in the normal developmental process of the testis.

6.4 | *Nr5a1* gene regulation in fetal and adult Leydig cells

Shima et al. identified a fetal Leydig cell enhancer (FLE) in the upstream region of the *Nr5a1* gene (Figure 1).[116] The FLE can induce gene expression exclusively in FLCs but not in ALCs. However, lineage tracing of FLCs revealed that FLCs contribute to at least part of the ALC population. Furthermore, disruption of the *Nr5a1* gene using FLE-Cre resulted in the loss of FLCs and a significant decrease in ALCs. Most interestingly, deletion of the FLE by genome editing led to the complete disappearance of both FLCs and ALCs.[114] These findings led us to consider two possibilities: first, that FLCs give rise to ALCs, and therefore the loss of FLCs leads to the loss of ALCs; and second, that NR5A1 expression in ALCs is regulated by a combination of the FLE and another ALC-specific enhancer. However, such a regulatory region for *Nr5a1* expression in ALC has not yet been identified.

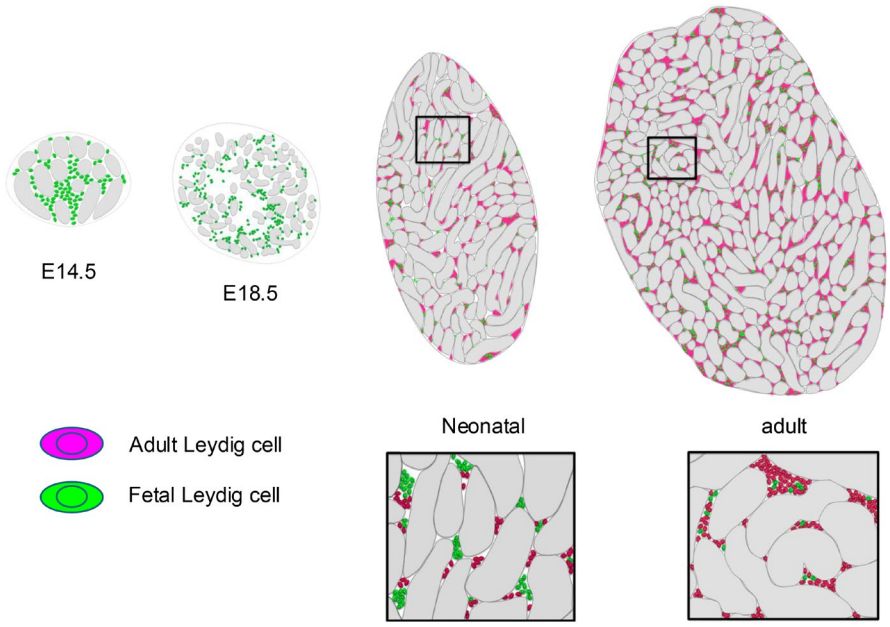

FIGURE 5 Two distinct Leydig cell populations in the developing testis. Schematic illustrations of fetal Leydig cells (green) and adult Leydig cells (magenta) in the developing testis. Fetal Leydig cells first appear at embryonic day 12.5 (E12.5) in the interstitial space of the testis and increase in number throughout fetal stages, such as E14.5 and E18.5. Adult Leydig cells emerge after birth and increase in number exponentially during puberty; however, a small population of fetal Leydig cells persists in the adult testis.

6.5 | NR5A1 function in Leydig cells

As previously mentioned, NR5A1 is essential for the functional differentiation of both FLCs and ALCs. However, the overall picture of which downstream gene NR5A1 regulates during the fate determination and differentiation of Leydig cells remains unclear. Similar to adrenocortical cells, analyses of cultured cells have shown that NR5A1 directly regulates genes necessary for testosterone synthesis, such as *Star*, *Cyp11a1*, *Cyp17a1*, and *Hsd3b1*. Indeed, when NR5A1 expression is suppressed in vitro or in vivo, *Cyp11a1* gene expression is significantly reduced, and lipid accumulation is observed in Leydig cells.[117] There is also a report that NR5A1 directly regulates Eps15 homology domain-containing protein 3 (EHD3), which is involved in endocytosis, thereby modulating testosterone production.[118] However, since these reports are fragmented, comprehensive genome-wide analyses, similar to those on the adrenal cortex, are anticipated for Leydig cells as well.

7 | OVARY

NR5A1 expression in the ovary is observed in granulosa cells, luteal cells, and theca cells. Among these, granulosa and theca cells are considered counterparts to testicular Sertoli and Leydig cells, respectively. Although there are some similarities between these counterparts, several important differences have also been reported.

7.1 | Heterogeneity and *Nr5a1* regulation in ovarian granulosa cells

Unlike Sertoli cells, lineage tracing analyses by Mork et al. suggested that murine granulosa cells consist of two developmentally distinct populations. According to their result, pre-granulosa cells migrated from the ovarian surface epithelium and contributed to follicle formation. The migration of surface epithelial cells has bimodal peaks during early fetal stages and neonatal stages. Early migrators contribute to the follicles in the medullary region, and some of these relatively larger follicles in the neonatal ovary contribute to the first cycle of ovulation and trigger the postpubertal ovulatory cycle. Meanwhile, surface epithelial cells that migrate postnatally contribute to primordial follicles in the cortex, close to the ovarian surface. These follicles serve as a reservoir for ovulation throughout life until menopause.[119]

The mechanisms underlying NR5A1 expression in the ovary have not been elucidated as thoroughly as those in the testis. What is known is that the expression of enhanced green fluorescent protein (EGFP) can be induced in all NR5A1-expressing cells throughout the body, including granulosa, theca, and luteal cells in the ovary, using an approximately 200-kb BAC clone containing the *Nr5a1* gene and neighboring gene loci (Figure 1). This suggests that the regulatory region for *Nr5a1* expression in the ovary is also included within this 200-kb region, and further analysis is expected in the future.

7.2 | Heterogeneity and *Nr5a1* regulation in ovarian theca cells

The study by Mork et al. suggests that ovarian interstitial cells, including theca cells, also migrate from the ovarian surface epithelium.[119] According to this hypothesis, there would be cell populations in the theca cells that arise at different developmental stages. Meanwhile, Miyabayshi et al. performed histological analysis of the ovaries of FLE-EGFP mice. In these mice, EGFP expression is induced in FLCs but not in ALCs in the testis, whereas EGFP expression is observed only in a subset of theca cells in the ovary. These results suggest the possibility that ovarian theca cells are composed of heterogeneous cell populations,[120] although the functional differences between cell populations have not been elucidated.

TABLE 1 Summary of the heterogeneity, functions, downstream genes of NR5A1, and upstream regulators of *Nr5a1* gene in NR5A1-expressing tissues.

Tissue	Function	Downstream genes	*Nr5a1* gene regulation
VMH	Migration and aggregation of VMH neurons	*A2bp1*, *Amigo2*, *Cdh4*, *Nptx2*, *Sema3a*, *Slit3*, *Netrin3*	Homeobox TFs which bind to the VMH enhancer in the intron 6
	Regulation of energy expenditure	*Cnr1*, *Bdnf* (?)	
	Regulation of female sexual behavior	Unknown	
	Diet-induced thermogenesis		
	Maternal behavior		
	Anxiety-like behavior	*Cnr1*	
Pituitary gonadotrope	Gonadotropin secretion	*Cga*, *Lhb*, *Fshb*, *Gnrhr*, *Spp1*, *Grem1*, *Tgfbr3l*	PITX2 that binds to the pituitary enhancer in intron 6 Estrogen receptor that binds to the α enhancer in intron 4 Musashi1 that binds to the 3′-UTR
Splenic vascular endothelium	RBC filtering (splenic sinus) Reaction to blood-borne antigens (marginal sinus)	Unknown	M33
Fetal cortex of adrenal gland (X-zone)	Catabolism of progesterone and 11-deoxycorticosterone (mice) Production of DHEA-S and 11-oxygenated androgens (humans)	*Akr1c18* (?)	HOX-PBX1-PREP1 and NR5A1 that bind to the fetal adrenal enhancer in intron 4
Definitive cortex of adrenal gland	Production of mineralocorticoid, glucocorticoid, and adrenal androgens	*Star*, *Cyp11a1*, *Hsd3b1*, *Cyp17a1*, *Cyp21a1*, *Cyp11b1*, *Cyp11b2*, *Akr1b*, *Scarb1*, *Mc2r*, *Nr0b1*, *Sult2a1*, *Adcy4* (mice) *VSNL1*, *ZIM2*, *PEG3*, *SOAT1*, *MTSS1* (humans)	Unknown
Sertoli cell	Initial differentiation and identity maintenance at later stages	Sox9 (mice) SRY (humans?)	WT-1, LHX9, USF1, USF2
Fetal Leydig cell	Masculinization of fetuses (androstenedione production)	*Star*, *Cyp11a1*, *Hsd3b1*, *Cyp17a1*, *Insl3*	Fetal Leydig enhancer in intron 6
Adult Leydig cell	Induction of male-specific secondary sexual characteristics, sperm maturation (testosterone production)	*Star*, *Cyp11a1*, *Hsd3b1*, *Cyp17a1*, *Hsd17b3*, *Insl3*, *Ehd3*	Unknown
Granulosa cell	Early differentiation of granulosa cells	Unknown	Unknown
Theca cell	Androgen production	*Star*, *Cyp11a1*, *Insl3*	Unknown

7.3 | NR5A1 function in granulosa and theca cells

NR5A1 plays an indispensable role in granulosa differentiation, as evidenced by the fact that granulosa cell-specific *Nr5a1* gene disruption results in impaired follicle development and infertility.[121] Recent studies have reported that NR5A1, in combination with other transcription factors, can transform human fibroblasts or iPS cells into granulosa-like cells, supporting the central role of NR5A1 in granulosa cell differentiation.[122,123]

Several studies have focused on the function of another nuclear receptor, liver receptor homolog-1 (LRH-1, officially NR5A2), in the ovary. This factor is expressed in granulosa and luteal cells but not in theca cells[124] and has been shown to activate parts of the steroid hormone synthesis pathways in vitro.[125] Conditional disruption of *Nr5a2* in granulosa cells does not affect primordial follicle formation at perinatal stages.[126] However, several studies have demonstrated the functional importance of LRH-1 in the late stages of follicle development, such as primordial follicle activation,[127] ovulation,[128] and luteinization.[129] These results contrast with those of NR5A1, which plays a key role in early granulosa cell differentiation and the establishment of follicular reserve that guarantees lifetime fertility in females.[126]

Ovarian theca cells are the main source of androgens, which are subsequently transported to granulosa cells and aromatized into estrogens. Similar to the adrenal cortex and testicular Leydig cells, NR5A1 regulates steroidogenic genes such as *Star*, *Cyp11a1*, and *Insl3* in response to LH in isolated theca cells.[130] Female mice with theca cell-specific *Nr5a1* gene deletion showed significantly lowered steroidogenic gene expression, confirming the importance of NR5A1 in theca cells in vivo.[131]

8 | CONCLUSION AND FUTURE PERSPECTIVE

In this review, we have summarized previous reports on the functional heterogeneity of cell populations and the potential differences in gene cascades centered on the *Nr5a1* gene in various tissues expressing NR5A1 (Table 1). The most well-documented examples include the adrenal cortex and testicular Leydig cells. These tissues have long been known to contain morphologically and functionally distinct cell populations, such as the fetal and definitive cortex in the adrenal gland and FLCs and ALCs in the testes. Recent analyses have revealed that the regulatory mechanisms of NR5A1 expression, a key factor in the development of these tissues, differ between the fetal and adult stages. This suggests that differences in gene cascades centered around the *Nr5a1* gene are crucial for the developmentally and functionally distinct differentiation of these cell populations. However, the regulatory regions of *Nr5a1* during the mature phase in the adrenal cortex and testicular Leydig cells—often referred to as adult enhancers—have not yet been identified, and the mechanisms for maintaining *Nr5a1* expression during the mature phase remain unclear. Furthermore, the developmental relationship between fetal and adult populations is not yet fully understood, necessitating further analysis, including cell lineage tracing.

In addition to the fetal and mature stages, recent attention has been drawn to the increase in steroid hormone production during the neonatal period. The increase in sex hormones during the neonatal period, known as mini-puberty, is prominent in males but also occurs in females.[132,133] Histological analysis of human neonatal testes suggests that neonatal-type Leydig cells are present during this period and are responsible for the increased production of androgens[134,135]; however, the origin and differentiation mechanisms of neonatal Leydig cells remain unclear. Similarly, the transient production of DHEA and DHEA-S in the adrenal cortex during the neonatal period, a phenomenon referred to as adrenarche, is thought to be induced by the activation of the zR of the adrenal cortex, but the molecular mechanisms triggering this process remain unknown.[136–138] Understanding the role of NR5A1 and its regulatory mechanisms in these transient neonatal phenomena is considered a significant topic for future research.

Functional heterogeneity of NR5A1-expressing cells is also strongly suggested in other tissues. For instance, differences in gene expression between individual VMH neurons have been reported. Additionally, in splenic vascular endothelial cells, it is anticipated that gene expression may differ between the marginal sinus and splenic sinus. In ovarian granulosa and theca cells, numerous reports suggest differences in the timing of differentiation and function between individual cells. Clarifying the regulatory mechanisms of *Nr5a1* gene expression, in conjunction with these findings, is considered one of the major challenges for future research in developmental biology, endocrinology, and reproductive biology.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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