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Review

Hormones and Signaling Pathways Involved in the Stimulation of Leydig Cell Steroidogenesis

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Abstract: Leydig cells, located in the testis interstitial space, are the primary source of testosterone in males. Testosterone plays critical roles in both reproductive and metabolic functions and therefore is essential for male health. Steroidogenesis must be properly regulated since dysregulated hormone production can lead to infertility and metabolic disorders. Leydig cell steroidogenesis relies on the coordinated interaction of various factors, such as hormones and signaling molecules. While luteinizing hormone (LH) is the main regulator of Leydig cell steroidogenesis, other molecules, including growth hormones (GH), prolactin, growth factors (insulin, IGF, FGF, EGF), and osteocalcin, have also been implicated in the stimulation of steroidogenesis. This review provides a comprehensive summary of the mechanisms and signaling pathways employed by LH and other molecules in the stimulation of Leydig cell steroidogenesis, providing valuable insights into the complex regulation of male reproductive and metabolic health.

Keywords: Leydig cell; steroidogenesis; testosterone; androgens; LH; STAR; fertility; testis; kinase; transcription factor

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1. Introduction

Steroid hormones play a vital role in various physiological processes, including growth and development, reproduction, and metabolism. Steroid hormones are synthesized by different endocrine glands and organs, and their secretion is tightly regulated to maintain homeostasis. Among the organs involved in steroid hormone synthesis, the testes play a crucial role in male physiology through the production of androgens by Leydig cells. Leydig cells, located in the testis interstitial compartment, are responsible for the production and secretion of testosterone and insulin like-3 (INSL3), two critical hormones for reproductive function and overall health in men. In addition to regulating male fertility, testosterone also plays essential roles in the development of secondary sexual characteristics, muscle mass, bone density, and red blood cell production (reviewed in [1]).

The homeostasis of steroidogenesis is of paramount importance for male health. Testosterone deficiency or excess can result in differences of sex development (DSD), as well as other pathologies in men, including infertility, erectile dysfunction, osteoporosis, and metabolic disorders. Testosterone synthesis and secretion in males is therefore tightly regulated by multiple signaling pathways, and not surprisingly, understanding these pathways remain a topic of active research.

In contrast to endocrine cells that produce polypeptide hormones, steroidogenic cells store minimal amounts of steroid hormones and lack secretory vesicles for rapid release (reviewed in [2]). Therefore, a prompt steroidogenic response involving the coordinated action of several pathways is required for rapid de novo synthesis of steroid hormones.

This review provides a comprehensive summary of the current state of knowledge of signaling pathways involved in the stimulation of Leydig cell steroidogenesis.

2. Main Factors Involved in the Stimulation of Leydig Cells

2.1. Luteinizing Hormone

Luteinizing hormone (LH) belongs to the glycoprotein hormone family, which also comprises follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH) [3]. LH is a glycoprotein heterodimer composed of an alpha and beta subunit. While the alpha subunits of LH, FSH, and TSH are identical, the beta subunit of each hormone is distinct and confers its unique biological effects [3].

Gonadotrope cells located in the anterior pituitary gland synthesize and secrete LH in a pulsatile manner due to hypothalamic GnRH, which is also secreted in a pulsatile manner. LH, along with FSH and placental chorionic gonadotropin (CG), are classified as gonadotropins due to their effects on the gonads. LH is a crucial component of the hypothalamic-pituitary-gonadal (HPG) axis that links the nervous system with the gonads. The HPG axis is controlled by a classic negative feedback loop where circulating testosterone secreted by the testes continuously feeds back to the hypothalamus and pituitary to adjust GnRH and LH output. In males, the LH receptor is primarily present in Leydig cells. Although CG is exclusively found in primates and equids, the LH receptor recognizes both LH and CG and is thus referred to as the luteinizing hormone/chorionic gonadotropin receptor (LHCGR) (reviewed in [4]). The LHCGR receptor, along with other glycoprotein hormone receptors, belongs to the G protein-coupled receptor superfamily. In Leydig cells, the binding of LH to LHCGR induces a conformational change in the receptor, initiating a signaling cascade that ultimately results in increased testosterone synthesis.

Multiple mouse models have been utilized to elucidate the functions of LH in Leydig cells, including GnRH-deficient mice and knockout mice for LH and LHCGR. GnRH-deficient hypogonadal (hpg) mice have significantly decreased pituitary and plasma gonadotropins, which directly affect the reproductive system. This condition results in the development of cryptorchidism, accompanied by underdeveloped testes and a significantly diminished number of Leydig cells (approximately 10% of normal values), and ultimately infertility [5,6]. Similarly, *Lhb* and *Lhcgr* knockout mice are infertile, have reduced levels of serum and intratesticular testosterone, smaller testes and accessory glands, and possess only a few Leydig cells [7–10] (reviewed in [11]). In addition, *Lhcgr* knockout mice exhibit cryptorchidism and increased serum LH levels [7,8]. Sexual differentiation and fetal gonadal development are, however, normal in the knockout models, indicating that, unlike humans, fetal testosterone production required for masculinization is not dependent on gonadotropins in mice.

Naturally occurring mutations and polymorphisms in the *LHB* and *LHCGR* genes have been identified in humans (reviewed in [12]). Most mutations are in *LHCGR* and only a few in *LHB* (reviewed in [11,12]). Inactivating mutations in *LHCGR* lead to a failure of Leydig cell differentiation, resulting in Leydig cell hypoplasia (LCH). Males with inactivating mutations in *LHB* are normally masculinized at birth but later present delayed or lack of spontaneous puberty that is accompanied by hypogonadism, low testosterone levels, and infertility (reviewed in [11,12]). As expected, the phenotype of the homozygous *Lhb* knockout male mice closely mimics that of humans harboring inactivating *LHB* mutations [9] (reviewed in [12]). These findings indicate that LH participates actively in the control of Leydig cell differentiation, steroidogenesis, and male fertility.

The binding of LH to LHCGR on the surface of Leydig cells activates multiple pathways, which trigger downstream signaling cascades via G-proteins (Figure 1). In the text that follows, we provide an overview of the primary pathways downstream of LH that promote Leydig cell steroidogenesis, which include PKA, CAMKI, ERK1/2, PKC and PKB/AKT.

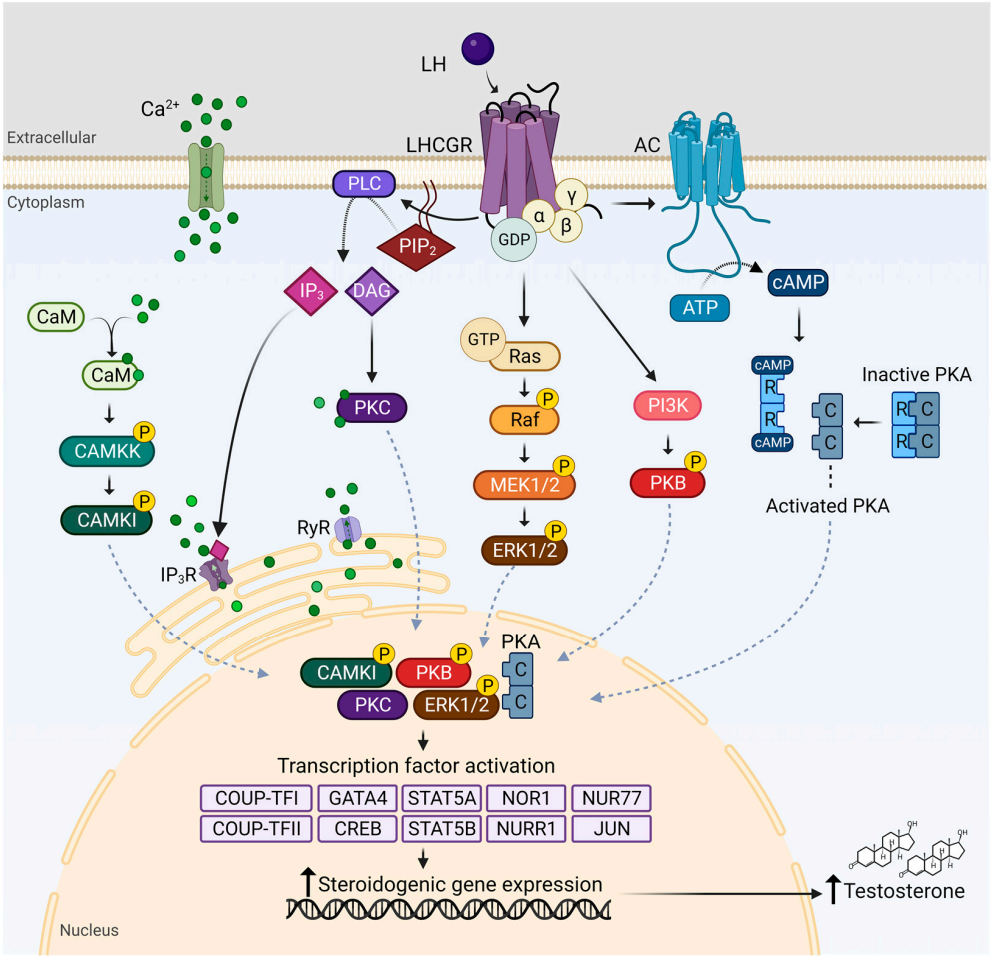

Figure 1. Key pathways in LH-mediated steroidogenesis in Leydig cells. Upon LH binding to LHCGR, multiple pathways are activated: (i) LHCGR activation stimulates adenylate cyclase (AC), increasing the conversion of ATP to cyclic AMP (cAMP). Elevated cAMP levels activate protein kinase A (PKA) by releasing its catalytic subunits (C) from its regulatory subunits (R). (ii) LHCGR activation triggers phospholipase C (PLC), resulting in the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) in diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). DAG activates protein kinase C (PKC), while IP_3 binds to its receptor (IP_3R) in the endoplasmic reticulum (ER), leading to calcium (Ca^{2+}) release. The ryanodine receptor (RyR) in the ER also facilitates Ca^{2+} release. Calcium can also enter the cell through plasma membrane channels. Calcium-bound calmodulin (CaM) activates calcium/calmodulin-dependent protein kinase kinase I (CAMKKI), which phosphorylates calcium/calmodulin-dependent protein kinase I (CAMKI). (iii) LH binding to its receptor activates Ras, subsequently activating the MAPKKK Raf. This activation triggers the phosphorylation of MAPKKs (MEK1/2) and MAPKs (ERK1/2). (iv) The activation of LHCGR activates phosphatidylinositol-3-kinase (PI3K), which phosphorylates protein kinase B (PKB). Once activated, these kinases translocate to the nucleus (indicated by grey dashed arrows), where they phosphorylate various transcription factors, such as COUP-TFI, COUP-TFII, GATA4, CREB, STAT5A, STAT5B, NOR1, NURR1, JUN and NUR77. This leads to the upregulated expression of genes involved in steroidogenesis and, ultimately, increased testosterone production. See text for references.

2.1.1. cAMP-Dependent Protein Kinase

Activation of LHCGR/G-proteins stimulates adenylate cyclase (AC), which increases the conversion of ATP to cyclic AMP (cAMP). cAMP acts as a second messenger, activating cAMP-dependent protein kinase A (PKA). This signaling pathway is commonly known as the Gs/AC/cAMP/PKA pathway (reviewed in [13]). PKA is a ubiquitous serine/threonine protein kinase that recognizes a consensus sequence RRXS*/T*Hpo in target proteins,

where "*" represents the phosphorylation site and "Hpo", a hydrophobic residue [14] (reviewed in [15]).

Since its discovery in 1968 [16], PKA has been linked to several cellular functions such as growth and cell division [17], metabolism and regulation of energy balance (reviewed in [18]), cell differentiation [19], transcriptional regulation [20], and spermatogenesis and sperm motility [21]. In an inactive state, PKA exists as a tetrameric holoenzyme composed of two regulatory subunits (R) bound to two catalytic subunits (C) (reviewed in [15]). The binding of cAMP to the PKA regulatory subunits leads to the release of the catalytic subunits from the regulatory subunits [22,23]. Over the years, two different forms of PKA, referred to as PKA type I and type II, have been identified. These isoforms share the same catalytic subunit but possess different regulatory subunits (RI and RII) [24].

In mice, inactivation of the *Prkaca* gene, which encodes the catalytic alpha subunit (Cα) of PKA, leads to partial lethality, with less than 30% of animals surviving to adulthood [25]. While testis size in these mice appears normal compared to their body weight, they nonetheless exhibit a reduction in sperm count and an increase in abnormal spermatozoa [25]. In humans, around 65% of Carney complex (CNC) tumor patients exhibit haploinsufficiency of the RIα gene (*PRKAR1A*), which correlates with a notable decrease in fertility among male CNC patients [26,27].

In the mouse MA-10 Leydig cell line, active PKA translocates to the nucleus, where it phosphorylates multiple transcription factors, such as GATA4 and bZIP family members. Phosphorylation of GATA4 at Ser261 leads to increased GATA4-dependent activation of several gene promoters such as *Star*, *Cyp17a1*, *aromatase*, and *Inha* (*inhibin α*) [28,29]. More recently, we have shown that PKA also cooperates with COUP-TFII (NR2F2) and STAT5B to activate the *Star* promoter in MA-10 Leydig cells [29].

2.1.2. Calcium

LHCGR activation in Leydig cells triggers the production of inositol 1,4,5-triphosphate (IP_3), which in turn binds to its receptor (IP_3R), resulting in the release of calcium (Ca^{2+}) [30]. Ca^{2+} release alters cytosolic Ca^{2+} concentration, playing a crucial role in facilitating steroidogenesis. Calcium serves as a key second messenger, controlling various cellular functions such as autophagy, apoptosis, and cell proliferation (reviewed in [31]). The levels of cytosolic Ca^{2+}, directly and indirectly, regulate the activity of proteins and enzymes. Cells actively store Ca^{2+} at high concentrations, particularly in the endoplasmic reticulum (ER), due to its essential role in intracellular processes (reviewed in [32]). The release of Ca^{2+} from the ER is facilitated by the ryanodine receptor (RyR) and the IP_3R, acting as the primary channels responsible for this process [30]. These channels rapidly release Ca^{2+} into the cytoplasm, providing the cell with the necessary Ca^{2+} for the execution of various intracellular processes, including fertilization, gene transcription, muscle contraction, exocytosis and cell differentiation, proliferation and motility (reviewed in [33]). Extracellular Ca^{2+} can also enter through plasma membrane channels, thus serving as an additional source of Ca^{2+} for the cell.

Studies using rat Leydig cells have revealed that both LH and dibutyryl cyclic AMP (db-cAMP), an analog of cAMP, increase intracellular Ca^{2+} concentration through extracellular and intracellular sources [34,35]. Additionally, stimulation of MA-10 Leydig cells with forskolin (Fsk) increases intracellular Ca^{2+} levels, resulting in enhanced steroid production [36]. Conversely, inhibition of RyRs in these cells leads to reduced steroidogenesis. This inhibition specifically impacts the activity of the cAMP-induced *Star* promoter, leading to a decrease in STAR production and subsequently affecting the translocation of cholesterol from the outer to the inner mitochondrial membrane [36].

Calmodulin (CaM), a protein highly responsive to Ca^{2+} levels, serves as an upstream activator for several calcium-dependent proteins, including calcium/calmodulin-dependent protein kinases I, II and IV (CAMKI, CAMKII, and CAMKIV) (reviewed in [37]). Among the calcium/calmodulin-dependent protein kinases, CAMKI has been identified as the main CAMK that significantly influences steroidogenesis in MA-10 Leydig cells [38].

Calcium/Calmodulin-Dependent Protein Kinase I

CAMKI, the smallest member of the Ca^{2+}/CaM-dependent protein kinase family, is a versatile serine/threonine-specific multifunctional protein kinase. It recognizes a consensus sequence BXRXX(S/T)XXXB, where B represents a hydrophobic amino acid, and X can be any amino acid [39]. Structurally, CAMKI is a monomeric enzyme consisting of an N-terminal catalytic domain and a C-terminal regulatory domain. The N-terminal domain includes the ATP-binding domain (residue 1–100) and the substrate-binding domain (residue 101–275). In the absence of Ca^{2+}, CAMKI remains in an autoinhibited state due to the folding of the regulatory domain over the catalytic domain (reviewed in [40]). The key factor governing the maximal activation of CAMKI involves two crucial steps. First, there must be an elevation in the intracellular concentration of Ca^{2+}. Second, CAMKI kinase (CAMKKI) phosphorylates a specific residue, Thr177, in CAMKI [41]. These two events act as limiting factors in achieving peak CAMKI activation.

CAMKI is the most recently identified kinase in Leydig cells and has emerged as a subject of interest in recent studies, particularly in MA-10 Leydig cells. Although our understanding of CAMKI action in Leydig cell function is still in its early stages, emerging evidence suggests it is involved in crucial cellular processes. Previous studies have revealed that CAMKI acts as an activator, collaborating with multiple proteins, including transcription factors, to enhance the expression of genes associated with steroid hormone production [29] (reviewed in [42,43]). Some of the transcription factors found to cooperate with CAMKI include members of the nuclear receptor family NR4A (NUR77/NR4A1, NURR1/NR4A1, NOR1/NR4A3), NRF2 members (COUP-TFI/NR2F1 and COUP-TFII/NR2F2), GATA4, STAT5B, CREB, and cJUN. These findings highlight the multifunctional nature of CAMKI and its potential role in regulating steroidogenesis downstream of LHCGR activation in Leydig cells.

2.1.3. Protein Kinase C

Protein kinase C (PKC) is another important kinase that is activated by the binding of LH/hCG to its receptor. One of the signaling pathways triggered by LHCGR activation involves phospholipase C (PLC). Through the process of hydrolysis, PLC breaks down phosphatidylinositol 4,5-bisphosphate (PIP_2), resulting in the release of diacylglycerol (DAG) and IP_3 [44]. DAG plays a vital role as a potent activator of PKC, while IP_3 leads to an increase in Ca^{2+} levels, as mentioned above (see calcium section).

PKC is a family of serine/threonine protein kinases that are involved in several cellular functions, including cell proliferation and differentiation (reviewed in [45]), autophagy and apoptosis [46], and regulation of chloride channel in the human kidney [47].

The PKC family comprises several isozymes that are classified into four subfamilies based on their activation mechanism: cPKC, nPKC, aPKC and PKN [48]. Each subfamily shares a common structural organization consisting of a regulatory domain, a highly conserved catalytic domain, and variable regions (reviewed in [48–50]). Importantly, all PKC isozymes recognize the consensus phosphorylation sites (R/K)X(S/T), (R/K)(R/K)X(S/T), (R/K)XX(S/T), (R/K)X(S/T)(R/K), and (R/K)XX(S/T)XR/K in target proteins (reviewed in [50]).

The conventional or classic cPKC subfamily proteins consist of cPKCα, βI, βII, and γ, which are activated by DAG, phosphatidylserine, and Ca^{2+}. In contrast, the novel or non-classic nPKC subfamily proteins include nPKCδ, ε, η, and θ, which are activated by DAG and phosphatidylserine but not Ca^{2+} due to the absence of Ca^{2+} coordinating residues. The atypical subfamily of aPKC proteins comprises aPKCζ, ι, and λ. These aPKC members contain a PBI domain that confers specificity in intracellular signaling by interacting with scaffold proteins. Unlike other subfamilies, aPKC kinases are not activated by DAG, as their C1 domain does not bind DAG. Lastly, the PKN subfamily includes PKN1, PKN2, and PKN3, which all contain an HRI domain that mediates their activation by Rho proteins (reviewed in [48–50]).

MA-10 Leydig cells express several PKC isozymes, including PKCα, βI, βII, δ, γ, ε, θ and ζ [51,52]. Furthermore, studies in MA-10 Leydig cells have shown that phorbol 12-myristate 13-acetate (PMA), an analog of DAG, can increase the levels and phosphorylation of PKCα, δ, and ε, which was also associated with an increase in the level of STAR protein and progesterone production [51,53]. In R2C Leydig cells, inhibition of PLC and PKC leads to a significant decrease in steroid production and phosphorylation of cAMP-responsive element binding (CREB) [54]. This decrease is accompanied by a reduction in *Star* gene transcription [54]. The PKC pathway also influences the orphan nuclear receptor DAX1/NR0B1, which modulates *Star* expression [55].

2.1.4. Extracellular Signal-Regulated Kinase

Extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2) are serine/threonine kinases, members of the MAP kinase (MAPK) signaling cascade. This pathway is involved in many cellular functions such as growth, proliferation, cell differentiation, mitosis, metabolism and apoptosis (reviewed in [56,57]). Although ERK1 and ERK2 have minor differences, they share numerous functions and are collectively referred to as ERK1/2. The activation of ERK1/2 is mediated through a sequential cascade of reactions known as the Ras/Raf/MEK/ERK1/2 signaling pathway. In response to extracellular stimuli, Ras is activated, subsequently activating the MAPKKK Raf. This, in turn, triggers the activation of two MAPKKs, MEK1 and MEK2, which phosphorylate the MAPKs ERK1/2. Once activated, ERK1/2 translocates to the nucleus, where it phosphorylates and activates transcription factors that influence gene expression, recognizing preferentially a consensus sequence PXS/TP (reviewed in [58,59]).

Several studies have explored the role of ERK1/2 in Leydig cells, revealing its involvement in steroidogenesis and the proliferation of postnatal Leydig cells [55,60,61] (reviewed in [62]).

A *Cyp17a1*-iCre mouse line, which expresses the Cre recombinase in steroidogenic tissues, including Leydig cells, was utilized to conditionally inactivate *Mek1* and *Mek2* [61,63]. MEK1/MEK2-deficient male mice exhibit a reduction in the number of Leydig cells compared to control animals. Moreover, testosterone levels upon hCG stimulation are reduced in these mice, along with a significant decrease in the expression of key steroidogenic genes, such as *Star*, *Hsd3b6*, *Cyp17a1* and *Hsd17b3* [61,63]. These findings demonstrate that ERK1/2 plays a pivotal role in the regulation of steroidogenesis.

In various Leydig cell models, including MA-10, MLTC-1, and primary rat Leydig cells, it has been observed that activation of LHCGR induces Ras activation, subsequently leading to the phosphorylation of ERK1/2 [64,65]. In addition, treatment of MA-10 and MLTC-1 Leydig cells with two different MEK inhibitors, U0126 and PD98059, significantly reduces LH-induced steroidogenesis while increasing STAR protein levels, but not its phosphorylation [55,65].

In MA-10 Leydig cells, ERK1/2 has been found to functionally cooperate with STAT5B and GATA4 to activate the *Star* promoter [29]. The ERK1/2-GATA4 cooperation on *Star* is consistent with a study conducted in rat primary cardiomyocyte cells, which revealed that ERK1/2 phosphorylates GATA4 at Ser105 [66]. Interestingly, mice carrying a GATA4 S105A mutation exhibit a substantial decrease in plasma and intratesticular testosterone levels, indicating the crucial role of this phosphorylation in the regulation of testosterone [67].

2.1.5. Protein Kinase B

Following LHCGR activation, another kinase called protein kinase B (PKB), also known as AKT is activated. PKB is a widely expressed serine/threonine protein kinase that comprises three isoforms: PKBα/AKT1, PKBβ/AKT2, and PKBγ/AKT3 [68–71]. The PKB/AKT isoforms phosphorylate target proteins at a consensus sequence known as RXRXX(S/T) (reviewed in [72]). Since its discovery, PKB/AKT has been associated with several cellular processes, including glucose metabolism, cell proliferation, apoptosis, gene transcription, and cell migration (reviewed in [72]). The activation of PKB/AKT

depends on the upstream kinase phosphatidylinositol-3-kinase (PI3K), a lipid kinase that phosphorylates PKB/AKT on Thr308 and Ser473 [73].

In MA-10 Leydig cells, hCG stimulation leads to the activation of PKB/AKT, which is associated with increased levels of several genes and proteins important for steroidogenesis, including STAR, JUNB, and NUR77/NR4A1 [74]. Consistent with this, activation of PKB/AKT in MA-10 Leydig cells results in increased production of steroid hormone [74].

2.2. Growth Hormone (GH)

In addition to LH, other hormones are known to stimulate Leydig cell steroidogenesis (Figure 2), including growth hormone (GH). GH is a crucial peptide hormone that regulates several essential physiological processes in the body. It is primarily secreted by somatotrope cells located in the anterior pituitary, but local production of GH by many tissues has also been reported (reviewed in [75]). GH mediates its effects by binding to the GH-receptor (GHR) and, in some species, including humans, to the prolactin receptor (PRLR) [76,77] (reviewed in [78]).

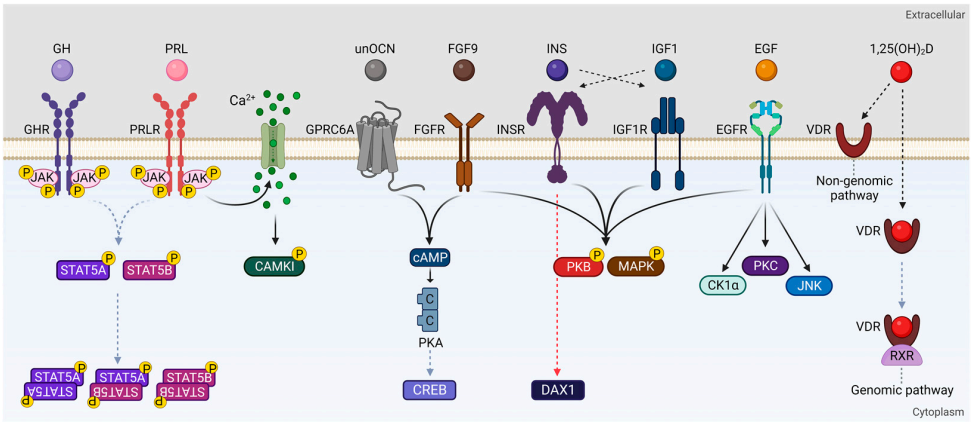

Figure 2. Additional signaling pathways implicated in the stimulation of Leydig cell steroidogenesis. Several hormones and molecules contribute to increased steroid production in Leydig cells. Upon growth hormone (GH) binding to the GH-receptor (GHR), the receptor becomes activated, leading to the formation of GHR dimers. Prolactin receptor (PRLR) is also activated by prolactin (PRL) binding. Both active GHR and PRLR induce the activation of JAKs, which phosphorylate STAT5A and STAT5B transcription factors. Phosphorylated STAT5s hetero- and homodimerize, and translocate to the nucleus where they regulate gene transcription. In addition to GH, other growth factors involved in steroidogenesis are insulin (INS), insulin-like growth factor 1 (IGF1), fibroblast growth factor (FGF), and epidermal growth factor (EGF). Insulin and IGF1 actions are mediated through the activation of two related receptors: INS receptor (INSR) and IGF1 receptor (IGF1R). Both INS and IGF1 can bind to both receptors, albeit with different affinities. INS, IGF1, FGF9, and EGF trigger a series of events upon binding to its tyrosine kinase receptors, resulting in the activation of at least two pathways, phosphatidylinositol-3-kinase (PI3K)/protein kinase B (PKB) and MAPKs. Conversely, high levels of insulin activate DAX1/NR0B1, indicated by red dashed arrows, which represses hormone-induced steroidogenesis. FGF9 can also activate the protein kinase A (PKA) pathway. EGF receptor (EGFR) can activate protein kinase C (PKC), cJun N-terminal kinases (JNK) and casein kinase 1α (CK1α). In addition, undercarboxylated osteocalcin (unOCN) binds to and activates G protein-coupled receptor family C group 6-member A (GPRC6A) coupled to adenylate cyclase (AC). This leads to increased cAMP production, activation of PKA, and subsequent activation of the downstream transcription factor, CREB. Activated vitamin D ($1,25(OH)_2D$) acts through two pathways: non-genomic and genomic. In the non-genomic pathway, $1,25(OH)_2D$ binds to the vitamin D receptor (VDR) on the cell membrane, activating phospholipases, second messengers, and kinases. In the genomic pathway, $1,25(OH)_2D$ enters the cell, binds to the VDR in the cytoplasm, and forms a complex with the retinoid X receptor (RXR). This complex acts as a transcription factor, recognizing vitamin D response elements (VDREs) in target gene promoter regions. See text for references.

In contrast to dimeric glycoproteins such as gonadotropins, two receptors are needed to establish a trimeric structure composed of two membrane receptors and the GH molecule (reviewed in [79]). While the majority of human GHR is found in the liver, it is also abundant in all cellular components of the human reproductive system [80]. In males, GHR and GH binding proteins are present in the testes (Leydig and Sertoli cells), seminal vesicles, epididymis, vas deferens, and prostate [80].

GH acts both directly and indirectly to induce anabolic and metabolic responses in multiple target tissues. Directly, GH acts via the GHR, while indirectly, GH stimulates the production of insulin-like growth factor 1 (IGF1) not only in the liver but also in peripheral target tissues (reviewed in [78,81]).

Studies using animal models have revealed the importance of GH in male reproductive health (reviewed in [81]). GH-deficient male rats and mice exhibit smaller testes, underdeveloped secondary sex organs, delayed puberty, and reduced fertility rates [82–85]. In addition, male mice lacking GH have lower intratesticular testosterone levels and their ability to produce testosterone in response to LH is also diminished [82–84]. Stimulation of Leydig cells with GH increases *Star* gene expression and testosterone production [86–88]. Moreover, GH improves Leydig cell responsiveness to physiological hCG concentration (reviewed in [89]). In prepubertal male rats, treatment with recombinant hGH results in an increase in body weight, early onset of puberty, activation of spermatogenesis, Leydig cell differentiation and testosterone production [90]. These findings suggest that in the testis, GH mediates its effects, at least in part, by acting directly on Leydig cells.

Janus Kinase

Janus kinase (JAK) is a distinct family of tyrosine kinases that comprises four members: JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2). In mammals, JAK1, JAK2, and TYK2 are ubiquitous, while JAK3 is found mainly in bone marrow, the lymphatic system, endothelial cells, and vascular smooth muscle cells (reviewed in [91]). Members of the JAK family exhibit a unique structure with more than one kinase domain, contributing significantly to their functional versatility and regulatory capabilities (reviewed in [92]). In mice, inactivation of *Jak1* or *Jak2* leads to embryonic lethality, emphasizing their critical roles [93–95].

In the canonical pathway, binding of GH to GHR leads to receptor activation and formation of GHR dimers. This triggers the transphosphorylation of JAKs, which subsequently phosphorylate tyrosine residues on the bound receptor, creating a docking site for members of the Signal Transducers and Activators of Transcription (STATs) family of transcription factors. Upon docking, JAKs phosphorylate STATs, which then dissociate from the receptor. Phosphorylated STATs dimerize and translocate to the nucleus to regulate gene transcription. STATs bind to the promoter region of target genes, specifically to the γ-interferon-activated sequence (GAS; TTCNNNGAA) (reviewed in [91,96]).

Among the STAT family members, STAT5A and STAT5B have been identified in Leydig cells [97]. In MA-10 Leydig cells, GH treatment induces phosphorylation of STAT5B, facilitating its translocation to the nucleus [97]. A recent study conducted in MA-10 Leydig cells showed that activated-STAT5B increases *Star* transcription by directly binding to a GAS sequence and by cooperating with cJUN [87]. In addition, activated-STAT5B activates the *Nr4a1/Nur77* promoter in MA-10 Leydig cells [87]. Consistent with this, inhibition of JAK by tofacitinib in Leydig cells decreases STAT5B phosphorylation [98].

2.3. Prolactin

Prolactin (PRL) is a peptide hormone that is predominantly produced and secreted by lactotrope cells of the anterior pituitary gland. The structural similarities between the PRL and GH genes and receptors suggest a shared evolutionary origin, likely derived from a common ancestral precursor [99]. The role of PRL in females has been extensively studied and is mainly associated with lactation and mammary gland development. In the context of male reproductive physiology, several studies have provided evidence for

the involvement of PRL, particularly in Leydig cell steroidogenesis (reviewed in [100]). However, it is important to acknowledge that there is conflicting data regarding the precise role of PRL in Leydig cells, which can be attributed to the functional state of Leydig cells and the timing and dosage of PRL exposure.

In vitro studies have shown that PRL plays a significant role in male reproduction by potentially stimulating testicular steroidogenesis. PRL indirectly contributes to testosterone production in Leydig cells by modulating the release of gonadotropins from the pituitary gland (reviewed in [100]). PRL is also thought to directly regulate steroidogenesis by increasing the number of LH receptors in Leydig cells and enhancing the sensitivity of these cells to LH stimulation [101–105]. For instance, when plasma PRL levels are reduced, LH receptor levels in rat Leydig cells are also reduced [106–108]. In addition, in MA-10 Leydig cells, the influence of PRL on hCG-induced steroidogenesis was found to be biphasic; at low concentrations of PRL, steroidogenesis is stimulated, whereas, at high concentrations of PRL, steroidogenesis is inhibited [109]. On the other hand, genetic deletion of either the hormone (PRL-KO) or its receptor (PRLR-KO) in the mouse had no impact on male reproductive functions [110,111]. This lack of male reproductive phenotype could be due to compensation by other hormones, cytokines, or homologous receptors (reviewed in [100]).

The membrane receptor for PRL, PRLR, is present in Leydig cells across various species, including humans, mice, rats, and rams [112–115]. However, in humans, it appears that Leydig cells do not exhibit detectable PRL binding to interstitial cells [116]. Similar to GHR, activation of PRLR involves ligand-induced sequential receptor dimerization (reviewed in [117]). Upon PRL binding to PRLR and subsequent dimerization, a signaling cascade is initiated with the activation of JAK2, which in turn phosphorylates STAT5A and STAT5B (Figure 2). As described above, for GH action, phosphorylated STAT5B then translocates to the nucleus, where it activates gene transcription (reviewed in [117]). STAT5 was found to be involved in PRL signaling in the MA-10 Leydig cell line. However, the role of STAT5 in steroidogenesis in primary Leydig cell cultures depends on the developmental status of Leydig cells. While STAT5 is not involved in PRL signaling in primary Leydig cell cultures from juvenile rats [97], in primary Leydig cell cultures from adult rats, PRL increases STAT5 phosphorylation and *Lhcgr* mRNA levels [98]. Conversely, the JAK inhibitor tofacitinib reduces PRL-mediated phosphorylated STAT5 and *Lhcgr* mRNA levels in primary Leydig cell cultures from adult rats [98]. PRL also regulates Ca^{2+} uptake in Leydig cells via increased Ca^{2+} entry [118]. It is well known that free calcium activates steroidogenesis via the CAMKI pathway, as described above. Therefore, it is likely that PRL regulates steroidogenesis via both the JAK/STAT and the CAMKI pathways.

2.4. Growth Factors

Several growth factors also contribute to Leydig cell steroidogenesis, including insulin, insulin-like growth factor (IGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, TGFα, as well as IGF-binding proteins (IGFBPs). In the following sections, the roles and contributions of a subset of these factors that have been widely studied in Leydig cells is summarized (Figure 2).

2.4.1. Insulin Family of Growth Factors

The insulin family of growth factors, including insulin, insulin-like growth factor 1 (IGF1), IGF2, and relaxin, are small polypeptides that play critical roles in controlling growth, metabolism, and reproductive functions. Among them, insulin and IGF1 have been extensively studied in the context of testicular function. Insulin and IGF1 actions are mediated through the activation of two related tyrosine kinase receptors, insulin receptor (INSR) and IGF1 receptor (IGF1R). These receptors are composed of two extracellular α subunits and two transmembrane β subunits, which come together to form heterotetrameric glycoproteins (reviewed in [119]). Interestingly, both insulin and IGF1 can bind to both receptors, albeit with different affinities. Insulin exhibits a high affinity for INSR but can

also bind to IGF1R, albeit with a lower affinity. Similarly, IGF1 exhibits preferential binding to IGF1R while also displaying a lower affinity for INSR (reviewed in [119]).

Insulin

Insulin is a hormone produced primarily by the β-cells of the pancreas in response to elevated blood glucose levels [120] (reviewed in [121]). It acts by binding to its receptor INSR present in several tissues, leading to lower blood glucose levels by promoting cellular uptake and utilization of glucose. Insulin also stimulates the synthesis and storage of glycogen and fat and inhibits the breakdown of stored glycogen, fat, and protein in muscles (reviewed in [121]). Insulin deficiency affects the entire metabolism, including male fertility [122]. The role of insulin in the regulation of metabolic processes is often investigated in patients with type 1 diabetes, who produce very little or no insulin, and type 2 diabetes, characterized by insulin resistance.

Several animal models were developed to investigate the molecular mechanisms and histopathological processes involved in insulin function, including spontaneously diabetic rodent strains, streptozotocin (STZ)-induced diabetes, *Ins2* and *Insr* knockout mice, among others [123–126]. In diabetic rats, Leydig cell function is impaired, resulting in lower testosterone levels [124,126,127], a condition partially reversed by insulin treatment [126]. Similarly, male patients with type 2 diabetes exhibit significantly lower serum INSL3 concentrations [128]. This is consistent with data from diabetic rats where lower INSL3 levels are also observed, levels that are increased in insulin-treated rats [126]. In mice, double knockout of *Insr* and *Igf1r* results in a dramatic reduction in the number of Leydig cells that are unresponsive to hCG stimulation, while steroidogenesis in individual knockout male mice is not affected [129]. This indicates a functional redundancy between the two receptors and pathways in post-natal Leydig cells.

INSR is present in Leydig cells, and the addition of insulin to Leydig cells in primary culture and to TM3 Leydig cells increases steroidogenesis [130,131]. Conversely, several studies have reported the negative impact of insulin deficiency on Leydig cells [124–126]. Very high levels of insulin, such as those observed in obesity and type 2 diabetes, have negative effects on steroidogenesis through the induction of the atypical nuclear receptor DAX1/NR0B1, which represses hormone-induced steroidogenesis [132].

Insulin triggers a series of events upon binding to its tyrosine kinase receptor INSR, resulting in the activation of at least two pathways, PI3K/PKB and Raf/Ras/MEK/ERK1/2 (reviewed in [133]). In Leydig cells, both pathways appear to play a role in mediating insulin action. However, the exact mechanism of insulin action in the regulation of Leydig cell steroidogenesis remains to be fully characterized.

Insulin-like Growth Factor 1

Insulin-like Growth Factor 1 (IGF1) is a small peptide hormone produced primarily by the liver in response to GH stimulation, but almost all tissues, including the testes, can synthesize IGF1 ([134,135], reviewed in [78]). In the testes, IGF1 and its receptor (IGFR) are present in Sertoli, Leydig, germ, and peritubular cells [119,130,135,136]. In rodent Leydig cells, IGF1 secretion and upregulation of IGFR can be stimulated by LH, hCG, and GH [137–140].

Several studies involving animal models have shown that IGF1 is essential for male reproduction [141] (reviewed in [78,119,142]). In fact, IGF1-deficient male mice are infertile [141]. Consistent with this, studies have shown that IGF1 is critical for the proliferation, development and functionality of Leydig cells [130,136,143,144]. This growth factor exerts its effects through both para- and autocrine action [143]. Specifically, IGF1 stimulates the proliferation of Leydig cell precursors [136,145] (reviewed in [78]). Conversely, IGF1 deficiency results in altered proliferation and differentiation of Leydig cell precursors, leading to fewer and smaller Leydig cells and lower serum testosterone levels in adulthood [141,146]. As mentioned previously in the section on insulin, inactivation of both *Insr*

and *Igf1r* leads to a drastic reduction in Leydig cell number and size and steroidogenic failure [129].

Similar to INSR, activated-IGFR leads to the activation of PI3K/PKB and Raf/Ras/MEK/ERK1/2 pathways. In primary cultures of immature rat Leydig cells, IGF1 stimulates the phosphorylation of AKT and ERK1/2 [147,148]. In addition, IGF1 stimulates fetal Leydig cell proliferation through the MEK/ERK1/2 pathway [149].

2.4.2. Fibroblast Growth Factor 9

Fibroblast growth factor 9 (FGF9) belongs to the FGF family, which comprises at least 22 members found in a wide range of cell types (reviewed in [150]). FGF members interact with the extracellular domain of the FGF receptor (FGFR), leading to the activation of the intracellular tyrosine kinase domain, followed by the activation of signaling cascades. Although there are four known FGFRs, there are at least seven functionally distinct receptors due to alternative splicing events [150]. The different isoforms of FGFRs suggest a distinct function in each system. FGF9 is the main FGF family member present in the mammalian testis and binds to FGFR2IIIc, FGFR3IIIb, FGFR3IIIc, and FGFR4 (reviewed in [151]). Within the testes, FGF9 is present in a specific spatiotemporal pattern in Leydig, Sertoli, and germ cells [152–155]. The presence of FGF9 and its receptors in Leydig cells suggests that FGF9 functions as an autocrine factor in the regulation of Leydig cell steroidogenesis. Indeed, its effects are believed to be primarily mediated through autocrine and paracrine signaling within the testicular microenvironment rather than systemic effects via circulation [156].

Although *Fgf9* knockout mice die at birth, analysis of embryos revealed partial or complete XY gonadal sex reversal [157]. A similar phenotype is observed in humans harboring an FGF9 variant (D195N) associated with 46 XY Difference of Sex Development (DSD) [158].

In mouse primary Leydig cells and MA-10 Leydig cells, treatment with FGF9 increases testosterone production [154,156,159], and this involves phosphorylation and activation of AKT, MAPK and PKA pathways [156,159]. Although the downstream targets of FGF9 action in Leydig cells remain to be identified, the importance of the FGF9 pathway for Leydig cell function is well-established.

2.4.3. Epidermal Growth Factor (EGF) Family

The EGF family consists of 11 members divided into four sub-groups based on their receptor binding specificity. Most EGF family members, including EGF, transforming growth factor α (TGFα), amphiregulin (AREG), epigen (EPGN), heparin-binding EGF-like growth factor (HB-EGF), epiregulin (EREG), and betacellulin (BTC), recognize and bind to the EGF receptor (EGFR) also known as ErbB1. Upon ligand binding, EGFR homodimerizes with another EGFR or heterodimerizes with other EGFR/ErbB family members (reviewed in [160,161]). EGF family members have been implicated in the regulation of cell proliferation, differentiation, survival, and motility in several tissues, including the male gonad (reviewed in [160]).

To better understand the function of EGF, numerous mouse knockout models have been developed (reviewed in [161]). While single and even triple (EGF, AREG, TGFα) knockout mice are viable and fertile, disruption of the *Egfr* gene leads to pre-implantation or post-natal lethality [162,163]. This indicates that the various EGF family members can compensate, at least in part, for the absence of some family members [162–164].

Leydig cells of several species, including humans and rodents, contain both EGF and EGFR [165,166]. The impact of EGF on Leydig cell steroidogenesis is controversial. Current data suggest that its effects are subject to various factors, such as cell maturity and experimental conditions, including treatment duration and the cell line used. Several studies reported a stimulatory role for EGF on Leydig cell steroidogenesis. In MA-10 Leydig cells, a time-dependent effect of EGF was observed [167]. Exposure to EGF initially decreases the activity of adenylate cyclase activated by hCG within the first hour. In the next 7 h, EGF stimulates steroidogenesis via a cAMP-independent pathway. Finally, at 8 h

post-EGF treatment, a reduction in LHCGR levels is observed. Other studies also support a stimulatory role for EGF in Leydig cell steroidogenesis [148,166,168–171]. Furthermore, EGFR signaling is required for early hCG-induced steroidogenesis in mouse MLTC-1 Leydig cells, suggesting a crosstalk between EGFR and LHCGR [168]. In addition, male mice treated with an EGF inhibitor (AG1478) exhibit a substantial reduction in serum testosterone levels [168]. Conversely, an inhibitory role for EGF in testosterone production in Leydig cells has also been reported [172]. EGF was found to reduce steroidogenic gene expression and steroid production in progenitor Leydig cells (PLCs) [173]. Moreover, EGF was found to stimulate the proliferation of stem Leydig cells (SLCs) and PLCs while blocking their differentiation into mature adult Leydig cells [173].

Mechanistically, binding of EGF to its receptor EGFR induces phosphorylation of EGFR and ERK1/2 through the extracellular calcium-sensing receptor (CASR). This observation was made in Rice H500 rat non-metastasizing Leydig tumor cells treated with Ca^{2+}. In these tumor cells, activated-CASR increases the production of parathyroid-hormone-related peptide (PTHrP) via multiple signaling pathways, including PKC, MEK, and JNK [174]. In MLTC-1 Leydig cells, treatment with EGF increases testosterone production and STAR protein levels without any significant change in phospho-STAR levels [166]. More recently, LH-mediated EGFR activation was found to activate casein kinase 1α (CK1α), leading to increased testosterone synthesis [175]. Consistent with this, conditional and partial inactivation of the *Csnk1a1* gene (encoding CK1α) in steroidogenic cells in the mouse results in a significant reduction in the expression of several steroidogenic genes leading to lower testosterone levels and decreased male fertility [175].

Although these findings support a role for EGF in Leydig cell steroidogenesis, conflicting data on the effects of EGF on Leydig cell function highlights the complexity of this pathway. Additional work is necessary to fully decipher the role of EGF/EGFR in steroidogenesis.

2.5. Vitamin D

Vitamin D (VitD) is a steroid hormone, and its active form 1,25 dihydroxyvitamin D (1,25$(OH)_2$D) is essential for maintaining calcium and phosphate homeostasis and promoting skeletal health (reviewed in [176,177]). In the classical pathway (genomic), VitD binds to the vitamin D receptor (VDR), which forms a heterodimer with the retinoid X receptor (RXR). This VitD/VDR-RXR heterodimer acts as a transcription factor, recognizing vitamin D response elements (VDREs) present in the promoter regions of various target genes (reviewed in [178,179]). VitD also acts through non-genomic pathways involving phospholipases, second messengers, and kinases (reviewed in [178,179]).

While the liver and kidneys are commonly recognized as the primary organs involved in VitD metabolism, the presence of VitD metabolizing enzymes is not confined exclusively to these organs. In tissues other than the liver and kidneys, VitD was shown to have paracrine/autocrine functions (reviewed in [177,180]).

The male reproductive system, including the testis, contains several VitD metabolizing enzymes as well as VDR (reviewed in [178,181]). In the human testis, the enzyme CYP2R1 is predominantly found in Leydig cells [182], where its expression is hCG-dependent [183]. Another essential enzyme, CYP27B1, is also present in Leydig cells. A transgenic reporter mouse model with a 1.5 kb fragment of the human *CYP27B1* promoter revealed significant expression in the testis, including Leydig and Sertoli cells [184,185]. VitD action is dependent on the presence of VDR. This receptor is present in Leydig cells and other cells of the male reproductive system, such as Sertoli cells, germ cells, spermatozoa, and epithelial cells (reviewed in [176]). In the human testis, VDR is present in Leydig cells from both the fetal and adult population [186–188]. These studies collectively suggest that Leydig cells locally metabolize VitD, indicating an intracrine/autocrine role in testicular function, particularly in steroidogenesis.

Consistent with this, numerous studies have explored the relationship between VitD levels and steroidogenesis (reviewed in [181,189]). For instance, some studies have reported

a correlation between decreased 25-hydroxyvitamin D (25-OHD) levels (the immediate precursor of VitD) and lower testosterone concentrations [190–192] (reviewed in [189,193,194]). In addition, CYP2R1, CYP27B1, and VDR levels are lower in infertile men compared to those with normal testicular function (reviewed in [181]). A study using testis samples from young men affected by testicular disorders revealed lower levels of 25-OHD compared to those with normal testicular function [182].

To better elucidate the relationship between VitD and male reproduction status, studies using mice models were performed. In CYP27B1- and VDR-deficient mice, reproductive and endocrine function was impaired [195–197]. More specifically, in *Vdr* knockout mice (*Vdr*KO), reproductive performance and sperm quality declined, resulting in smaller litter size, lower live birth rate, and lower number of successful breeding performances [197–200]. The absence of VDR also affected testes morphology, with smaller adipocytes and reduced lipid droplet accumulation [197]. These results suggest that VDR is a regulator of lipid metabolism and essential for maximal male fertility. Moreover, expression of *Hsd3b1* and *Cyp11a1* was significantly decreased in the testes of *Vdr*KO mice [197,201]. Interestingly, male rats receiving a VitD-deficient diet for 3 months presented a decrease in the production of testosterone, reduced testis volume, and decreased number of spermatids and spermatocytes, suggesting an essential role of VitD for male testicular function [202]. In the mouse TM3 Leydig cell line, downregulation of VDR results in a notable reduction in the expression of crucial steroidogenic genes, such as *Cyp11a1*, *Hsd3b1*, *Star*, *Nr5a1*, and *Prkaca* [197,201]. Conversely, the upregulation of VDR enhances the expression of the majority of these genes. VDR was found to directly regulate *Hsd3b1* expression by binding to a VDRE in the proximal promoter region [201]. The modulation of VDR levels also has a significant impact on genes associated with lipid metabolism [197]. Moreover, the knockdown of VDR in TM3 Leydig cells impairs steroid production, further emphasizing its importance in this process [197,201].

Despite the presence of VDR and its associated enzymes in Leydig cells, our understanding of the role and mechanism of VitD action in steroidogenesis remains limited. In addition, there is currently no evidence supporting VitD supplementation for improving testosterone levels (reviewed in [193]). The current literature on the correlation between sex hormone production and systemic changes in VitD levels presents conflicting findings, especially due to compensatory mechanisms (reviewed in [181]). More work is needed to fully comprehend how VitD influences steroid hormone production in Leydig cells.

2.6. Osteocalcin

Osteocalcin (OCN) or bone γ-carboxyglutamic acid protein is a bone-derived factor produced primarily by osteoblasts (reviewed in [203]). Post-translationally, osteocalcin is γ-carboxylated and considered biologically inactive [204]. A fraction of osteocalcin undergoes decarboxylation and reaches the circulation, acting as a hormone. In target tissues, the undercarboxylated (unOCN) form of osteocalcin mainly binds to the G protein-coupled receptor family C group 6-member A (GPRC6A), while in the central nervous system, it binds to GPR158 as well [205,206].

In vivo studies using null mouse models have demonstrated that osteocalcin is essential for male fertility. In fact, osteocalcin knockout mice exhibit reduced testis weight, oligospermia, and low testosterone levels and produce smaller litter sizes than wild-type animals [207]. A similar phenotype was found in *Gprc6a* knockout mice [205,208]. The correlation between osteocalcin and male reproduction was also investigated in humans (reviewed in [209]). Using populational-based samples and bone disorders patient-based samples, osteocalcin was found to be positively correlated with testosterone serum concentration; patients with lower OCN had lower testosterone levels and vice-versa [210]. Moreover, Oury and collaborators [205] found in a cohort of patients with primary testicular failure two individuals carrying a missense mutation in the *Gprc6a* locus. Consistent with this, a recent study strongly suggests a role for the unOCN/GPRC6A axis in the regulation of testosterone production [211].

In the testis, the osteocalcin receptor *Gprc6a* is highly expressed in Leydig cells [208]. The binding of unOCN was shown to regulate steroidogenesis independently of the HPG axis [205]. The GPRC6A receptor is coupled to adenylate cyclase, and once activated, results in increased cAMP production, activation of signaling pathways, and ultimately the activation of downstream effectors such as CREB, which then upregulate the expression of multiple genes encoding essential steroidogenic proteins and enzymes such as STAR, CYP11A1, CYP17A1, and 3βHSD [207] (Figure 2).

unOCN is also implicated in the regulation of vitamin D metabolism in mouse MA-10 Leydig cells, where it was found to stimulate *Cyp2r1* gene expression [212], which codes for the CYP2R1 enzyme involved in the conversion of vitamin D into its active form $1,25(OH)_2D$ (see the section on vitamin D).

3. Conclusions

As described in this review, several pathways are involved in the stimulation of Leydig cell steroidogenesis. Recent advances in technology and molecular biology tools (gene editing to easily generate animal models, more efficient genetic screening of patients, development of high throughput functional assays to study receptors/kinases/transcription factors) have led to significant progress in our understanding of the mechanisms and signaling pathways involved. However, further research is needed to fully comprehend the complex interplay between signaling pathways in Leydig cell steroidogenesis. Leydig cell-specific knockout models are a promising tool that will help better understand the roles of specific receptors, signaling cascade proteins, and kinases in the regulation of steroidogenesis. The development and use of new drugs that target these signaling pathways also hold significant potential for the treatment of disorders related to steroid hormone production. Overall, the field of reproductive endocrinology is constantly evolving, and new tools and technologies are leading to a better understanding of the regulation of steroidogenesis.

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