Advances in stem cell research for the treatment of primary hypogonadism

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Abstract | In Leydig cell dysfunction, cells respond weakly to stimulation by pituitary luteinizing hormone, and, therefore, produce less testosterone, leading to primary hypogonadism. The most widely used treatment for primary hypogonadism is testosterone replacement therapy (TRT). However, TRT causes infertility and has been associated with other adverse effects, such as causing erythrocytosis and gynaecomastia, worsening obstructive sleep apnoea and increasing cardiovascular morbidity and mortality risks. Stem-cell-based therapy that re-establishes testosterone-producing cell lineages in the body has, therefore, become a promising prospect for treating primary hypogonadism. Over the past two decades, substantial advances have been made in the identification of Leydig cell sources for use in transplantation surgery, including the artificial induction of Leydig-like cells from different types of stem cells, for example, stem Leydig cells, mesenchymal stem cells, and pluripotent stem cells (PSCs). PSC-derived Leydig-like cells have already provided a powerful in vitro model to study the molecular mechanisms underlying Leydig cell differentiation and could be used to treat men with primary hypogonadism in a more specific and personalized approach.

Reduced serum levels of testosterone, a condition known as male hypogonadism, or testosterone deficiency, affects millions of men[1]. Hypogonadism has been linked to a number of metabolic and quality-of-life changes that include infertility, cardiovascular disease, altered mood, fatigue, decreased lean body mass, reduced bone mineral density, increased visceral fat, metabolic syndromes, decreased libido and impaired sexual function[2–4]. In male mammals, testosterone is predominantly produced by Leydig cells in the testis and is under the control of pituitary luteinizing hormone (LH; also known as lutropin)[5] (FIG. 1). Small amounts of testosterone are produced by the adrenal gland and are under the control of adrenocorticotropic hormone (ACTH)[5]. A lack of response or a reduced response to LH that affects the ability of the testis to synthesize testosterone is referred to as primary hypogonadism, whereas a reduction in serum levels of LH that affects production of testosterone is referred to as secondary hypogonadism[6]. Primary hypogonadism is frequently associated with genetic causes, ageing, the consequences of drug treatment (for example, chemotherapy) and exposure to viruses (for example, HIV), testicular trauma, or stress[6].

Testosterone replacement therapy (TRT) is the first choice for treating primary hypogonadism, as exogenous administration of testosterone can largely reverse low serum levels of this hormone and ameliorate hypogonadism-associated symptoms[3]. However, adverse effects of TRT on fertility have been reported, mainly as a result of negative feedback in the hypothalamic–pituitary–gonadal (HPG) axis that leads to reduced levels of the gonadotropins LH and follicle-stimulating hormone (FSH), with subsequent suppression of spermatogenesis[7] (FIG. 1a). Thus, owing to the suppressive effects on fertility, TRT is not suitable for patients who wish to maintain fertility during treatment. Other potential adverse effects of TRT include causing erythrocytosis and gynaecomastia, worsening obstructive sleep apnoea, elevating prostate-specific antigen (PSA) and increasing cardiovascular morbidity and mortality risks[8].

Owing to the adverse effects of TRT, demands for alternative therapies to TRT are noticeably increasing. Stem-cell-based therapy has, therefore, begun to gain widespread attention. The success of clinical trials of stem-cell-based therapy in other fields, such as the nervous system, bone and cardiovascular diseases[9,10], suggests that stem-cell-derived Leydig cells are feasible as a new method for treating testicular failure. Moreover, these cells provide ideal tools for modelling primary hypogonadism and screening new compounds for correcting Leydig cell dysfunction, which could help to speed up discovery of the molecular mechanisms underlying hypogonadism and the identification of new chemical entities that target mechanisms controlling testosterone production.

In this Review, we describe the types and functions of Leydig cells and present studies in the stem-cell field, including in vitro derivation of Leydig cells, allograft and

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Key points

- Primary hypogonadism is mainly treated using testosterone replacement therapy (TRT). However, TRT has adverse effects and is unsuitable for men with hypogonadism wishing to maintain fertility.
- Stem-cell-based therapy, in which a cell lineage can be re-established in human bodies to produce testosterone normally, would be the ideal choice for treating primary hypogonadism.
- Stem Leydig cells, mesenchymal stromal cells, pluripotent stem cells and fibroblasts are newly discovered sources of Leydig cells.
- Stem-cell-derived Leydig cells have many potential applications, including understanding the underlying mechanisms of primary hypogonadism, treating primary hypogonadism using transplantation therapy, and discovering drugs aimed at recovering Leydig cell function.
- Research is needed in the applications of Leydig cells, including constructing 3D testicular organoids, promoting in vitro culture conditions of Leydig cells, and exploring the in vivo transplantation locations of Leydig cells.

xenograft transplantation of stem-cell-derived Leydig cells in animals, models of primary hypogonadism and drug discoveries.

Development of Leydig cells

The development of Leydig cells involves cell proliferation, morphological differentiation and functional maturation. Owing to the scarcity of human sources, our knowledge about Leydig cells is mostly obtained from non-human mammals, such as rats and mice; however, species differences are considerable[1]. Unlike most mammalian Leydig cells, which have a biphasic development (fetal and pubertal-to-adult stages), human and non-human primate Leydig cells undergo a triphasic development, including fetal, neonatal and adult stages; the adult stage begins at puberty (FIG. 2).

Fetal and neonatal Leydig cells

Fetal Leydig cells originate and mature during the fetal stage of development. These cells produce hormones, such as testosterone and androstenedione, which are crucial for the development of male ductal structures, external genitalia, brain, and the gubernaculum ligament[11].

The developmental process of fetal Leydig cells in human fetal testes can be divided into three stages: the differentiation stage (gestational weeks 8–14), the maturation stage (gestational weeks 14–18) and the involution stage (gestational weeks 18–38)[12] (FIG. 2a). Fetal Leydig cells are thought to originate from mesenchymal cells present in the mesonephros, coelomic epithelial cells, neural crest cells, or cells present in the adrenogonadal primordium[11]. The precursors of fetal Leydig cells migrate into the gonads, which are segregated into testicular cords and interstitium at gestational week 7 (REF.[13]). Around gestational week 8, when fetal Leydig cells first appear in the interstitium and are identifiable by their large size and substantial number of lipid droplets[13], they begin to differentiate. At gestational week 9, they can synthesize testosterone[13]. From gestational weeks 8–14, they undergo morphological differentiation and attach to each other to form clusters[14]. From gestational weeks 14–18, their number and testosterone synthesis capacity reach a peak, which makes them occupy most of the testicular interstitium and is associated with increasing testosterone levels[12,15–17]. Mature fetal Leydig cells grow round in shape with an enormous quantity of smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER) and mitochondria with tubulovesicular cristae, and also develop round and large-volume lipid droplets in the cytoplasm[18,19]. Notably, although fetal Leydig cells are ultrastructurally similar to adult Leydig cells in most aspects, they still differ substantially in the number and size of lipid droplets, which are increased in number and present in larger sizes in fetal Leydig cells than in adult Leydig cells[20,21]. From gestational week 18 to the time of full-term birth, the fetal Leydig cell population gradually regresses[16].

From a mechanistic viewpoint, the differentiation of fetal Leydig cells starts with the activation of steroidogenic factor 1 (SF-1), which is strictly regulated by fetal Leydig cell-specific enhancer (FLE)[21], in the pre-gonadal mesonephric mesenchyme (FIG. 2a). SF-1 then regulates the expression of the sex-determining region Y (*SRY*) gene, which induces the differentiation of testicular Sertoli cells, the epithelial cells residing in the seminiferous tubules that are essential for male sexual development and spermatogensis[22,23], and elevates the expression of SF-1 in fetal Leydig cells[24–26]. After cell differentiation, SF-1 further contributes to the functional maturation of Leydig cells, in which SF-1 controls the steroidogenic pathway of testosterone biosynthesis by manipulating the expression of critical steroidogenic enzymes, such as the upregulation of cytochrome P450 family 11 subfamily A, polypeptide 1 (CYP11A1) and cytochrome P450 family 17, subfamily A, polypeptide 1 (CYP17A1) in Leydig cells[27].

Once the maturation stage begins, fetal Leydig cells first show expression of 3β-hydroxysteroid dehydrogenase/△5/△4 isomerase (3β-HSD) followed by the induction of the entire testosterone biosynthetic pathway, including the upregulation of CYP11A1, 3β-HSD, CYP17A1 and 17β-hydroxysteroid dehydrogenase/ketosteroid reductase (17β-HSD) (FIG. 2a). Notably, 17β-HSD, which catalyses the last step of testosterone biosynthesis from androstenedione to testosterone, is present at much higher levels in Sertoli cells than in fetal Leydig cells, unlike the adult testis, where 17β-HSD3 (an isotype of 17β-HSD) is exclusively expressed in Leydig cells[28,29]. Thus, testosterone biosynthesis in the fetal stage is understood to be fulfilled by both Leydig cells and Sertoli cells[22,30]. In addition to steroidogenic enzymes (for example, 3β-HSD), steroidogenic acute regulatory protein (STAR), platelet-derived growth factor receptor alpha (PDGFRα) and insulin-like factor 3 (INSL3) are considered markers for fetal Leydig cells owing to their crucial roles in either the development of fetal Leydig cells or their steroidogenesis[11].

The number of Leydig cells is increased during the neonatal stage, which occurs at the postnatal age of 2 months and peaks at 3 months postnatally, when cells produce a comparable level of testosterone with that of the pubertal stage (generally 10–14 years old)[1,31]. This testosterone peak is associated with neural development, neural sexual behaviour differentiation and cell imprinting in the brain, prostate and kidney enabling the

Ductal structures
Embryos contain two sets of ductal structures, the Wolffian and Müllerian ducts, which develop into the male and female reproductive tracts, respectively.

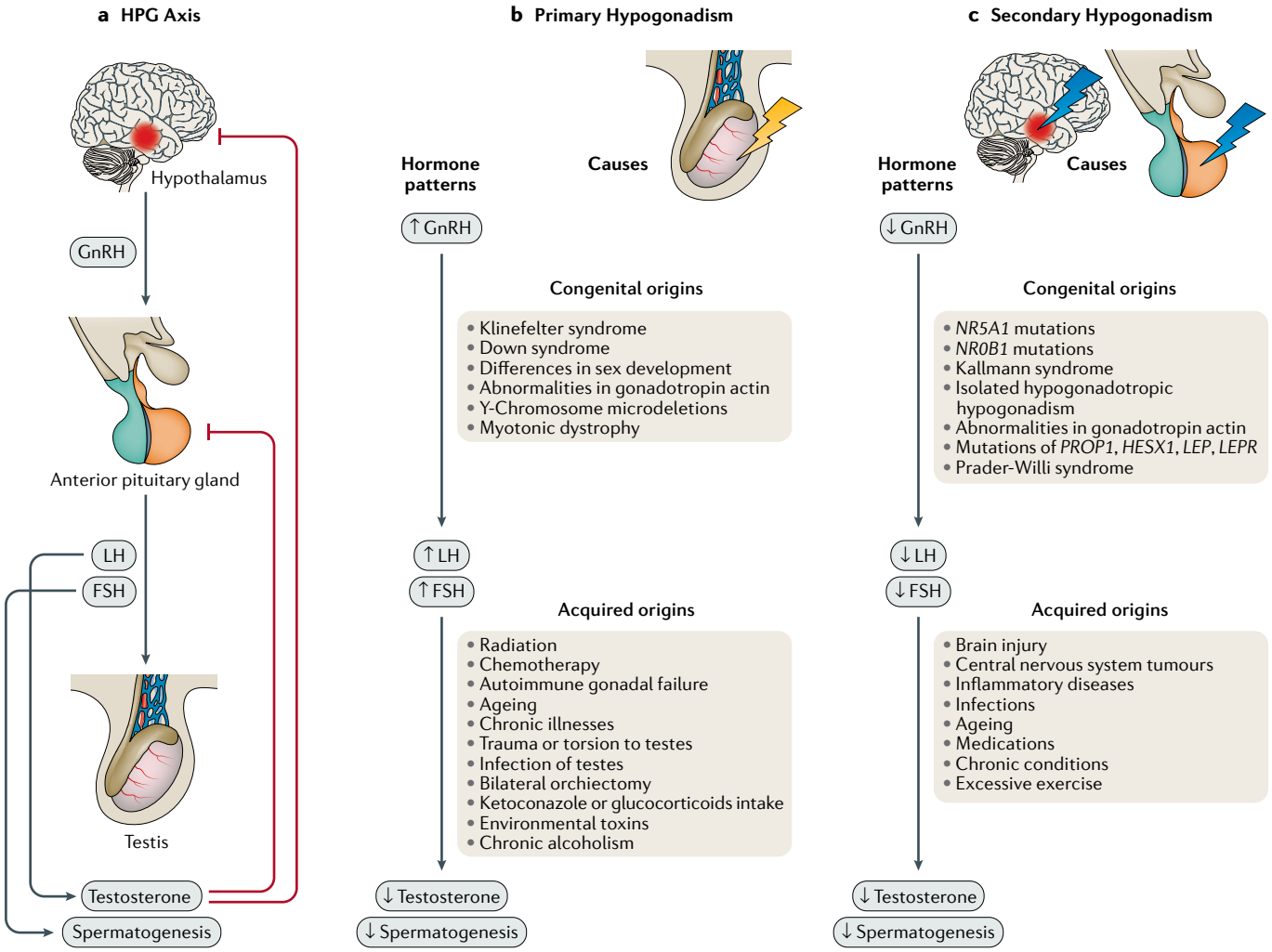


Fig. 1 | **The hypothalamus–pituitary–gonadal axis and pathophysiology of male hypogonadism. a** | In humans, the hypothalamic–pituitary–gonadal (HPG) axis controls testosterone synthesis. Gonadotropin-releasing hormone (GnRH) released from the hypothalamus stimulates the anterior pituitary gland to release luteinizing hormone (LH). LH triggers Leydig cells within the testes to produce testosterone, which reversely inhibits the release of GnRH and LH. GnRH also triggers the anterior pituitary gland to release follicle-stimulating hormone (FSH), which activates Sertoli cells, thereby supporting spermatogenesis. **b** | In patients with primary hypogonadism, the response of Leydig cells to LH stimulation is weak, and these cells consequently produce reduced amounts of testosterone. Primary hypogonadism is caused by a malfunction at the level of the testes and is of either congenital or acquired origin. **c** | In patients with secondary hypogonadism, the malfunction occurs at the level of either the hypothalamus or the pituitary, thereby reducing serum levels of GnRH and LH, leading to reduced production of testosterone. Secondary hypogonadism can also be of either congenital or acquired origin.

response of these tissues to androgen stimulation during adulthood[6,16]. The possible origins of neonatal Leydig cells include non-degraded fetal Leydig cells and newly formed stem Leydig cells, which are probably derived from testicular mesenchymal cells[16,21,32,33] (FIG. 2b). From 4 months to 1 year postnatally, the neonatal Leydig cell population rapidly drops to a nadir[16]. Thereafter, neonatal Leydig cells gradually regress during the pre-pubertal stage from the ages of 1–10 years[6,16] (FIG. 2b).

Adult Leydig cells

Adult Leydig cells are the Leydig cell population established by the end of puberty (~14 years old)[12]. Fetal and neonatal Leydig cells produce testosterone, which is essential for male genital development and brain masculinization during prenatal and neonatal stages[1]. Adult Leydig cells also produce testosterone, which is crucial for the establishment of secondary sexual characteristics, the initiation and maintenance of spermatogenesis, the development of the accessory sex glands at puberty and the maintenance of secondary sexual characteristics and fertility throughout adulthood[16,34]. During the first year of life, the adult stem Leydig cell population expands rapidly and is then maintained at a stable cell number until puberty[1,12]. During puberty, the phenotype of adult Leydig cells gradually changes from stem-like to mature, when they finally become the main source of testosterone and bring serum testosterone to peak levels[1]. In humans, this developmental process of the adult Leydig cell population is divided into four stages

based on the differences in Leydig cell morphology, gene expression patterns and steroid molecules synthesized at each stage: stem Leydig cells, progenitor Leydig cells, immature Leydig cells, and ultimately adult Leydig cells (FIG. 2c).

Stem Leydig cells are the first developmental stage of adult Leydig cells. Stem Leydig cells are thought to originate from a combination of fetal Leydig cells, testicular mesenchymal cells, peritubular cells (located at the outer face of the seminiferous tubules) and perivascular cells (located in the testicular blood vessels)[11,35,36] (FIG. 2b,c). LH secreted from pituitary glands and factors derived from Sertoli cells and peritubular cells are considered to initiate stem Leydig cell differentiation[16]. Fetal Leydig cells and testicular mesenchymal cells share the same ontogeny; however, fetal Leydig cells are considered to redifferentiate into neonatal Leydig cells at the postnatal age of 2 months[16,21], and testicular mesenchymal cells continue to proliferate or are at rest until the postnatal stage (FIG. 2a and b). Testicular mesenchymal cells give rise to most of the cell types in the testicular interstitium, including peritubular and perivascular cells, which are the best-validated source of stem Leydig cells[37–40]. Stem Leydig cells from testicular seminiferous tubules of adult rats that were treated with ethane dimethane sulfonate (EDS), which selectively depletes Leydig cells, are able to differentiate into Leydig cells in vitro[41,42], confirming that these peritubular stem Leydig cells are the progenitors of adult Leydig cells. Stem Leydig cells are distinguishable from other adult Leydig cell populations by their spindle shape and sparse SER. They can also be identified by the surface marker PDGFRα, subunit alpha V (CD51), Thy-1 cell surface antigen (CD90) and p75 neurotrophin receptor[42].

In rats, stem Leydig cells proliferate and differentiate into progenitor Leydig cells from postnatal days 7 to 14 and these cells predominate in the testes from postnatal days 14–21 (REF.[12]). Around postnatal day 35, the progenitor Leydig cells divide several times and differentiate into immature Leydig cells[12]. These immature Leydig cells increasingly express CYP11A1, 3β-HSD and CYP17A1 and, therefore, are increasingly capable of synthesizing testosterone[43–46]. However, the major steroid produced by immature Leydig cells is 5α-androstane-3α, 17β-diol, as testosterone is largely metabolized by 3α-HSD and 5α-reductase, which are highly expressed in immature Leydig cells and convert testosterone into 5α-androstane-3α, 17β-diol, which is thought to have no androgenic effects in androgen-regulated organs, but can stimulate prostate cell proliferation[34,47].

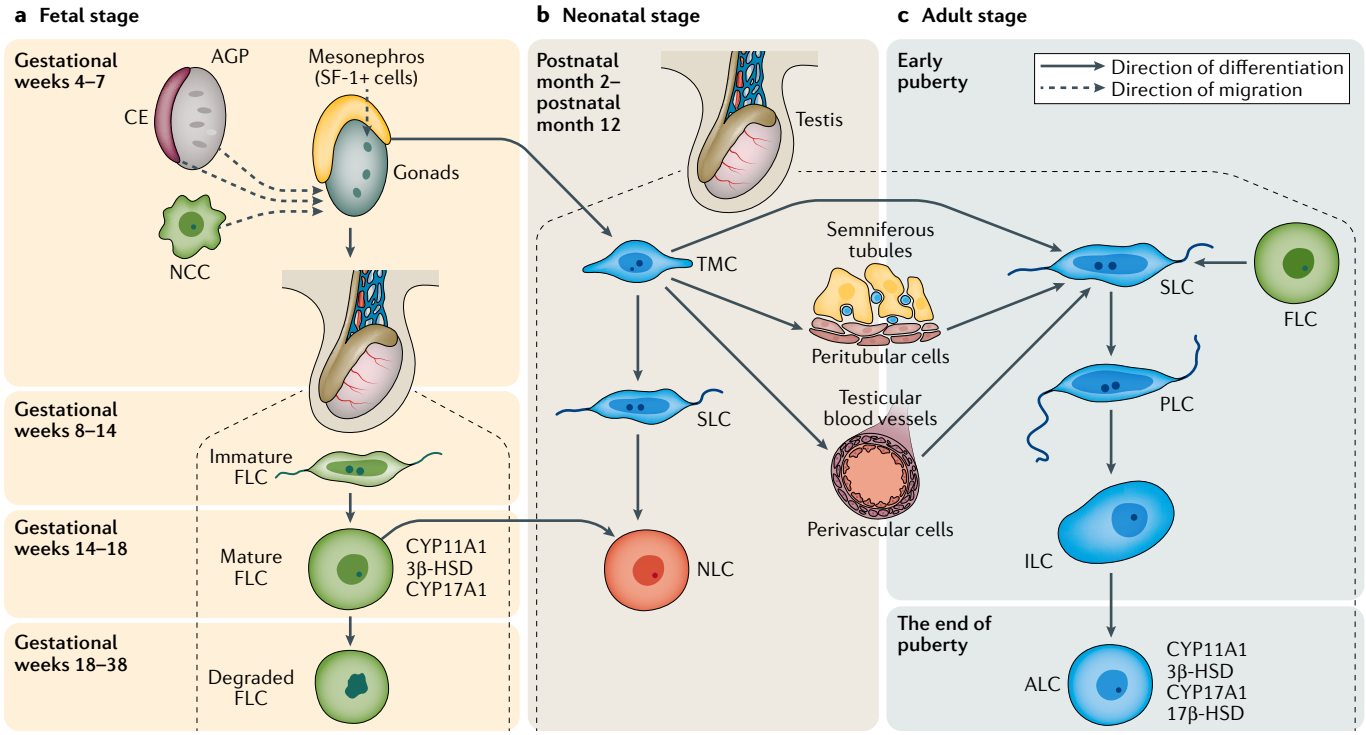


Fig. 2 | **The development of human Leydig cells. a** | Fetal Leydig cells (FLCs) are cells that produce testosterone before birth. They originate from the mesenchymal cells present in the mesonephros, coelomic epithelial (CE) cells, neural crest cells (NCC), or cells present in the adrenogonadal primordium (AGP). The precursors of FLCs, such as mesenchymal cells expressing steroidogenic factor 1 (SF-1), migrate into the gonads and form FLCs. **b** | Some cells present in the mesonephros differentiate into testicular mesenchymal cells (TMCs), which continue to proliferate or are at rest until the postnatal age. At the postnatal age of 2 months, a second wave of testosterone is produced by neonatal Leydig cells (NLCs), which are derived from either non-degraded FLCs or newly formed stem Leydig cells (SLCs). After postnatal year 1, NLCs gradually regress. **c** | Adult Leydig cells (ALCs), which are established during puberty (10–14 years old), are the third developmental stage of human Leydig cells. The differentiation of ALCs is generally divided into four stages, namely SLCs, progenitor Leydig cells (PLCs), immature Leydig cells (ILCs), and ALCs. SLCs originate from non-degraded FLCs, TMCs, peritubular cells located on the outer face of seminiferous tubules, or perivascular cells associated with testicular blood vessels. 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase/ketosteroid reductase; CYP11A1, cholesterol side-chain cleavage enzyme, mitochondrial; CYP17A1, steroid 17α-hydroxylase/17,20 lyase.

From postnatal days 55–90, immature Leydig cells divide once and then differentiate into adult Leydig cells[12]. Owing to the scarcity of human specimens, the development of human adult Leydig cells remains unknown. However, adult Leydig cell development in humans is considered to be similar to that in rodents except for a longer time span at each stage[16]. The expansion and differentiation of human stem Leydig cells into progenitor Leydig cells happens at the onset of puberty (~10 years old). The derivation of immature Leydig cells to adult Leydig cells then happens during puberty[16] (FIG. 2c).

Adult Leydig cells contain more SER but fewer lipid droplets than immature Leydig cells[46]. As the most noticeable organelle, SER in adult Leydig cells appears as branching and anastomosing tubules, meshed networks, or even has a myelin sheath-like morphology[46]. Mitochondria, which are in a polymorphic shape with tubular or lamellar cristae inclusions, are also abundant in adult Leydig cells[46], whereas they are less polymorphic in immature Leydig cells and show flattened cristae[48]. In these cells, upregulated expression of testosterone biosynthetic enzymes, including CYP11A1, 3β-HSD, CYP17A1 and 17β-HSD (FIG. 2c), and reduced expression of testosterone-metabolizing enzymes, including 3α-HSD and 5α-reductase, enable adult Leydig cells to produce considerably more testosterone than immature Leydig cells[45,49–51]. Testosterone is the predominant androgen produced by adult Leydig cells[21].

The development of adult Leydig cells is regulated by a complicated signalling network involving numerous factors. One of the most prominent factors is LH, which is produced by the anterior pituitary in response to gonadotropin-releasing hormone (GnRH) produced by the hypothalamus (FIG. 1a). LH stimulates both the proliferation and differentiation of postnatal Leydig cells, demonstrated by increases in the number of Leydig cells, expression of steroidogenic enzymes, the number of steroidogenesis-associated organelles and testosterone production by Leydig cells at different stages of differentiation following the exogenous administration of LH compared with baseline levels[12,52–59].

Human chorionic gonadotropin (HCG), which is secreted by the placenta during pregnancy, binds to the luteinizing hormone–choriogonadotropin receptor (LHCGR) and regulates the differentiation and function (steroidogenesis) of fetal Leydig cells before birth[21]. After birth, experimental treatments with hCG show that this hormone can stimulate both the proliferation and differentiation of Leydig cells[12,60,61]. Another factor that regulates the development of Leydig cells is insulin-like growth factor I (IGFI), which is secreted by the testis and exerts its most obvious effects during puberty[62,63]. IGFI stimulates both proliferation and differentiation of Leydig cells, and especially promotes the transition from immature to adult Leydig cells[64]. This transition is further increased by LH, which increases the expression of IGFI receptor in rat Leydig cells[65–67]. Sertoli cells secrete desert hedgehog protein (DHH) and platelet-derived growth factor (PDGF), both of which are also essential for Leydig cell development[68]. Specifically, DHH and the PDGF ligands PDGF-AA and PDGF-BB have all been shown to stimulate the in vitro proliferation of rat Leydig cells, whereas only DHH and PDGF-AA induce their differentiation[34,41]. Other factors that contribute to the development of adult Leydig cells include fibroblast growth factor 2 (FGF2), activin, androgens, transforming growth factor β (TGFβ), parathyroid hormone-related protein (PTHrP), leukaemia inhibitory factor (LIF), thyroid hormone (triiodothyronine) and lithium[34,42,69,70]. A combination of LH (1 ng/ml), PDGF-BB (10 ng/ml), triiodothyronine (1 nM) and IGF1 (70 ng/ml) is able to induce in vitro differentiation of human stem Leydig cells[71].

Male hypogonadism

The testes of a healthy man generally produce 3–10 mg of testosterone every day, roughly corresponding to serum concentrations of 300–1,000 ng/dl (10.4–34.7 nmol/l)[72]. Testosterone deficiency, the threshold of which can vary among laboratories and assays, is suggested to be <280–300 ng/dl (9.8–10.4 nmol/l) for total testosterone levels and 5–9 ng/dl (0.17–0.31 nmol/l) for free testosterone levels[73]. Reduced testosterone production can be caused by either primary hypogonadism, in which Leydig cells produce low levels of androgens owing to a weak response of Leydig cells to LH stimulation (FIG. 1b), or secondary hypogonadism, in which reduced serum levels of LH leads to reduced production of androgens, especially testosterone[74] (FIG. 1c). Serum hormone levels in men with reduced testosterone production can help to distinguish between primary and secondary hypogonadism. Patients with primary hypogonadism have elevated serum levels of gonadotropins (LH and FSH) and correspondingly higher levels of hypothalamic GnRH than healthy men[75] (FIG. 1b), whereas men with secondary hypogonadism attributable to hypothalamic–pituitary disorders have low levels of both gonadotropins and GnRH[75] (FIG. 1c).

Reduced serum levels of testosterone affect both health and quality of life in men with hypogonadism[2–4]. As testosterone is crucial for the initiation and maintenance of spermatogenesis, low testosterone levels are one of the main factors associated with male infertility. Reduced testosterone levels can also lead to the loss of secondary sex characteristics (including body and facial hair) and sexual dysfunction, owing to the diminution of testosterone metabolites, such as dihydrotestosterone and 3α-androstenediol glucuronide. Other manifestations of hypogonadism include obesity, cardiovascular disorders, fatigue, altered mood and decreased libido, lean body mass, bone mineral density, muscle mass and strength, in comparison to patients with normal testosterone levels[2–4].

Both primary and secondary hypogonadism can have congenital and/or acquired origins[6,76–81] (BOX 1, FIG. 1b and 1c). The prevalence of primary and secondary hypogonadism varies with age. A study evaluating 231 men (mean age 47.2 years), who were diagnosed with low libido or hypogonadism, showed that 7.4% and 42.4% had primary and secondary hypogonadism, respectively[82]. Another study evaluating a general population of men aged 40–79 years showed that 2.5% and 11.8% of men manifested symptoms of primary and secondary hypogonadism, respectively[6,83]. However, among

Total testosterone
All testosterone in the blood, including free testosterone and testosterone bound to albumin and sex hormone binding globulin (SHBG).

Free testosterone
Testosterone in the blood not bound to any proteins.

Box 1 | **Causes of male hypogonadism**

Primary hypogonadism can have both congenital and acquired causes[6,76–78].

Congenital causes

- Disorders of sex development include
  - Sawyer syndrome, in which 15–30% of patients have mutations (such as *SRY* mutations) or alterations in the Y chromosome
  - Complete androgen insensitivity syndrome (mutations of the androgen receptor)
  - Congenital adrenal hyperplasia and disorders of androgen biosynthesis (deficiencies of 3β-HSD, CYP17A1, or STAR)
  - Galactosaemia (a deficiency in galactose-1-phosphate uridylyltransferase)
  - Testicular regression syndrome (testicular torsion or trauma to scrotal contents before birth)
- Trisomies (Klinefelter syndrome and Down syndrome)
- Abnormalities in gonadotropin action (mutations of *LHCGR*)
- Y-Chromosome microdeletions (deletions of Azoospermic Factor a, b and c)
- Myotonic dystrophy (a mutation of dystrophia myotonica protein kinase gene)

Acquired causes

- Radiation
- Chemotherapy (such as alkylating and anti-neoplastic agents)
- Autoimmune gonadal failure
- Ageing
- Chronic illnesses (such as HIV and kidney disease)
- Trauma or torsion of the testes
- Infection of the testes
- Bilateral orchiectomy
- Ketoconazole (the anti-fungal drug) or glucocorticoids
- Environmental toxins
- Chronic alcoholism

Secondary hypogonadism can also be of both congenital and acquired origin[78–81].

Congenital causes

- Mutations of genes includes
  - *NR5A1* gene
  - Nuclear receptor subfamily 0 group B member 1 gene
  - Genetic causes of Kallmann syndrome: anosmin 1 gene, fibroblast growth factor receptor 1 gene and prokineticin 2 gene
  - Genetic causes of isolated hypogonadotropic hypogonadism: gonadotropin-releasing hormone receptor gene and Kisspeptin and G protein-coupled receptor 54 gene
  - Paired-like homeobox 1 gene
  - HESX homeobox
  - Leptin and leptin receptor gene
- Genetic defects of chromosomes (such as Prader-Willi syndrome)

Acquired causes

- Brain injury (surgery and trauma)
- Tumours of the central nervous system
- Inflammatory diseases (such as histiocytosis and tuberculosis)
- Infections (such as HIV)
- Ageing
- Medications (such as androgens and GnRH analogue intake)
- Chronic conditions (such as diabetes, heart diseases, obesity, anorexia nervosa, malnutrition, obesity and hyperprolactinaemia)
- Excessive exercise

boys manifesting delayed puberty (the onset of puberty occurred later than the age of 14 years), 7% had primary hypogonadism, 10% had secondary hypogonadism, and 20% had hypogonadism resulting from both primary and secondary causes[6,84]. In addition to age, the prevalence of primary and secondary hypogonadism might be associated with the prevalence of obesity and diabetes[85].

Testosterone replacement therapy

The most common method of treating male hypogonadism is with testosterone replacement therapy (TRT)[86]. Exogenous testosterone can be administered via various preparations with corresponding dosing intervals[87], including long-acting preparations (10 weeks–6 months) (subcutaneous pellets and injectable intramuscular undecanoate), intermediate-acting preparations (1–3 weeks) (intramuscular testosterone cypionate, enanthate, propionate and ester combinations), daily-acting preparations (transdermal patch and topical gel) and short-acting formulations (oral undecanoate, buccal system and intranasal pump). Exogenous administration of testosterone can restore serum testosterone levels and greatly ameliorate the symptoms of male hypogonadism[86]. However, most formulations, with the exception of the short-acting preparations, impair spermatogenesis by repressing the HPG axis[87–92]. In healthy men, testosterone production is precisely regulated by the HPG axis: the hypothalamus initiates the process of testosterone production by secreting GnRH to stimulate the release of LH from the anterior pituitary gland, which stimulates Leydig cells to produce testosterone[7] (FIG. 1a). In parallel, GnRH stimulates the release of FSH from the anterior pituitary gland, which activates Sertoli cells, thereby facilitating spermatogenesis[7] (FIG. 1a). In men with hypogonadism who receive TRT, elevated circulating levels of testosterone suppress both the hypothalamus and the pituitary gland, therefore inhibiting the release of GnRH, LH and FSH. The reduction in LH levels results in the repression of endogenous production of testosterone by Leydig cells, causing a decline in intratesticular testosterone levels, which (together with the reduced FSH levels) leads to the inhibition of sperm production[87–92]. Such spermatogenic impairment can be reversed after TRT has been discontinued, but most testosterone preparations are not suitable for men wishing to maintain fertility during treatment. Other adverse effects of non-short-acting testosterone include polycythaemia, serious pulmonary oil microembolism reactions, anaphylaxis and transference leading to secondary exposure to testosterone[86,87].

Owing to the concern regarding infertility, short-acting testosterone therapy becomes an alternative choice for the treatment of hypogonadism[87]. Intranasal administration of exogenous short-acting testosterone (given every 8–12 h) enables serum testosterone to fluctuate around normal mean levels, potentially reducing the duration of exposure to supraphysiological testosterone levels, which are often caused by other formulations[87]. This short-acting testosterone potentially minimizes the suppression of exogenous testosterone on HPG axis function and, therefore, preserves fertility[87]. A single-centre, open-label, single-arm trial that evaluated 33 men with hypogonadism (18–55 years old; total testosterone <300 ng/dl) after they received intranasal testosterone therapy for 6 months, showed that such treatment did not affect sperm concentrations compared with baseline (mean difference of −5.5 million/ml, 95% CI: −11.6 to 0.5, P = 0.081) and maintained a total motile sperm count of over five million in 93.9% of men[93], suggesting that intranasal testosterone did not suppress spermatogenesis in a substantial manner. Other short-acting formulations include a testosterone buccal system that causes small fluctuations of serum testosterone levels, but is administered at longer time intervals (given every 12 h) and higher doses (60 mg/day) than intranasal testosterone therapy (22–33 mg/daily), and oral testosterone undecanoate, although this preparation causes fluctuating testosterone serum levels[87], which could lead to potential inhibitory effects on male fertility[86,94]. Other adverse effects of short-acting testosterone formulations include irritation and high blood pressure (with testosterone undecanoate) and intranasal administration is contraindicated in men with nasal disease[86,87]. Collectively, longer and larger studies on short-acting testosterone are needed to confirm the therapeutic efficacy, safety and effects on fertility preservation.

Men receiving TRT are at a higher risk of cardiovascular events than men not receiving TRT, likely owing to the fact that TRT can cause excessive erythrocytosis (which results in augmentation of blood-flow resistance) and expansion of extracellular volume (which predisposes men with cardiac disease to fluid overload)[95]. A study that evaluated men with hypogonadism undergoing angiography found a higher risk in men treated with TRT (n = 1,223, mean age = 64 years) than men without TRT (n = 7,486, mean age = 61 years) for combined cardiovascular events of myocardial infarction, stroke and death (HR = 1.29, 95% CI: 1.04–1.58, P = 0.02)[96]. Another study of 55,593 men with an average age of 54 years found that TRT could increase their risk of having a non-fatal myocardial infarction compared with the risk during the pre-TRT period (RR = 1.36, 95% CI: 1.03–1.81)[97]. Notably, the relative risk of myocardial infarctions for TRT increased from 0.95 (95% CI: 0.54–1.67) for men aged <55 years old to 3.43 (95% CI: 1.54–7.66) for men aged >75 years old[97], demonstrating a stronger link between TRT and myocardial infarctions in older patients. The link between TRT and an increase in cardiovascular events has been an extensively debated topic. Although the benefits of TRT are widely supported in cases of true hypogonadism (either classical or age-related), the use of TRT by men without hypogonadism aiming to increase their testosterone levels is questionable, likely related to reported cardiovascular events, and is, therefore, not recommended[98,99].

Prostate-related events, such as elevation of PSA and prostate cancer, are also potentially stimulated by TRT[100]. A meta-analysis across 19 studies identified an OR of 1.8 (95% CI: 1.07–2.95) of having a prostate-related event in 651 men with hypogonadism treated with TRT compared with 433 men receiving placebo treatment[100]. Another meta-analysis showed that short-term TRT can increase PSA levels; levels above 10 ng/ml indicate a 42–64% likelihood of having prostate cancer[101,102]. This analysis found that men receiving transdermal testosterone therapy (n = 45) had higher PSA values (mean difference = 0.33, 95% CI: 0.21–0.45, P < 0.00001) than men in the placebo group (n = 51), suggesting that TRT might initiate prostate cancer[101,102]. Nevertheless, for quality-of-life reasons, TRT can cautiously be offered to men at a low risk of prostate cancer or those men who have been treated for prostate cancer, with no evidence that treatment instigates disease recurrence[103].

TRT can cause problems owing to transdermal administration via gels and other products. Skin irritation can occur and the testosterone can be transferred via skin contact to children and partners, resulting in potentially undesirable adverse effects of increased testosterone levels, such as virilization in young children and hyperandrogenism in women[104]. TRT also fails to meet the criteria of precision medicine, as testosterone levels resulting from exogenous administration of testosterone are difficult to adjust to match the circadian rhythm of the serum testosterone levels, which generally peaks during sleep and reaches a nadir in the afternoon[98,105,106]. Thus, alternative therapeutic methods are desired by men affected by primary hypogonadism who wish to be treated comprehensively.

Gonadotropin therapy

Alternative therapies to TRT, such as administration of exogenous gonadotropins (hCG or LH) with or without FSH, can be used to restore fertility in patients with secondary hypogonadism[72]. Administration of gonadotropins is not indicated for men with primary hypogonadism, as their Leydig cells respond weakly to gonadotropins. hCG and LH share the same receptor (LHCGR) and promote the testosterone biosynthesis of Leydig cells, but hCG has a longer half-life (36 h) than LH (30 min), making hCG a more commonly used gonadotropin for treating secondary hypogonadism[107]. Notably, hCG is the only FDA-approved non-testosterone compound for the treatment of male hypogonadism[108]. In comparison to TRT, hCG therapy can preserve spermatogenesis. hCG is generally given by subcutaneous or intramuscular injection, with an initial dose of 1,000–1,500 IU twice or three times a week[109,110]. FSH alone cannot reverse infertility in hypogonadal men, but FSH can be given (via subcutaneous injection, 75–150 IU three times a week) to patients when hCG administration alone fails to restore spermatogenesis[109]. A study including 75 men diagnosed with secondary hypogonadism showed that 38 men became fathers after

receiving 116 cycles of hCG therapy with an initial hCG dose of 1,500 or 2,000 IU given twice a week followed by 75 IU of FSH given three times a week, if no sperm were detected following 6 months of hCG treatment[110]. The median concentration of sperm to achieve pregnancy was 8.0 million/ml (95% CI: 0.2–59.5 million/ml) after 2.3 years of treatment[110]. hCG administration is also used in combination with TRT to maintain fertility during treatment, or to re-establish fertility after TRT[107], and has minimal adverse effects, except for gynaecomastia[111]. However, the required twice-per-week injections are inconvenient for the patients. Thus, hypogonadal men who do not seek to preserve fertility might choose alternative therapies[111].

Stem-cell-based therapy

Cell-based therapy, in which the Leydig cell lineage can be re-established in human bodies to produce testosterone normally in response to the HPG axis, is the ideal choice for treating primary hypogonadism. Cell-based (mostly stem-cell-based) therapies for treating nervous system diseases and rescuing damaged organs are increasingly available[112]. Although research is still ongoing, the full potential of these therapies is getting closer to being fulfilled.

Two types of natural stem cells are frequently used in stem-cell-based therapy: embryonic stem cells (ESCs), a type of pluripotent stem cell (PSC), which are dissociated from the inner cell mass of a blastocyst, and adult stem cells, which exist in the body after birth[113–115]. Compared with adult stem cells, ESCs are superior owing to their unlimited self-renewal capacity and pluripotency — the capacity to differentiate into any type of somatic cell in the body. Adult stem cells show more restricted differentiation capacity than ESCs and can exist as either multipotent stem cells (precursors of a limited number of cell types, such as haematopoietic stem cells) or as unipotent stem cells (precursors of a single cell type, such as spermatogonial stem cells). Unlike ESC, which can only be isolated from blastocysts, adult stem cells are comparatively easily obtained, as they are distributed throughout the body and are present as a variety of cell types[112].

In addition to these natural sources of stem cells, stemness can be artificially induced in some types of cells. In 2006 and 2007, mouse and human ESC-like cells, respectively, were successfully generated by genetically reprogramming adult fibroblasts[116,117]. These cells possess the characteristics of PSCs, including unlimited self-renewal and pluripotency and, therefore, are considered a type of PSC. These cells are generated through artificial induction, and so they are called induced pluripotent stem cells (iPSC). Whether clinically relevant differences exist between iPSCs and ESCs that might hinder their applications in transplantation surgery remains unclear. However, a study in 2013 comparing autologous with allogeneic transplantation of iPSC-derived neurons showed that autografted human iPSC-derived neurons survived better in vivo and only led to minimal immune response after transplantation, whereas allografted iPSC-derived neurons had poorer survival and more serious immune rejection[118]. In addition to the prospect of therapies based on cells of the patient's own genetic background, human iPSCs also present advantages in that the source cells (exfoliated renal epithelial cells in urine) can be obtained in a non-invasive manner, avoiding additional trauma to patients and the destruction of human blastocysts[119]. Thus, human iPSCs overcome both the immune rejection and ethical issues associated with other cell sources, and could be a valuable resource for replacing diseased or damaged tissues[120].

Inducing stem cells into Leydig cells

Leydig cells are the main source of testosterone in the human body; therefore, Leydig cell transplantation is a reasonable solution to treating primary hypogonadism[71]. Owing to difficulties in obtaining human adult Leydig cells, stem cells become an alternative source for deriving these cells. Different types of stem cells, including human adult stem Leydig cells, mesenchymal stem cells, pluripotent stem cells and fibroblasts have been successfully induced into Leydig-like cells (FIG. 3).

Adult stem Leydig cells. Among all the cells that can potentially give rise to Leydig cells, adult stem Leydig cells, which reside in the testes of adult men and are already able to regenerate new adult Leydig cells (FIG. 3a), are the first choice for cell-based therapies for hypogonadism[71,121]. Adult stem Leydig cells are thought to be multipotent, as they can form other lineages, such as chondrocytes, adipocytes and osteoblasts[122].

Early in 2004, transplantation of testicular interstitial stem cells (a population that has also been shown to include adult stem Leydig cells) resulted in a significant increase in circulating testosterone levels in LH receptor-knockout mice versus mice receiving no cells ($P<0.01$)[121]. However, the researchers could not determine which stem lineage in transplanted animals gave rise to the final testosterone-secreting cells, as multiple lineages (stem adult Leydig cells, spermatogonial stem cells and myoid stem cells) were included in the testicular interstitial stem cell population (TABLE 1). From 2013 to 2016, three studies performed in rats and one in humans definitively showed that seminiferous tubules were the source of stem adult Leydig cells[41,123–125]. Researchers have now successfully isolated stem adult Leydig cells from seminiferous tubules of rats, monkeys and humans using specific expression markers (e.g. CD51, CD90 and p75)[34,71,126]. These cells have been expanded in vitro using factors promoting their proliferation (e.g. PDGF-AA and/or PDGF-BB, activin, DHH and FGF2), and stem adult Leydig cells have differentiated into adult Leydig cells using factors essential for their differentiation (e.g. PDGF-AA, DHH and lithium) (TABLES 1 and 2).

The successful isolation, expansion and in vitro culture of human Leydig cells, as shown by the fact that human stem Leydig cells could proliferate for at least eight passages and expand approximately 5,000 times in vitro, makes them a promising resource for treating primary hypogonadism[71] (TABLE 2). However, concerns remain about the sources of human stem Leydig cells, as most are obtained either from cadavers or from patients

Blastocyst
Mammalian preimplantation embryos consisting of an inner cell mass (giving rise to embryos) and trophectoderm (giving rise to the placenta).

Stemness
The ability of a cell to proliferate, to differentiate and to keep a balance between proliferation, regeneration and quiescence.

with spermatogenic failure and testicular dysfunction[71]. Moreover, a relatively rare population (approximately 0.3–1.79% of total testicular cells) of primary stem Leydig cells exists in the testicular tissue and the purity of the isolated Leydig cell population after in vitro expansion remains questionable[127].

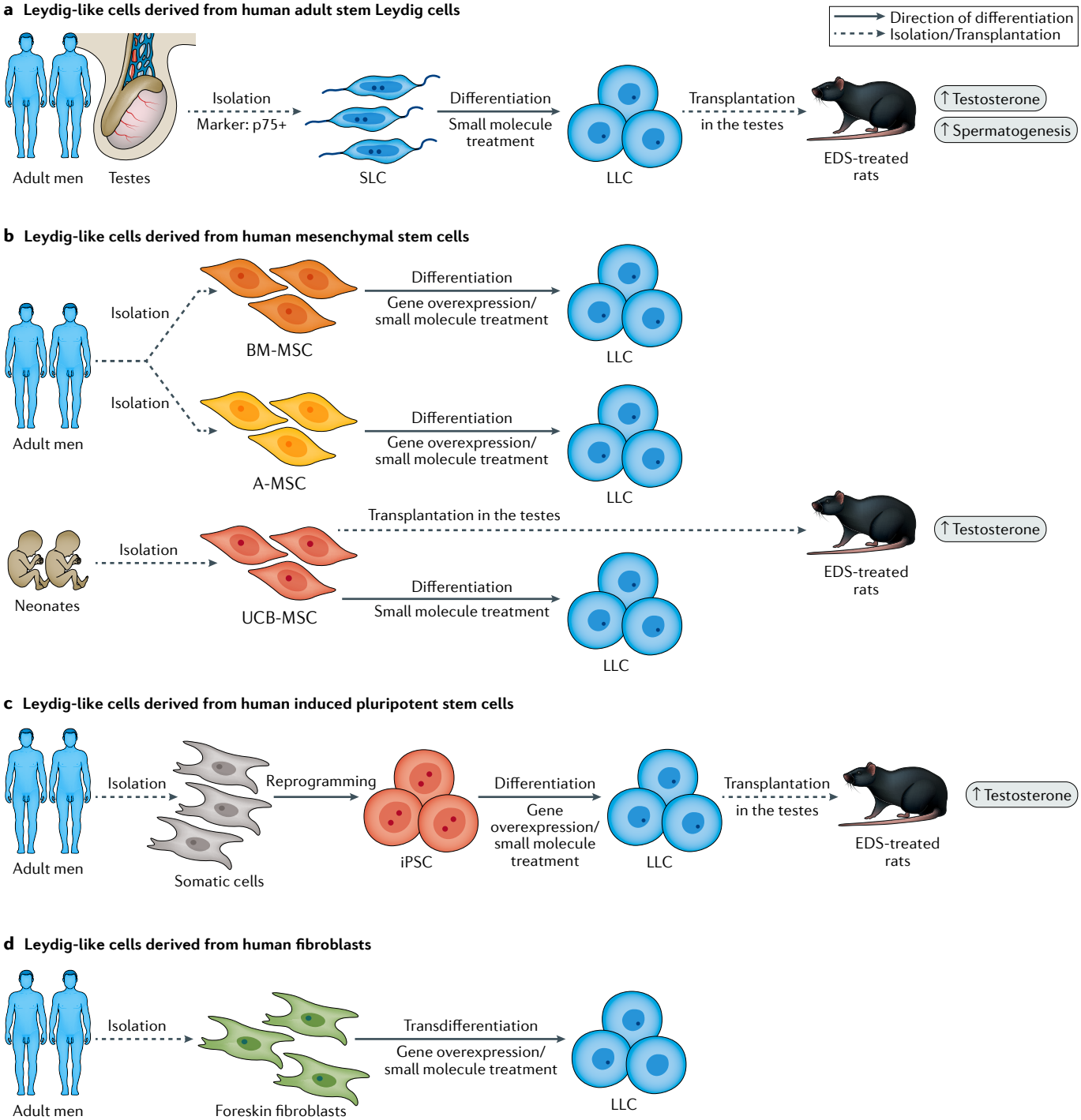


Fig. 3 | **Induction of human Leydig-like cells from different cell types.** Human Leydig-like cells (LLCs) can be induced through the differentiation of different types of stem cells. **a** | Human adult stem Leydig cells (SLCs), which reside in the testes of adult men, can be isolated using the marker $p75^+$ and induced into LLCs with treatment of small molecules, such as thyroid hormone, luteinizing hormone, insulin-like growth factor 1 and platelet-derived growth factor-BB. These LLCs rescued serum levels of testosterone and spermatogenesis when they were transplanted in the testes of ethane dimethane sulfonate (EDS)-treated rats[71]. **b** | Human mesenchymal stem cells (MSCs), which are isolated from bone marrow (BM-MSCs), adipose (A-MSCs), or umbilical cord blood (UCB-MSCs), can be induced into LLCs with the ectopic expression of certain genes (such as *NR5A1*) and treatment of small molecules, or through the transplantation of isolated cells into EDS-treated rats[140,141,143,144,147,148]. **c** | Human induced pluripotent stem cells (iPSCs), which can be reprogrammed from somatic cells of adult men, can be induced in LLCs using small molecules with or without the forced expression of *NR5A1*. These LLCs rescued serum levels of testosterone when they were transplanted in the testes of EDS-treated rats[163]. **d** | Human foreskin fibroblasts collected from adult men can be induced into LLC through transdifferentiation.

Table 1 | **Summary of preclinical trials that derive Leydig-like cells from rodent stem cells**

Cells	Origins	Study design	Main results	Study type	Ref.
Testicular interstitial stem cells	Mouse	Testicular cells with "Hoechst dim"[a] criterion transplanted into LHCGR-knockout mice	Restoration of circulating testosterone level	In vivo	121
Seminiferous peritubular cells (SLCs)	Rat	Dissociation of seminiferous tubules from testicular interstitium and separate culture of cell types	Testosterone-producing cells appeared on the surface of seminiferous tubules	In vitro	210
Seminiferous peritubular cells (SLCs)	Rat	Seminiferous tubule cells incubated with ITS, LH or NGF after EDS treatment	Testosterone-producing cells greatly induced by NGF	In vitro	123
Seminiferous peritubular cells (SLCs)	Rat	Incubation of seminiferous tubule cells with PDGF-AA or PDGF-BB after EDS treatment	Testosterone-producing cells induced by PDGF-AA	In vitro	124
Seminiferous peritubular cells (SLCs)	Rat	Incubation of seminiferous tubule cells with different factors after EDS treatment	Testosterone-producing cells induced by DHH, lithium and activin	In vitro	41
PDGFR$^+$ interstitial cells (SLCs)	Rat	Incubation of isolated cells with thyroid hormone, IGFI and LH; transplantation of isolated cells into EDS-treated rats	Testosterone-producing cells obtained in vitro and 3β-HSD$^+$ cells detected in vivo	In vitro; in vivo	42
Nestin$^+$ interstitial cells (SLCs)	Mouse	Incubation of isolated cells with defined conditions and transplantation of isolated cells into EDS-treated young adult mice and rats or ageing mice	Induced cells produced testosterone and restored serum testosterone levels	In vitro; in vivo	128
CD51$^+$ interstitial cells (SLCs)	Mouse	Incubation of isolated cells with defined conditions and transplantation of isolated cells into EDS-treated rats	Induced cells produced testosterone and restored serum testosterone levels	In vitro; in vivo	129
Seminiferous peritubular cells (SLCs)	Mouse	Subcutaneous transplantation of SLCs with Sertoli cells and myoid cells into mice and evaluation of the effects of DHH on SLCs	Restored serum testosterone level; DHH promoted SLC differentiation	In vivo	135
Bone marrow MSCs	Mouse	Isolation of CYP11A1$^+$ cells	Induced cells expressed CYP11A1, SF-1, 3β-HSD and LHCGR	In vitro	139
Bone marrow MSCs	Mouse	Forced expression of *Nr5a1* and treatment with cAMP	Induced cells produced testosterone	In vitro	139
Adipose MSCs	Rat	Subcutaneous injection of adipose MSC into ageing rats	Elevated serum testosterone and high serum LH levels	In vivo	146
ESCs	Mouse	Forced expression of *Nr5a1* and treatment with cAMP and retinoic acid	Induced cells produced progesterone	In vitro	156
ESCs	Mouse	Embryoid body formation and forced expression of *Nr5a1*with cAMP treatment and LIF withdrawal	Induced cells produced testosterone and oestradiol	In vitro	157
ESCs	Mouse	Mesenchymal cell induction, forced expression of *Nr5a1* with LIF treatment and gelatine coating	Induced cells produced corticosterone and testosterone	In vitro	159
ESCs	Mouse	Forced expression of *Nr5a1* with cAMP and forskolin treatment; transplantation of induced cells into EDS-treated rats	Elevated serum testosterone levels	In vitro; in vivo	160
Embryonic or dermal fibroblasts	Mouse	Forced expression of *Nr5a1*, *Dmrt1*, and *Gata4*; transplantation of induced cells into endogenous Leydig cell-deleted mice	Elevated serum testosterone levels or restored serum testosterone levels	In vitro; in vivo	170
Embryonic fibroblasts	Mouse	Treatment with small molecules; transplantation of induced cells into endogenous Leydig cell-deleted mice	Elevated serum testosterone levels or restored serum testosterone levels	In vitro; in vivo	171

3β-HSD, 3β-hydroxysteroid dehydrogenase; cAMP, cyclic adenosine monophosphate; CYP11A1, cholesterol side-chain cleavage enzyme, mitochondrial; DHH, desert hedgehog protein; EDS, ethylene dimethane sulfonate; ESCs, embryonic stem cells; IGFI, insulin-like growth factor I; ITS, insulin, transferrin and selenium; LH, luteinizing hormone; LHCGR, luteinizing hormone/choriogonadotropin receptor; LIF, leukaemia inhibitory factor; MSCs, mesenchymal stromal (or stem) cells; NGF, nerve growth factor; PDGF-AA and PDGF-BB, platelet-derived growth factor ligands; SF-1, steroidogenic factor 1; SLCs, stem Leydig cells. [a]Hoechst dim: the capacity of cells to exclude the Hoechst dye, a fluorescent dye used for labelling DNA.

Studies conducted in mice and rats show that isolated stem Leydig cells can be transplanted back into testes and then generate new adult Leydig cells to replace chemically injured or senescent ones, therefore, rescuing testosterone production[42,128,129] (TABLE 1). In mice, transplanted stem Leydig cells were found to be able to differentiate into testosterone-producing Leydig cells and promote proliferation of spermatogenic cells[128,129]. In humans, stem Leydig cells can be isolated from testes using the surface marker p75 (REF.[71]) (TABLE 2). When p75$^+$ stem Leydig cells were transplanted into the testes of EDS-treated rats, they differentiated and replaced the injured adult Leydig cells, therefore, increasing serum testosterone levels and accelerating the recovery of spermatogenesis as shown by the presence of significantly more synaptonemal complex protein 3 (a commonly

used marker to evaluate spermatogenesis) positive cells ($P < 0.01$), an increased sperm number ($P < 0.01$) and higher sperm mortality ($P < 0.01$) in EDS-treated rats receiving p75$^+$ cells, compared with those rats receiving saline[71] (FIG. 3a). p75$^+$ cell transplantation also promoted a significant increase in reproductive organ weight compared with saline-treated rats ($P < 0.01$), suggesting that these cells could rescue the atrophy of reproductive organs caused by testosterone deficiency[71]. Another group that isolated p75$^+$ stem Leydig cells from monkeys also demonstrated that autologous stem Leydig cells could ameliorate testosterone deficiency-related syndromes and rescue spermatogenesis[126]. This study showed that transplantation of 1.15–2.13×10^7 stem Leydig cells per testis could significantly increase levels of total testosterone from 628 ng/dl (21.79 nmol/l) to 1267 ng/dl (43.96 nmol/l) ($P < 0.05$) at week 4. The levels of free testosterone, bioavailable testosterone and the ratio of testosterone to LH were also elevated. However, proliferation of Leydig cells was detected at week 8 after transplantation, but levels of testosterone had decreased, suggesting that transplanted Leydig cells only maintained normal function up to 8 weeks[126].

Endosialin$^+$ cells isolated from the human testis are also probably stem adult Leydig cells, as they can differentiate into Leydig cells and produce testosterone in response to LH stimulation after in vitro induction and in vivo transplantation[127]. However, the capacity of these

Table 2 | **Summary of preclinical trials that derive Leydig-like cells from stem cells of humans and monkeys**

Cells	Origins	Study design	End steroids	Study type	Ref.
Testicular peritubular cells (SLCs)	Human	Incubation of isolated cells with forskolin and PDGF-BB	Induced cells produced progesterone	In vitro	125
p75$^+$ SLCs	Human	Defined conditions for Leydig cell induction; transplantation of isolated cells into EDS-treated rats	Induced cells produced testosterone and/or restored serum testosterone levels	In vitro; in vivo	71
p75$^+$ SLCs	Monkey	Defined conditions for Leydig cell induction; autologous transplantation of isolated cells into testosterone-deficient monkeys	Induced cells produced testosterone and/or restored serum testosterone levels	In vitro; in vivo	126
Endosialin$^+$ SLCs	Human	Defined condition for Leydig cell induction; transplantation of isolated cells into immunodeficient mice	Induced cells produced testosterone	In vitro; in vivo	127
Bone marrow MSCs	Human	Forced expression of *NR5A1* and collagen type I coating	Induced cells produced testosterone and cortisol	In vitro	140
Bone marrow MSCs	Human	Forced expression of *LHCGR* and cAMP treatment	Induced cells produced testosterone, cortisol and aldosterone	In vitro	141
UCB- MSCs	Human	Forced expression of *NR5A1* and/or cAMP treatment	Induced cells produced testosterone and oestradiol	In vitro	147
Bone marrow MSCs	Human	Forced expression of *NR5A1* and cAMP treatment	Induced cells produced testosterone and cortisol	In vitro	143
UCB-MSCs	Human	Forced expression of *NR5A1* and cAMP treatment	Induced cells produced testosterone and cortisol	In vitro	143
Bone marrow MSCs	Human	Treatment with HMG, LH, HCG, PDGF and IL-1α	Induced cells produced testosterone	In vitro	144
UCB MSCs	Human	Incubation of UCB-MSCs with Leydig cell-conditioned media or defined differentiation-induced media	Induced cells expressed CYP11A1, 3β-HSD, CYP17A1 and LHCGR	In vitro	145
UCB-MSCs	Human	Transplantation of isolated cells into EDS-treated rats	Elevated serum testosterone levels	In vivo	148
iPSCs	Human	Induction of mesenchymal progenitors with forced expression of *NR5A1*, collagen type I coating, and DHH, cAMP and HCG treatment	Induced cells produced testosterone	In vitro	162
iPSCs	Human	Induction of Leydig cells with iPSC differentiation-induced and enrichment media	Induced cells produced testosterone and elevated serum testosterone levels	In vitro; in vivo	163
Urine-derived stem cells	Human	Forced expression of *NR5A1* and activation of PKA and LHRH pathways	Induced cells produced testosterone and cortisol, and expressed low amounts of LHCGR	In vitro; in vivo	172
Foreskin fibroblasts	Human	Forced expression of *NR5A1* and treatment with small molecules	Induced cells produced testosterone	In vitro	173

3β-HSD, 3β-hydroxysteroid dehydrogenase; cAMP, cyclic adenosine monophosphate; CYP11A1, cholesterol side-chain cleavage enzyme, mitochondrial; CYP17A1, steroid 17α-hydroxylase/17,20 lyase; EDS, ethylene dimethane sulfonate; HCG, human chorionic gonadotropin; HMG, human menopausal gonadotropin; IL, interleukin; iPSCs, induced pluripotent stem cells; LH, luteinizing hormone; LHCGR, luteinizing hormone/choriogonadotropin receptor; LHRH, luteinizing hormone-releasing hormone; MSCs, mesenchymal stromal (or stem) cells; PDGF, platelet-derived growth factor; PDGF-BB, a PDGF ligand; PKA, protein kinase A; SLCs, stem Leydig cells; UCB; umbilical cord blood.

cells to replace injured adult Leydig cells in EDS-treated rats and accelerate the recovery of spermatogenesis needs to be investigated.

The administration of gonadotropins to the recipients who receive Leydig cell transplantation potentially could help to maintain testosterone biosynthesis. Administration of 24–36 μg LH per day in hypogonadal rats, who received endogenous LH-suppressive treatment, is sufficient to maintain testosterone production of Leydig cells at or above the baseline[130]. However, no direct in vivo evidence supports the role of gonadotropin in the survival, proliferation and differentiation of transplanted stem Leydig cells. The same is true for the type (that is, LH or hCG), dose, time interval and duration of gonadotropin administration in Leydig cell transplantation therapy.

Successful autologous therapy using stem Leydig cells in primates substantially increases the feasibility of using stem Leydig cells for treating patients with primary hypogonadism. However, direct injection of stem Leydig cells into the testes might not be feasible for clinical therapy, as repeated transplantation in the testes to maintain the size of the Leydig cell population is impractical. Moreover, the testicular microenvironment of ageing men, with the commonly observed increase in macrophage number and activated inflammatory processes, including the increase in inflammatory cytokine interleukin 1 beta (IL-1B) levels and increased testicular reactive oxygen species (ROS) production, is probably unsuitable for Leydig cell transplantation[131–133]. IL-1B increases the expression of cyclooxygenase 2 (COX2), a key enzyme in the biosynthesis of the pro-inflammatory mediator prostaglandins, and increased ROS impairs cell membrane lipids, proteins and protein–protein interactions critical for cholesterol import into mitochondria, the rate-limiting step of testosterone production in Leydig cells[131–134].

In 2019, a strategy was developed that enables stem Leydig cells to be precisely directed to differentiate into adult Leydig cells in vivo outside the testes[135] (TABLE 2). Researchers mixed stem Leydig cells with Sertoli cells and myoid cells, encapsulated them in Matrigel and transplanted these encapsulated cells subcutaneously into the abdomen of castrated mice. After 1 month, mice that had received the mixed cell autograft showed high serum levels of testosterone and reduced serum levels of LH, whereas autografts containing only stem Leydig cells did not survive[135]. These observations suggest that transplanted stem Leydig cells need to be accompanied by niche cells, which release paracrine signals (for example, DHH and LIF) important for Leydig cell development[34,41,42], to support their in vivo survival and differentiation if they are transplanted outside the testes[135]. Furthermore, this research group found that differentiation of stem Leydig cells into adult Leydig cells was improved if the stem cells were pre-incubated with a DHH agonist, but differentiation was inhibited by pre-treatment with a DHH antagonist. These results impressively showed the essential role of DHH in the development of adult Leydig cells and demonstrated the feasibility of using stem Leydig cells in transplantation therapy, even when the testicular environment of the recipient is not suitable for the survival and differentiation of stem Leydig cells when transplanted alone[135].

The main limitations of using human stem Leydig cells in transplantation therapy are immune rejection in allogenic transplantation and the necessarily invasive process of collecting stem Leydig cells for autologous therapy. The small number of human stem Leydig cells in the testes and the restricted proliferative ability of human stem Leydig cells during in vitro expansion are also challenges in using these cells therapeutically in the clinic[128]. Thus, other types of stem cells with improved availability that can be collected less invasively than with testicular biopsy are required for clinical use, especially in autologous therapy.

Mesenchymal stem cells. Mesenchymal stem (or stromal) cells (MSCs) are non-haematopoietic progenitor cells with the capacity for self-renewal and multipotency that can differentiate into various mesenchymal cell lineages[136]. MSCs can be isolated from bone marrow, umbilical cord blood, adipose tissue and other tissues[137] (FIG. 3b). Owing to their high expansion ability and wide availability, MSCs have become one of the most popular sources for deriving Leydig cells in vitro[138].

In 2007, rat bone marrow-derived MSCs were shown to differentiate into Leydig-like cells after being transplanted into testes[139] (TABLE 1). Moreover, mouse bone marrow MSCs could also be induced to differentiate into Leydig-like cells by a cell-sorting method based on enrichment for *CYP11A1* promoter-driven signals or a culture method combining transfection with *Nr5a1* (encoding SF-1) and cyclic adenosine monophosphate (cAMP) treatment[139]. However, the same methods in human bone marrow MSCs failed to generate Leydig-like cells but rather directed differentiation towards an adrenal-like phenotype[139]. Many groups have reported other methods of differentiating human bone marrow MSCs into steroidogenic cells, but the differentiated cells expressed both LH and ACTH receptors, and secreted both testosterone and cortisol (characteristics corresponding to Leydig and adrenal gland cells, respectively)[140–143] (FIG. 3b and TABLE 2). A pure population of induced human Leydig-like cells derived from bone marrow MSCs was lacking until 2016, when the first successful induction of human bone marrow MSC into Leydig-like cells, which express the steroidogenic enzyme 3β-HSD and produced testosterone, was achieved by gonadotropin treatment combined with other factors[144] (TABLE 2). However, this study still left questions as to whether cortisol or other adrenal-cell-specific steroids were produced by these cells, as the researchers did not perform any experiments to detect these steroids in the culture supernatants[144].

Adipose-derived MSCs and umbilical cord blood-derived MSCs can also be induced to differentiate into Leydig-like cells in vitro[143,145–147] (FIG. 3b). However, the differentiation capacity varies across species and MSC types. Comparison studies showed an ambiguous capacity for human bone marrow MSCs and umbilical cord blood MSCs to differentiate into steroidogenic cells[143,147] (TABLE 2). A study that overexpressed *NR5A1* in cells in

Myoid cells
Smooth muscle cells surrounding the seminiferous tubule.

combination with cAMP treatment could induce human umbilical cord blood MSCs to differentiate into cells producing low amounts of testosterone[147]. However, these cells also showed characteristics of granulosa-luteal cells, which are large luteal cells derived from luteinized granulosa cells in the ovary, including secretion patterns of steroids similar to granulosa-luteal cells (progesterone and oestradiol) and expression of granulosa-luteal cell-specific PPARG coactivator 1 alpha (*PPARGC1A*) gene[147]. Another study showed that both human umbilical cord blood MSCs and human bone marrow MSCs could be differentiated into cells producing testosterone and cortisol, but the umbilical cord blood MSC-derived cells secreted higher levels of steroids and had higher expression levels of steroidogenic genes than bone marrow-derived MSCs[143]. Human umbilical cord blood MSCs transplanted into hypogonadal male rats successfully restored serum testosterone levels, suggesting that these cells could potentially be used for treating patients with primary hypogonadism[148] (FIG. 3b and TABLE 2).

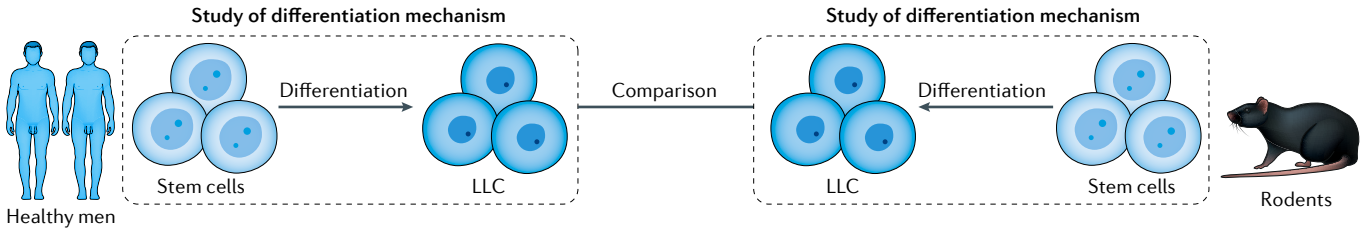
The main limitations of the widespread application of therapies based on MSC-derived Leydig cells are the invasive cell collection procedure and the limited self-renewal capacity of MSCs[137,149]. In addition, serial passage and long-term culture of MSCs have been shown to cause both genetic and epigenetic mutations, which will affect their therapeutic efficacy in treating primary hypogonadism[150–152].

Pluripotent stem cells. Owing to the limitations of adult stem Leydig cells and MSCs in transplantation therapy, PSCs (including ESCs and iPSCs), a stem cell type with the advantages of unlimited proliferative capacity and the potential to differentiate into all types of somatic cells, have emerged as an alternative choice for treating primary hypogonadism[137]. Specifically, iPSCs, the artificially induced PSCs, can be generated from exfoliated renal epithelial cells in urine samples using a non-invasive collection procedure[153], which dramatically expands the scalability of iPSC-based therapy. Both ESC and iPSC have been successfully induced into Leydig-like cells using different protocols adapted to the availability of PSC sources and differences in the differentiation potential of various PSC lines[154,155].

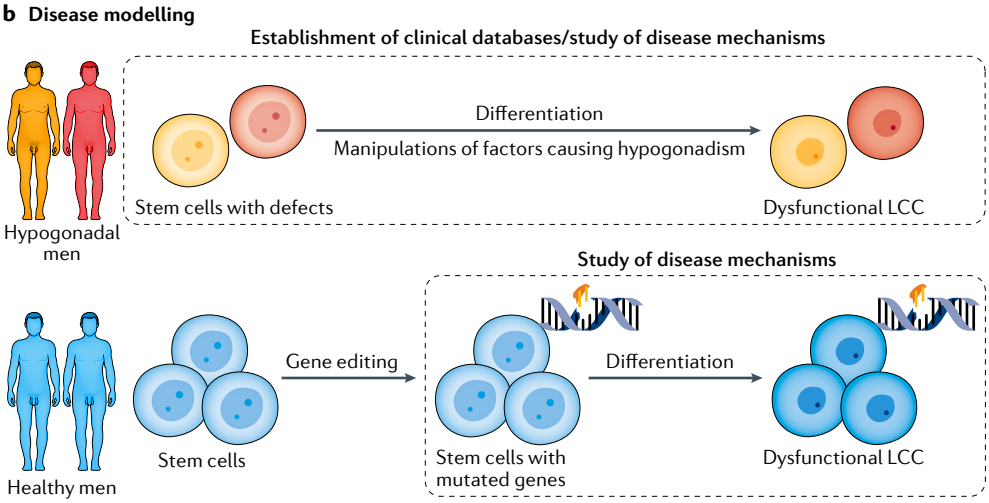
Early in 1997, ectopic expression of *Nr5a1* in mouse ESCs was demonstrated to induce these cells to develop into steroidogenic cells[156] (TABLE 1). However, induced cells only expressed CYP11A1 and produced progesterone[156]. Thereafter, other groups adopted the strategies of inducing ESCs and iPSCs to develop into Leydig-like cells via a method more similar to the development of adult Leydig cells, in which ESC and iPSC are induced to differentiate into intermediate mesoderm cells or MSCs, and then induction of these intermediate mesoderm cells or MSCs occurs to develop into Leydig-like cells[137]. In 2011, two groups successfully induced mouse ESCs to develop into steroidogenic cells via embryoid body formation (the method of inducing 3D aggregates in suspension that consist of three germ layers)[157,158] and mesenchymal cell derivation[159] (TABLE 1). However, the differentiation efficiencies of these processes were quite low, as indicated by the chaotic gene expression patterns (the expression of both the Leydig cell-specific gene *Hsd17b* and the adrenal gland-specific gene *Cyp21b*) and the various types of end steroids produced (testosterone, oestradiol, cortisol, and aldosterone) by the induced cells. In 2015, a robust way of inducing mouse ESCs to develop into Leydig-like cells was established by introducing *Nr5a1* into ESCs by lentiviral particles and treating them with cAMP and forskolin (a labdane diterpenoid, which activates adenylate cyclase and increases cAMP production in Leydig cells)[160] (TABLE 1). These cells produced progesterone and testosterone in vitro and further differentiated into functional adult Leydig cells in vivo; transplantation of these cells into EDS-treated mice restored serum levels of testosterone[160]. However, this method failed to induce human ESCs and iPSCs to develop into Leydig-like cells[161]. In 2019, two separate groups reported protocols using transfection-based (that is, forcing overexpression of SF-1 in cells by transient transfection in combination with treatment with cAMP, DHH and hCG) and small molecule-based (that is, adding different small molecule compounds sequentially to the differentiation-inducing medium to induce the commitment of Leydig cells) strategies to obtain human iPSC-derived Leydig-like cells[162,163] (FIG. 3c). Human Leydig -like cells induced by small molecule treatment showed the ability to rescue serum testosterone levels and increase testis weight in EDS-treated rats compared with EDS-treated rats receiving saline injection (FIG. 3c; TABLE 2), but the possibility that adrenal cells were present in the end population could not be ruled out, as aldosterone and cortisol production by these cells had not been measured[163]. By contrast, the end population of cells derived by the transfection-based protocol was predominantly human Leydig-like cells as opposed to adrenal cells, as the researchers confirmed that these cells had low expression of adrenal-specific genes and negligible amounts of aldosterone and cortisol production[162] (TABLE 2). However, the survival and testosterone biosynthetic capacity of these cells in vivo remains to be determined. Recovery effects on spermatogenesis after transplantation of PSC-derived Leydig-like cells also need to be evaluated.

Human PSCs possess unprecedented advantages in developmental studies, disease modelling and transplantation therapy, but the use of human PSCs (both ESCs and iPSCs) in clinical applications remains problematic. The legal issues, such as laws in Germany and Austria banning the use of embryos for research[164], and ethical concerns, such as views against abortion and destruction of embryos to obtain ESCs, have delayed human ESC studies and applications[165]. De novo mutations in iPSCs due to the reprogramming process (which can be detected by whole-genome sequencing and preferentially happens in condensed heterochromatic domains) could introduce high-risk mutations (for example, cancer-associated mutations) in iPSCs, which raises serious safety concerns. The epigenetic modifications in iPSCs in comparison to ESCs, such as differences in microRNA expression and lineage-specific DNA methylation signatures, can affect the differentiation potential of iPSCs and limit their applications in generating

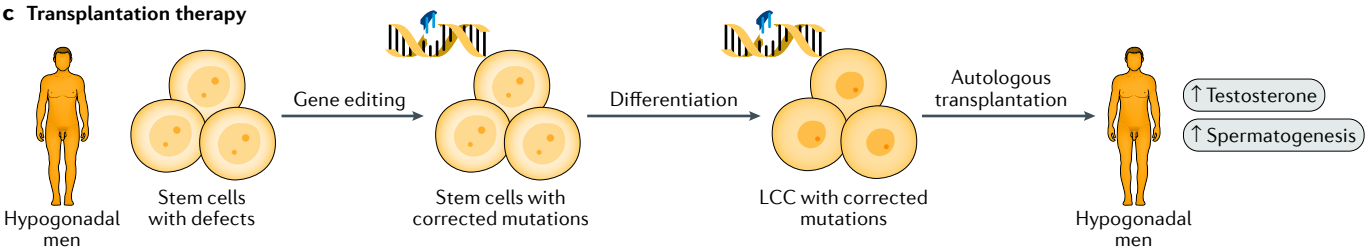
a Developmental study



b Disease modelling



c Transplantation therapy



d Drug screening and personalized therapy

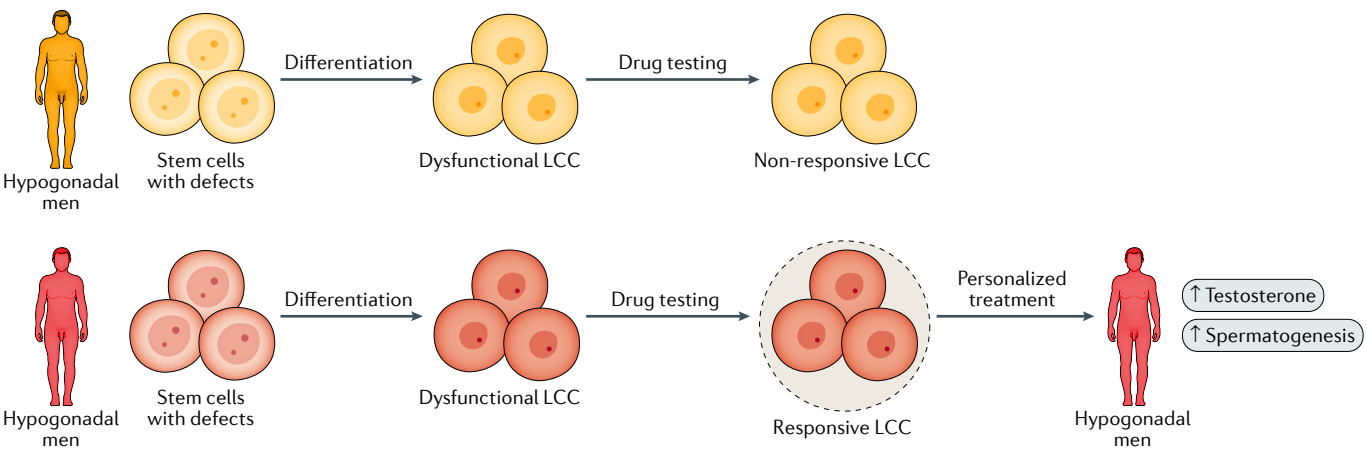


Fig. 4 | **Applications of stem cell-derived, Leydig-like cells for the study and treatment of primary hypogonadism. a** | Leydig-like cells (LLCs) derived from stem cells of healthy men can be used to study Leydig cell development in vitro. A comparison of successful induction strategies used in rodents and humans might improve understanding of why differentiation of human Leydig cells differs from that in other species. **b** | LLCs derived from stem cells of hypogonadal men can be used for disease modelling through the manipulation of genetic factors, environmental exposures and lifestyle. Candidate genes that might cause primary hypogonadism can be manipulated in healthy stem cells, which are further differentiated into LLCs to provide a model for the investigation of the pathophysiology of male hypogonadism. **c** | LLCs derived from patient-specific stem cells could undergo gene editing to correct genetic mutations and be transplanted back to patients to ameliorate the symptoms of primary hypogonadism. **d** | Drugs can be tested on LLCs derived from men with primary hypogonadism. Different patients with primary hypogonadism might show different responses to the same drug. Only the patient whose stem cell-derived LLCs are responsive to the drugs will receive the treatment with these drugs. Those patients whose stem cell-derived LLCs show weak or no response to drugs will not adopt such treatment. This approach can be used to select appropriate drugs and to limit unnecessary adverse effects for individual patients.

desired organs[166]. Accumulated mutations and decreasing pluripotency of iPSCs associated with the patient donor cells and spontaneous mutations and genomic instability as a result of the prolonged culture of PSC reduces their differentiation efficiency and therapeutic efficacy[167]. Tumorigenicity owing to the intrinsic properties of unlimited proliferation of PSCs, which is shown by the formation of a teratoma-like tumour after transplantation, also hampers their clinical use[168]. Elimination of residual PSCs in grafted cells, which can prevent tumour formation, is still a major challenge for PSC-based transplantation[168]. The future use of PSCs in human therapies will depend on our ability to overcome these issues.

Other types of stem cells. In contrast to normal differentiation, wherein a stem cell gives rise to its natural downstream lineage, transdifferentiation is the process by which one cell lineage switches to another lineage[169]. Natural transdifferentiation includes a stepwise conversion of one lineage to dedifferentiate to their precursors that can then differentiate into a new lineage; however, experimental transdifferentiation can convert one lineage directly into another without the intermediate precursor stage[169]. In 2017, a method of transdifferentiating mouse embryonic fibroblasts into Leydig-like cells was reported, by introducing transcription factors *Dmrl*, *Nr5a1* and *Gata4* into fibroblasts[170]. The same group subsequently reported transdifferentiation of mouse adult dermal fibroblasts into Leydig-like cells using small molecules including LH, forskolin, 20α-hydroxycholesterol and SB431542 (the inhibitor of TGFβ type I receptor kinase (ALK-5))[171] (TABLE 1). These two methods directly convert fibroblasts to Leydig cells without a transient dedifferentiation to a precursor such as PSCs or MSCs. These fibroblast-derived cells had a global transcriptomic expression pattern similar to that of Leydig cells, especially with regard to crucial enzymes in the testosterone biosynthetic pathway. More importantly, when transplanted into Leydig-cell-depleted mice, the transdifferentiated cells restored serum testosterone levels[170,171], showing similar effects of rescuing testosterone deficiency to Leydig cells derived from other types of stem cells.

The successful conversion of human urine-derived stem cells (from exfoliated renal epithelial cells) into steroidogenic cells by the forced expression of *NR5A1* via lentiviral infection[172] has also been reported. These cells produced both testosterone and cortisol, but were defined as adrenal-like cells rather than Leydig-like cells because they secreted cortisol in response to ACTH stimulation and expressed extremely low levels of LHCGR[172].

Studies have also demonstrated the successful induction of human foreskin fibroblasts to develop into Leydig-like cells via forced expression of *NR5A1* and treatment with small molecules[173] (FIG. 3d and TABLE 2). These cells expressed Leydig-cell-specific steroidogenic enzymes and produced testosterone. However, the testosterone biosynthetic capacity of these cells and their capacity for recovering spermatogenesis after transplantation still needs to be determined.

Although human Leydig-like cells can be derived from adult stem Leydig cells, MSCs, PSCs or fibroblasts, each cell source has advantages and disadvantages in terms of stem cell-based therapy. Stem Leydig cells are the natural precursors of Leydig cells that provide the safest sources, but their clinical use is hampered by the lack of testicular tissue donors and the limited self-renewal capacity of the cells. MSCs and fibroblasts can provide plentiful cell materials for Leydig cell induction, but the invasiveness of the collection methods is a major drawback in using these as the primary cells[127,174]. The concern of invasive collection regarding stem cell-based therapy can be overcome by deriving iPSCs from urine stem cells[153]. However, the tumorigenicity of iPSCs raises the same concern as with ESCs and dramatically reduces their safety in transplantation therapy[168]. Moreover, all established stem cell-based methods cannot rule out the contamination of other lineages in the end population and are incapable of maintaining grafted Leydig cells in vivo for more than 8 weeks[126]. Thus, an ideal method of deriving Leydig cells that are feasible for clinical use, should eliminate all of these concerns and generate a pure end population with 100% adult Leydig cells, which can continuously produce testosterone under the regulation of the HPG axis.

Future applications of induced Leydig cells

Unlike previous studies that were largely conducted in experimental animals, in vitro induction of human Leydig-like cells from different sources of stem cells by forced expression of genes and treatment with small molecules important for Leydig cell development unprecedently revealed properties unique to human Leydig cells, as well as showing the dynamic changes in human Leydig cell differentiation, including morphological and/or ultrastructural changes, and alterations in transcriptomic or proteomic expression patterns[71,138,162,163,173] (FIG. 4). A comparison study of successful induction strategies used in rats or mice and humans might provide us with the first opportunity to understand why differentiation of human Leydig cells differs from that in other species (FIG. 4a). Understanding these differences will be the key to producing clinically acceptable Leydig-like cells.

New iPSC technology enables researchers to perform reprogramming using clinical material derived from patients, which comes with the context of genetic and epigenetic background correspondence to genetics and epigenetic status of patients (FIG. 4b). The behaviour of patient-specific iPSCs, including their differentiation towards particular cell types that have dysfunctional counterparts in vivo, can be used to identify disease-specific cellular phenotypes and disease-associated mutations[175,176] (FIG. 4b). For example, iPSC-derived neuron and astrocytes from patients with Alzheimer's disease revealed amyloid-β peptide (Aβ) accumulation, which is a common pathological feature of Alzheimer's disease, leading to endoplasmic reticulum and oxidative stress phenotypes in these iPSC-derived cells[177]. Further investigation revealed that amyloid precursor protein (APP)-E693Δ mutation could be a cause of Aβ accumulation[177], highlighting the potential of iPSC-derived cells as a powerful tool to study mechanisms underlying diseases. Moreover, information obtained from patient-specific iPSCs and iPSC-derived lineages can be used to establish a comprehensive knowledge database that integrates the genomics status, ethnicity, environmental exposure and lifestyle of patients, as seen in a iPSC library of sickle cell disease established in 2017, which provides unique information for investigating this disease and developing novel therapeutic strategies[178,179]. Likewise, Leydig-like cells derived from men with hypogonadism can be used to establish a database to enable researchers to identify genetic mutations and epigenetic modifications associated with hypogonadism in men and help clinicians to predict both the risk of hypogonadism in their patients and their responses to treatments[180,181] (FIG. 4b).

Patient-specific iPSCs have been used to model the pathophysiology of various diseases[182,183]. iPSCs from men with hypogonadism could be used to model this disorder (FIG. 4b). Genetic factors, environmental exposures and lifestyle are also considered to contribute to hypogonadism in male patients[184,185] (BOX 1, FIG. 1b and 1c), but their effects and underlying mechanisms remain to be elucidated. The successful generation of iPSC-derived Leydig-like cells enables the importance of these factors for the occurrence, development and severity of hypogonadism in men to be fully investigated via the intentional manipulation of these factors during the production of Leydig-like cells derived from men with late-onset hypogonadism (FIG. 4b). Some genes are known to regulate the development and steroidogenic potential of Leydig cells, such as *NR5A1*, nuclear receptor subfamily 2 group F member 2 (*NR2F2*) and *LHCGR*[186–190], but the scarcity of human testicular biopsy samples limits their use in research and hampers the discovery of new treatment targets. Gene editing, which enables the manipulation of genes including their expression pattern, their dosage and the time course of dynamic changes in expressions and the generation of mutations carried by patients' cells in normal iPSCs, in combination with in vitro Leydig cell differentiation, could provide an ideal tool for us to establish in vitro models of male hypogonadism (FIG. 4b). Such modelling systems will improve our understanding of the roles of hypogonadism-associated genes in Leydig cell development, especially regarding congenital and early-onset Leydig cell dysfunction, which are precipitated by events in embryonic development that can be recapitulated during iPSC differentiation[182,183].

Stem cells provide abundant resources for generating tissues; therefore, many clinical trials have begun to explore the use of human PSC-derived or MSC-derived cells in transplantation therapy, such as using PSC-derived retinal pigment epithelium for treating age-related macular degeneration and MSC-derived neuronal cells for treating neurodegeneration[191,192] (FIG. 4c). A search of the ClinicalTrials.gov database conducted on 8 May 2021 (www.clinicaltrials.gov) revealed 29 clinical trials involving ESCs and ESC-derived cells, 26 clinical trials involving iPSCs and iPSC-derived cells and over 1,000 clinical trials involving MSCs and/or MSC-derived cells. The large-scale application of stem cell-derived cells to clinical therapy suggests that once functional Leydig cells can be induced from healthy stem cells or fixed stem cells, in which hypogonadism-inducing genetic mutations (such as *SRY* mutations and *LHCGR* mutations) have been corrected[169], they should be suitable for autologous transplantation surgery to treat primary hypogonadism by replacing damaged or dysfunctional Leydig cells (FIG. 4c). Results from Leydig cell transplantation in rodents and monkeys indicate that this treatment can restore serum testosterone levels, spermatogenesis and testicular function, supporting the practicality of Leydig cell transplantation for treating hypogonadism in humans[71,126].

The cost and duration of preparing materials for Leydig cell transplantation, mainly owing to the cultural expansion of stem cells and the induction of Leydig cells (which generally requires high-quality cell culture materials, highly-skilled personnel and several months–years to obtain clinical-grade grafted cells), might hinder its clinical applications. However, owing to unique advantages of grafted Leydig cells (that is, the secretion of testosterone in response to the regulation of the HPG axis), Leydig cell transplantation is still a potential alternative therapy option for treating hypogonadism. Under the regulation of the HPG axis, testosterone released by transplanted Leydig cells might be maintained within a normal range that exerts no inhibitory effects on the axis, whereas the administration of exogenous testosterone can result in transient or prolonged inhibition of the HPG axis[87–92]. Moreover, transplanted Leydig cells display a normal circadian rhythm of testosterone secretion in response to pulsatile LH secreted from the recipients of Leydig cells[126,129], whereas the exogenous administration of testosterone (including intranasal testosterone) can never be regulated by LH and show such a rhythm. Furthermore, compared with TRT and other medications that are given daily or weekly, the potential sustainability of Leydig cell transplantation for longer periods of time is exciting.

The concerns regarding patients who are anorchid or whose testicular environment is not suitable for Leydig cell transplantation are largely dispelled by the successful subcutaneous transplantation of Leydig cells in rats[135].

Progress in pancreatic islet transplantation through a co-transplantation of microencapsulated islets with MSCs sheds further light on the future of Leydig cell therapy[193]. To create a suitable transplantation environment, together with prior vascular induction, a sealed nylon mesh pocket, in which a silicon plate was placed, was pre-transplanted in diabetic mice. Four weeks later, pancreatic islets encapsulated with alginate and MSCs mixed with pieces of petaloid recombinant protein (space between these proteins enables better penetration of substances to nourish cells) were co-placed inside the cavity within the mesh bag that was created by the pre-transplanted silicon plates. The co-transplantation system then increased revascularization induced by MSCs and reduced blood glucose blood levels in the recipient mice compared with those mice receiving encapsulated islets alone (230.2 ± 104.9 mg/dl versus 436.8 ± 68 mg/dl, $P < 0.05$)[193]. Furthermore, mesh bags, which encapsulate transplanted cells and enable their easy removal, weaken immune rejection and raise the possibility of replacing dysfunctional or apoptotic cells within the transplanted mesh bags. Thus, when Leydig stem cells are used in Leydig cell transplantation for male hypogonadism, uptake of this therapy should be widespread and reduce the cost of therapy for this disorder.

Limitations of transplantation therapy using stem cell-derived Leydig cells include concerns regarding the use of patient-derived donor cells, including the low availability of stem Leydig cells, potential trauma caused by collecting stem Leydig cells and MSCs, the risk of tumorigenesis and immune rejection associated with therapeutic use of PSCs and the imperfect establishment of induction protocols[194]. Moreover, Leydig cell therapy might not be applicable for treating secondary hypogonadism, given that secondary hypogonadism is mainly caused by hypothalamic and/or pituitary failures[6]. In the cases of mixed (primary and secondary) hypogonadism, either Leydig cell transplantation or gonadotropin administration could fail to recover functional Leydig cells. Until Leydig cell transplantation approaches and corresponding regulatory systems are well established, medications for treating hypogonadism are, therefore, still the primary therapeutic choice. So far, the available medications for treating primary hypogonadism, including TRT (FDA approved), aromatase inhibitors (not approved by the FDA), selective oestrogen receptor modulators (only available in clinical trials) and novel agents under investigation, such as anabolic and androgenic steroids, selective androgen receptor modulators and enclomiphene citrate, are aimed at supplanting testosterone production, fine-tuning the HPG axis, or modulating metabolism of tesosterone[111]. Given the known adverse effects of these therapies, especially negative feedback effects on the HPG axis, perhaps other kinds of medication promoting testosterone biosynthetic activity in human Leydig cells themselves should be developed[111].

In addition, knowledge of the molecular mechanisms underlying testosterone formation has made it possible to consider the use of pharmacological means of inducing testosterone formation by hypofunctional Leydig cells. A series of protein–protein interactions between cytosolic and mitochondrial proteins were identified as being crucial for the movement of the steroidogenesis substrate cholesterol from intracellular stores into mitochondria[1,195]. Two of these proteins were identified as excellent targets to control cholesterol movement and testosterone formation, namely the 18 kDa translocator protein (TSPO) and the voltage-dependent anion channel (VDAC). TSPO drug ligands were shown to increase testosterone formation in vitro and in vivo in both normal and hypogonadal animals[1,196–198]. However, TSPO is present in all steroidogenic cells of the body, limiting the use of these compounds. Peptides containing a VDAC1 sequence blocking the interaction between VDAC1 and the adaptor protein 14-3-3ε protein, which acts as a negative regulator of cholesterol transport into mitochondria, increased testosterone formation by Leydig cells in vitro as well as in vivo in hypogonadal animals, in whom LH and testosterone production had been blocked by the administration of a GnRH antagonist[198]. The fact that 14-3-3ε levels are 10-fold higher in Leydig cells than in adrenal cells provides tissue specificity for the action of these peptides. The possibility of generating biologicals and drugs able to recover testosterone production in hypofunctional Leydig cells is an area of active investigation, which will have to be replicated in both animal and human studies[1,134,198].

To accelerate drug discoveries, stem cell-derived products can be adopted as a novel tool for drug compound screening and toxicity testing (FIG. 4d), particularly when corresponding human cell lines are unavailable[199–201]. To the best of our knowledge, no human Leydig cell line has yet been established, meaning that most preclinical testing of candidate drugs for treating hypogonadism in men is conducted in experimental animals. Several studies have shown that human iPSC-derived cells with in vitro phenotypes reflecting those of the donor individuals have helped researchers to identify novel drugs through screening of compounds[202,203]. New compounds can only be tested in animals; therefore, stem cell-derived Leydig cells could be a highly feasible next step to determine the effects of new drugs on human Leydig cells (FIG. 4d) and to ascertain whether these agents should enter clinical trials. Moreover, for those patients with primary hypogonadism, their own iPSC-derived Leydig-like cells could provide a highly accurate and personalized reference model to help guide treatment decisions (FIG. 4d).

Additional applications for stem-cell-derived Leydig-like cells include in vitro spermatogenesis approaches supported by 3D testicular organoids[204]. 3D testis scaffolds could be printed using a combination of biomaterials, such as nanocellulose-alginate hydrogel with arginine-glycine-aspartate motives, and interstitial testicular cells, to structurally and functionally support in vitro spermatogenesis of seeding spermatogonia[204]. Compared with mouse materials, human stem-cell-derived testicular cells, such as Leydig cells, will be a greater source for establishing 3D-printed testicular organoids. MSCs, which have been used as feeder cells for short-term expansion of spermatogonial stem cells[205], stem cell-derived Sertoli cells, which interact

with Leydig cells and play essential roles in Leydig cell development[206,207] and extracellular matrix components (collagens, laminin and fibronectin) have all been shown to be essential for testicular development[137,208] and could be important elements that not only promote in vitro culture of Leydig cells but also facilitate their attachment to the scaffolds of 3D testicular organoids[209].

Collectively, despite much experimental work in human stem cell-derived Leydig cells, most proposed clinical uses of these cells are merely based on clinical work of other non-Leydig cell lineages, such as neuron and pancreatic islets[177,193]. Future clinical applications of stem-cell-derived Leydig cells will certainly benefit from a better understanding of their cell properties in comparison to their in vivo counterparts, and more data are needed from clinical trials adopting stem cell-derived Leydig cells as testing materials.

Conclusions

In studies of hypogonadism, stem cells have been used to model the pathophysiological processes of hypogonadism and for Leydig cell transplantation. However, the efficiency and safety of Leydig-like cells derived from stem cells remains largely unknown, and more extensive and precise investigations are required before they can be applied in reproductive medicine. The successful subcutaneous transplantation of stem Leydig cells in rats is one potential ectopic location where Leydig cells could be transplanted outside the testes, but further trials of this approach are required in primates and eventually in humans.

Many obstacles still impede the use of stem cells to study Leydig cell development and to treat primary hypogonadism in men. In less than two decades, this field has seen enormous progress, including providing an in vitro system for visualizing Leydig cell differentiation and raising the possibility of treating men with hypogonadism in a highly accurate and personalized way. Stem cell-based research into primary hypogonadism is, therefore, expected to provide ever more possibilities in the future for male reproductive medicine and abundant resources for transplantation therapy.

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L.L. and V.P. researched data for the article, made substantial contributions to the discussion of its content, wrote the article, and reviewed and edited the manuscript before submission.

Competing interests
V.P. is named co-inventor on patents reporting on new molecules inducing testosterone production by normal and hypofunctional Leydig cells issued and filed with U.S.P.T.O. and other international agencies. These patents were licensed by McGill University to IASO BioMed, Colorado, USA. V.P. has received stock from IASO BioMed. L.L. declares no competing interests.

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