Asian Journal of Andrology (2022) 24, 335–344
www.asiaandro.com; www.ajandrology.com

Open Access

REVIEW

Male Endocrinology

Generation of Leydig-like cells: approaches, characterization, and challenges

Zhao-Hui Li[1], Jun-Dong Lu[1], Shi-Jun Li[2], Hao-Lin Chen[3], Zhi-Jian Su[1,4]

Testosterone production by Leydig cells (LCs) plays a crucial role in male reproduction. The functional degeneration of LCs can cause testosterone deficiency, ultimately resulting in primary male hypogonadism. Transplantation of exogenous LCs with the ability to produce testosterone in response to the regulation of the hypothalamus–pituitary–gonad axis could be a promising alternative option to treat male primary hypogonadism. Recent studies have shown that it is possible to generate Leydig-like cells from stem cells by various approaches. In addition, somatic cells, such as embryonic or adult fibroblasts, have also been successfully reprogrammed into Leydig-like cells. In this review, we summarized the recent advances in the generation of Leydig-like cells, with an emphasis on comparing the effectiveness and safety of different protocols used and the cells generated. By further analyzing the characteristics of Leydig-like cells generated from fibroblasts based on small signaling molecules and regulatory factors, we found that although the cells may produce testosterone, they are significantly different from real LCs. For future *in vivo* applications, it is important that the steroidogenic cells generated be evaluated not only for their steroidogenic functions but also for their overall cell metabolic state by proteomics or transcriptomic tools.

Asian Journal of Andrology (2022) **24**, 335–344; doi: 10.4103/aja202193; published online: 11 January 2022

Keywords: fibroblasts; hypogonadism; Leydig-like cells; reprogramming; stem cells

INTRODUCTION

Androgens, particularly testosterone, are essential for maintaining not only reproductive functions but also the general physiological properties of males, such as muscle bulk, fat distribution, bone health, and red blood cell production capacity.[1,2] Testosterone is produced by Leydig cells (LCs) in the testes, and their steroidogenic functions are strictly regulated by the hypothalamic–pituitary–gonadal (HPG) axis.[3,4] Decreased production of testosterone by the testes in men is categorized as hypogonadism, which is classified as primary, secondary, or mixed depending on the etiology.[5,6] In most circumstances, hypogonadism is not due to central deficiencies and instead results from defects in testicular failure, especially failure of LC functions (**Table 1**).[7,8] At the cell level, decreases in total androgen output can be caused by a number of reasons, including decreases in LC numbers due to proliferation defects and/or increased apoptosis, or reduced LC steroidogenic functions due to congenital or acquired causes, or a combination of all of these.[1,3,9]

Clinically, testosterone replacement therapy (TRT) is the most widely used approach for managing male hypogonadism.[10–12] However, TRT is far from a perfect solution due to its side effects, including a potential reduction in spermatogenesis, an increase in cardiovascular complications, and an elevated risk of prostate cancer.[13–15] The use of HPG axis-targeting molecules, such as gonadotropin-releasing hormone (GnRH), human chorionic gonadotropin (hCG), or their analogs, may be helpful in relieving the symptoms of hypogonadism. However, these treatments can commonly develop side effects associated with abnormal hormone-related symptoms. Therefore, it is necessary to find a new and effective testosterone supplementation method that can mimic the physiological circadian rhythm and pulsatility and is also under the regulation of the HPG axis.

Breakthroughs in stem cell research in the last 20 years have brought about new possibilities for managing all kinds of disorders, including hormonal deficiencies. Different types of stem cells, including embryonic stem cells (ESCs), adult tissue stem cells (ASCs), mesenchymal stem cells (MSCs), and induced pluripotent stem cells (iPSCs), are differentiated into target cells. Moreover, somatic cells, such as fibroblasts, have also been used as candidate cells. Via modulation of epigenetic characteristics and gene regulatory networks, fibroblasts have been successfully reprogrammed directly into iPSCs or transdifferentiated into different functional cells.

In this review, we summarize the main approaches used for differentiating stem cells or fibroblasts from various sources into Leydig-like cells. In addition, to better characterize the resulting cells and to better understand the transdifferentiation process, we compared and analyzed the gene expression profiles of reprogrammed cells, starting cells (fibroblasts), and wild-type LCs. Finally, we also listed the key challenges in the generation and use of Leydig-like cells.

LC LINEAGE DEVELOPMENT AND STEROIDOGENESIS

The origin, development, and steroidogensis of LCs in rodents and humans have been well established and reviewed.[1,3,7,16–18] Multiple generations of LCs have been identified in mammalian testes. For rodents, two distinct LC populations, fetal Leydig cells (FLCs) and

[1]Guangdong Provincial Key Laboratory of Bioengineering Medicine, Department of Cell Biology, Jinan University, Guangzhou 510632, China; [2]Institute of Life Sciences, Wenzhou University, Wenzhou 325035, China; [3]Department of Gynecology and Obstetrics, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou 325027, China; [4]National Engineering Research Center of Genetic Medicine, Jinan University, Guangzhou 510632, China.
Correspondence: Dr. ZJ Su (tjnuszj@jnu.edu.cn) or Dr. HL Chen (chenhaolin@wmu.edu.cn)
Received: 02 April 2021; Accepted: 21 October 2021

Table 1: The common causes of primary hypogonadism

Laboratory value	*Disease*	*Pathological feature*
Decreased serum testosterone level, elevated luteinizing hormone, and follicle-stimulating hormone	Klinefelter syndrome	Congenital abnormality of the sex chromosomes, 47,XXY
	Undescended testicles	One or both of the testicles are not descended at birth
	Orchitis	Viral infection or unspecific orchitis
	Hemochromatosis	Too much iron in the blood
	Acquired anorchia	Surgical removal, trauma, tumor, torsion, inflammation
	Leydig cell hypoplasia	Leydig cells cannot develop
	Cancer treatment	Chemotherapy or radiation therapy
	DSD	Disturbed testosterone synthesis due to enzymatic defects of steroid biosynthesis, 46,XY
	Noonan syndrome	Cryptorchidism
	Secondary testicular dysfunction	Medication, drugs, toxins, systemic diseases

DSD: disorders of sexual development

adult Leydig cells (ALCs), have been found sequentially in fetal and adult testes. In rats, FLCs appear by gestational day 14 and their levels reach plateaus by gestational day 19. The androgens produced by FLCs in the embryonic period play crucial roles in the masculinization of the male fetal genital tract and brain.[19] In humans, fetal interstitial progenitor cells appear at approximately 6–7 weeks postfertilization and then differentiate into the Sertoli and Leydig lineages between week 7 and week 8. FLCs appear at approximately week 7, persist for testosterone production, and disappear after birth.[20,21]

ALCs, which share a common progenitor with myoid cells, appear during fetal stages in mice and at prepubertal/peripubertal stages in humans.[20–22] ALCs develop during puberty from stem Leydig cells (SLCs), which undergo four stages of development: SLCs, progenitor Leydig cells (PLCs), immature Leydig cells (ILCs), and ALCs. During the transition from SLCs to ALCs, LCs gradually lose the ability to proliferate while acquiring steroidogenic capacities (**Figure 1**). Interestingly, cells at different stages produce different steroidogenic products by expressing different androgen metabolic enzymes. SLCs do not produce androgens due to a lack of steroidogenic enzymes, while PLCs, ILCs, and ALCs produce androsterone (AN), 5α-androstane-3α,17β-diol (3α-DIOL), and testosterone as final products, respectively.

The production of testosterone is a multistep catalytic process. In a rat model, luteinizing hormone (LH), which is secreted by the pituitary gland, binds to the receptor LH/choriogonadotropin receptor (LHCGR) in the LC membrane and triggers the synthesis of cyclic adenosine monophosphate (cAMP). Then, cholesterol is transferred into the mitochondrial inner membrane by steroidogenic acute regulatory protein (StAR) and possibly by a transduceosome complex containing translocator protein. In the inner mitochondrial membrane, cholesterol is converted into pregnenolone by the cholesterol side-chain cleavage cytochrome P450 enzyme (P450scc/CYP11A1). Pregnenolone is then further converted to testosterone by a series of enzymes, including 3β-hydroxysteroid dehydrogenase 1 (HSD3B1), cytochrome P450 17-alpha-hydroxylase/17,20-lyase (CYP17A1), and type 3 17β-hydroxysteroid dehydrogenase (HSD17B3), in the smooth endoplasmic reticulum (**Figure 2**).[1]

GENERATION OF TESTOSTERONE-PRODUCING LEYDIG-LIKE CELLS *IN VITRO*

It has been well demonstrated that ALCs proliferate only slightly after their maturation. Stem cells in the tissue play an important role in maintaining the homeostasis of the population. During aging or pathological conditions, the ALC population may reduce their numbers or steroidogenic functions, resulting in hypogonadism. The *in vivo* transplantation of stem cells or steroidogenic cells has become a potential approach for treating hypogonadism. To test this possible strategy, various attempts have been made to generate Leydig-like cells from stem or somatic cells of the testis or other organs in the last 20 years (**Table 2**).

Differentiation of SLCs into Leydig-like cells in vitro

It is well established that a single intraperitoneal dose (75 mg kg^{-1}) of ethane-1,2-dimethanesulfonate (EDS) in male adult rats can induce apoptosis of all existing ALCs in 3 days and result in the regeneration of a new LC population with full steroidogenic function in 56 days.[23,24] These early studies provided strong evidence of the existence of stem Leydig cells in adult testis. The first successful isolation of stem Leydig cells was accomplished in 2004. The study isolated a group of "side-population" from mice testicular cells by flow cytometry that had stem cell characteristics and was capable of restoring serum testosterone upon their transplantation into the interstitium of luteinizing hormone/choriogonadotropin receptor-knockout (LHRKO) mice testes.[25] Subsequently, the precursor cells regenerating ALCs in EDS-treated rats were identified and characterized as SLCs.[26]

These cells were then isolated and induced to differentiate into steroid-producing cells in culture, further confirming their stem cell nature. Similar cells were also isolated from mice and human testis by fluorescence-activated cell sorting (FACS) technique. Like other adult stem cells, SLCs also have multilineage differentiation potential. In addition to LC lineage, mouse- and human-derived SLCs have also been successfully differentiated into neurons, astrocytes, osteocytes, adipocytes, and chondrocytes *in vitro*.[27–32]

One of the earliest attempts to isolate SLCs was performed using testes of neonatal rats.[33] Platelet-derived growth factor receptor alpha-positive (PDGFRα$^+$) cells were isolated and cultured with medium containing platelet-derived growth factor beta dimer (PDGF-BB), LH, thyroid hormone, and insulin-like growth factor-1 (IGF-1) as well as insulin-transferrin-sodium selenite (ITS) cell culture supplement. The cells could be differentiated *in vitro* into Leydig-like cells with the ability to express steroidogenic enzymes and produce testosterone. More importantly, transplantation of PDGFRα$^+$ cells into EDS-treated rats resulted in the establishment of cells in the interstitial compartment of the testis, and they began to express steroidogenic enzymes, suggesting that the cells can recognize their niche and differentiate *in vivo*.

The regulatory mechanisms of rat SLC commitment and differentiation were subsequently revealed.[34–36] As was seen *in vivo* after EDS treatment, the processes of proliferation and differentiation of SLCs *in vitro* also occurred in the 1st week and from the 2nd to 3rd week, respectively. Platelet-derived growth factor alpha dimer (PDGF-AA), PDGF-BB, basic fibroblast growth factor (bFGF),

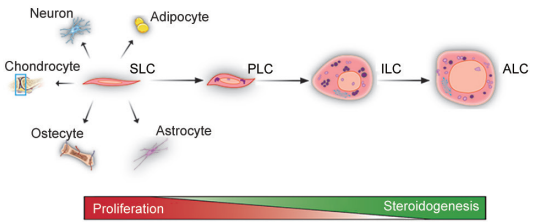


Figure 1: The differentiation and steroidogenic processes of ALCs. In rat testes, the differentiation of adult Leydig cells includes four stages: SLCs, PLCs, ILCs, and ALCs. SLCs are capable of self-renewal but not of producing androgens. PLCs can proliferate 4 rounds and synthesize androsterone as a major product. ILCs proliferate only once and produce 5α-androstane-3α,17β-diol as a major product. ALCs lose the ability to proliferate and produce testosterone as a final product. The wedge icons represent the proliferative or steroid-producing ability of Leydig cells. SLC: stem Leydig cell; PLC: progenitor Leydig cell; ILC: immature Leydig cell; ALC: adult Leydig cell.

desert hedgehog (DHH), and activin were found to stimulate SLC proliferation, while DHH, lithium ions, PDGF-AA, and activin promoted SLC differentiation. In addition, the Hedgehog signaling pathway was confirmed to be the critical commitment regulator that triggers the transition of SLCs into PLCs and thus differentiation into functional ALCs.[35,36]

Mouse SLCs have been isolated from adult C57BL/6 mouse testes by sorting nestin$^+$- or CD51$^+$-expressing cells.[27–30] The cells can be differentiated into Leydig-like cells using differentiation-inducing medium (M199 medium containing bovine serum albumin, ITS, LH and the smoothened agonist). Compared with rat cells, the mouse cells are easier and faster to differentiate *in vitro* under the same culture conditions.[36,37]

In a primate model, Cynomolgus monkey SLCs (CM-SLCs) were isolated based on the cell surface marker CD271$^+$.[38] After proliferation *in vitro* with the conventional cultivation method, CM-SLCs were transplanted back into the interstitium of the testis, and the cells were able to colonize, differentiate, and integrate into the host microenvironment. More importantly, CM-SLC transplantation significantly increased the serum testosterone level in a physiological pattern, recovered spermatogenesis, and ameliorated TD-related symptoms in elderly monkeys. Human SLCs were also purified using the same marker (CD217, also known as p75 neurotrophin receptor, p75NTR) as was used in the monkey model.[30] After culture in differentiation-inducing medium containing fetal bovine serum (FBS), ITS, thyroid hormone, LH, PDGF-BB, and IGF-1 for 4 weeks, most p75NTR$^+$ cells gradually differentiated into steroid-producing cells. After transplantation into the testes of EDS-treated rats, these p75NTR$^+$ cells developed into functional Leydig-like cells and secreted testosterone in a physiological pattern.

In addition, similar cells were also isolated from human testes using different markers. Eliveld *et al*.[31] isolated PDGFRα$^+$ cells from the human testicular interstitium and differentiated them into Leydig-like cells using different media. The differentiated cells expressed most steroidogenic enzymes except LHCGR. However, unlike the results of previous works based on other markers, human PDGFRα$^+$ cells transplanted into rat testes failed to differentiate into testosterone-producing cells. The authors suggested that LC development in humans might rely on different stimuli than that in rodents. Another surface protein marker, endosialin, has recently been applied for human SLC identification and isolation.[32] Endosialin-positive cells not only express all reported markers (PDGFRα, nestin, and nerve growth factor receptor) but are also capable of differentiating into Leydig-like cells *in vitro* and *in vivo*. However, most

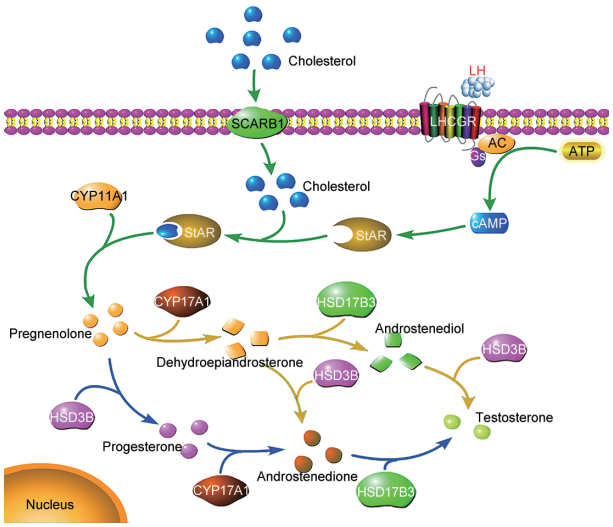


Figure 2: Diagram of the canonical steroidogenesis process in LCs. The steroidogenic signaling of ALCs are triggered by LH. LH binds to the LHCGR and activates adenylyl cyclase, resulting in the increase of cAMP concentration and cAMP-dependent phosphorylation of proteins through PKA. Cholesterol then is transferred into mitochondria and converted to pregnenolone by CYP11A1. In rodent model, the pregnenolone is mainly metabolized into progesterone, androstenedione, and testosterone by HSD3B1, CYP17A1, and HSD17B3, respectively, in the smooth endoplasmic reticulum (named Δ4 pathway). In human, the pregnenolone is mainly metabolized into dehydroepiandrosterone, androstenediol, androstenedione, and testosterone by HSD3B2, CYP17A1, and HSD17B3, respectively (named Δ5 pathway). The yellow and blue arrows represent the Δ5 pathway and Δ4 pathway, respectively. AC: adenylyl cyclase; ATP: adenosine triphosphate; LCs: Leydig cells; cAMP: cyclic adenosine monophosphate; CYP11A1: cholesterol side-chain cleavage cytochrome P450 enzyme; CYP17A1: cytochrome P450 17-alpha-hydroxylase/17,20-lyase; Gs: G proteins; HSD17B3: type 3 17β-hydroxysteroid dehydrogenase; HSD3B1: 3β-hydroxysteroid dehydrogenase 1; HSD3B2: 3β-hydroxysteroid dehydrogenase 2; LH: luteinizing hormone; LHCGR: luteinizing hormone/choriogonadotropin receptor; PKA: protein kinase A; SCARB1: scavenger receptor class b member 1; StAR: steroidogenic acute regulatory protein.

of them still retain the characteristics of stem cells, even 4 weeks after transplantation into the testicular interstitium. These findings suggest that there may be a number of precursor cells belonging to different stem cell populations that can form ALCs in the adult testis.

Differentiation of stem cells of nonsteroidogenic tissues into Leydig-like cells

Induction by cell type-specific transcription factors

As potential cell sources for regenerative medicine and model systems for studying LC development, cells from various nonsteroidogenic tissues have been tested for their ability to form Leydig-like cells. MSCs, ESCs, and iPSCs have more potential as sources of Leydig-like cells since they are easier to obtain and have more potential to expand than SLCs.[39] The orphan nuclear receptors of the nuclear receptor subfamily 5 group a (NR5A) family have been indicated to play essential roles in the development of the reproductive system and the differentiation of steroidogenic cells. Steroidogenic factor 1 (SF-1, also known as nuclear receptor subfamily 5 group a member 1, NR5A1) and liver receptor homolog 1 (LRH-1) are still the two most important transcription factors (TFs) for the successful differentiation of nonsteroidogenic tissue stem cells into Leydig-like cells *in vitro*.[40] Crawford *et al*.[41] demonstrated for the first time that transient expression of NR5A1 plus treatment with 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP) and

Table 2: The summary of Leydig-like cells generation using different cell types and approaches

Cell type	*Marker*	*Origin*	*In vitro/in vivo (transplanted subject)*	*Induction period in vitro (day)*	*Defined factors for induction*	*Reference*
SLCs	$CD51^+$	Mouse	+/+ (mouse, rat)	7–11	PDGF-BB, LH, thyroid hormone, IGF-1, ITS	27,29
SLCs	$CD51^+$	Mouse	+/–	4–8	ITS, LH, SAG	28
SLCs	$CD90^+$	Rat	+/–	21	ITS, LH, SAG	36
SLCs	$p75^+$	Human	+/+ (rat)	28	PDGF-BB, LH, thyroid hormone, IGF-1, ITS	30
SLCs	$PDGFR\alpha^+$	Rat	+/+ (rat)	7	PDGF-BB, LH, thyroid hormone, IGF-1, ITS	33
Testicular cells	Hoechst dye^-	Mouse	–/+ (LHRKO mouse)	-	-	25
Testicular cells	$PDGFR\alpha^+$	Human	+/+ (LuRKO mouse)	28	PDGF-BB, IGF-1, hCG, T_3, thyroxin, SAG, ITS	31
Testicular cells	$Endosialin^+$	Human	+/+ (NCG mouse)	14	SAG, LH, thyroid hormone, IGF-1, PDGF-AA, 25HC, ITS	32
Testicular cells	$CD271^+$	Cynomolgus monkey	+/+ (elderly monkey)	28	LH, thyroid hormone, IGF-1, PDGF-AA, ITS	38
BMSCs	$CD45^-$, $CD11b^-$, $CD34^-$, $CD31^-$, $CD44^+$, $CD105^+$	Human	+/–	21	NR5A1	45
BMSCs	$CD11b^-$, $CD34^-$, $CD45^-$, $SCA1^+$	Mouse	+/–	14–18	NR5A1, RA	44
BMSCs	-	Mouse, rat, human	+/+ (rat)	7	NR5A1, cAMP	42
ADSCs	$CD29^+$, $CD44^+$, $CD59^+$, $CD105^+$, $CD34^-$, $CD45^-$, HLA-DR^-	Human	+/+ (rat)	30	SAG, LH, 22R-OHC, PDGF-AA, lithium chloride, bFGF, androgen, ITS	54
UCMSCs	$CD59^+$, $CD105^+$, $CD34^-$, $CD45^-$	Human	+/–	35	SAG, LH, 22R-OHC, PDGF-AA, lithium chloride, bFGF, androgen, ITS	55
ESCs		Mouse	+/–	2–4	RA, cAMP, NR5A1	41
ESCs		Mouse	+/+ (rat)	14	NR5A1, cAMP, forskolin	46
ESCs		Human	+/–	17–28	NR5A1, cAMP	48
iPSCs		Human	+/–	22	NR5A1, cAMP, DHH, hCG	49
iPSCs		Human	+/+ (rat)	30	SAG, LH, 22R-OHC, IGF-1, PDGF-AA, lithium chloride, bFGF, androgen, RA, cAMP, ITS	53
Fibroblasts		Human	+/–	28	GATA4, NR5A1, NGFI-B	63
Fibroblasts		Human	+/–	14–28	NR5A1, forskolin, DAPT, purmorphami	64
Fibroblasts		Human	+/+ (castrated rat)	14–28	NR5A1, GATA4, DMRT1	73,74
Fibroblasts		Mouse	+/+ (rat)	14–21	Forskolin, 20α-OHC, LH, SB431542	77
Fibroblasts		Mouse	+/+ (mouse, rat)	14	NR5A1, DMRT1, GATA4, PDGF-AA, IGF-1	62

20α-OHC: 20a-hydroxycholesterol; 22R-OHC: 22R-hydroxycholesterol; 25HC: 25-hydroxycholesterol; ADSCs: adipose-derived stem cells; bFGF: basic fibroblast growth factor; BMSCs: bone marrow mesenchymal stem cells; DHH: desert hedgehog; DMRT1: doublesex and mab-3 related transcription factor 1; ESCs: embryonic stem cells; GATA4: GATA binding protein 4; IGF-1: insulin-like growth factor-1; iPSCs: induced pluripotent stem cells; ITS: insulin-transferrin-sodium selenite; LH: luteinizing hormone; LHRKO: luteinizing hormone/choriogonadotropin receptor-knockout; LuRKO: luteinizing hormone receptor-knockout; NGFI-B: nuclear receptor subfamily 4 group A member 1; NR5A1: nuclear receptor subfamily 5 group A member 1; PDGF-AA: platelet-derived growth factor alpha dimer; PDGF-BB: platelet-derived growth factor beta dimer; $PDGFR\alpha^+$: platelet-derived growth factor receptor alpha-positive; RA: retinoic acid; SAG: smoothened agonist; SLCs: stem Leydig cells; T_3: triiodothyronine; UCMSCs: umbilical cord mesenchymal stem cells; +: the finished experiments; –: no experiments; hCG: human chorionic gonadotropin; cAMP: cyclic adenosine monophosphate; NCG mouse: nonobese-diabetic coisogenic immunodeficient mouse

retinoic acid (RA) could successfully directly differentiate ESCs toward the steroid-producing cell lineage. Using a similar protocol, murine MSCs derived from bone marrow were also transformed into steroidogenic cells.[42] In addition to NR5A1, LRH-1 was confirmed to be able to induce MSCs into steroidogenic cells.[43–45] The critical effects of the NR5A family on stem cell transdifferentiation were also confirmed by a recent study showing that NR5A1 overexpression can induce differentiation of mouse ESCs into Leydig-like cells with the help of a different combination of small molecules (8-Br-cAMP and forskolin). Moreover, these cells are able to develop further *in vivo* and partially rescue serum testosterone levels when transplanted into the testes of hypogonadal rats.[46]

Similar results were also obtained for human stem cells, including bone marrow MSCs, ESCs, and iPSCs.[47,48] It is worth noting that the expression of NR5A1 alone was not enough to induce stem cells to fully differentiate into functional Leydig-like cells. To obtain highly differentiated steroid-producing cells, Li *et al*.[49] used a multistage induction protocol to differentiate human iPSCs. The first stage of the strategy is to induce the differentiation of iPSCs into early mesenchymal progenitors (EMPs) with feeder-free maintenance medium and animal component-free mesenchymal induction medium. Subsequently, EMPs were induced to overexpress NR5A1 and treated with collagen I substrate, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) containing FBS, cAMP, DHH, and hCG for 14 days to induce the formation of Leydig-like cells. Interestingly, human iPSCs were more likely to become Leydig-like cells than adrenal-like cells, suggesting a preference in the differentiation of iPSCs into steroidogenic cells.

Due to the critical role played by NR5A1 in the transdifferentiation of stem cells into steroidogenic cells, the regulatory mechanism was investigated *in vitro* and *in vivo*. In both murine and human ESCs, it has been shown that NR5A1 activates the transcription of octamer-binding transcription factor 4 (OCT-4) by binding its promoter and then inducing the expression of androgen-synthesizing enzymes.[50,51] In addition, during Leydig-like cell formation, NR5A1 promotes progenitor cell formation and survival. These results have been further confirmed in animal models. Leydig-specific *Nr5a1*

knockout mice have characteristic traits of infertility, cryptorchidism and hypoplastic testes.[52]

Induction by signaling molecules and regulatory factors

The generation of Leydig-like cells via the expression of transcription factors requires transfection of the cells with virus-related recombinant expression vectors, so these cells may not be suitable for therapeutic purposes because of safety considerations. The best solution is to reprogram the cells by adding exogenous induction factors, such as hormones, growth factors, and signaling modification molecules. Three studies reported recently by the Guo's group showed that stem cells from 3 different human sources were all successfully transdifferentiated into Leydig-like cells by external inducing factors.[53–55] The source cells included iPSCs, multipotent adipose-derived stem cells (ADSCs), and umbilical cord mesenchymal stem cells (UMSCs). The differentiation strategies were conducted via multiphase induction protocols. To start the differentiation, smoothened agonist (SAG), 22R-hydroxycholesterol, and lithium ions were added to the medium for 7 days. Then, the cells were switched to PDGF-AA- and bFGF-containing medium for 3 days to maintain proliferative activity. PDGF-AA, IGF-1, and androgen were then used for 1 week to enhance the differentiation and maturation process. Subsequently, PDGF-AA and bFGF were administered again for 3 days to maintain the dividing activity of the cells. For the last 5 days, LH, RA, and 8-Br-cAMP were added to the medium to stimulate the cells to differentiate into a final maturation state with the ability to produce testosterone. The ability of the resulting cells to modify testosterone concentrations *in vivo* was also tested by transplanting the cells into the testes of EDS-treated rats. Interestingly, cells from all 3 sources were able to survive in the rat testis and significantly elevated serum testosterone levels. Since transdifferentiation was induced entirely by external factors, the cells generated could have the highest safety. These results and regulatory mechanism of differentiation, if confirmed, could represent a major step in regenerative medicine in the field of andrology.

Direct reprogramming of fibroblasts into Leydig-like cells

Cell reprogramming was originally referred to a process that involves the dedifferentiation of terminally differentiated cells into totipotent or pluripotent cells via the expression of a series of TFs. The reprogrammed cells can then differentiate into different functional cells as normal stem cells. Somatic cells are normally in a stable state of high differentiation, but they may be directly converted into pluripotent stem cells via forced expression of a few key TFs. By forcing key gene expression or altering epigenetic modifications, researchers can directly lineage-switch fibroblasts into a variety of functional cells *in vivo* or *in vitro* without going through an intermediate pluripotent state.[56]

Fibroblast reprogramming by defined TFs

The most common method to reprogram fibroblasts is to transfect the cells with key TFs. In 2006, Takahashi and Yamanaka[57] demonstrated that the expression of four defined TFs, octamer-binding protein 3/4 (OCT-3/4), sex-determining region Y-box 2 (SOX-2), cellular myelocytomatosis oncogene (c-MYC), and kruppel-like factor 4 (KLF4), could induce human fibroblasts to differentiate into iPSCs. These generated iPSCs proved to be very similar to ESCs in terms of morphology, gene and protein expression profiles, epigenetic modification status, cell proliferation, and differentiation potential, demonstrating the key role of TFs in determining cell fate. Subsequently, with the rapid development of this technology, an increasing number of cell types, such as cardiomyocyte-like cells,[58] neural cells,[59] hepatocytes,[60] and Sertoli-like cells,[61] were generated via the induction of direct lineage switching through overexpression of cell lineage-specific TFs.

Our group succeeded in directly reprogramming fibroblasts into Leydig-like cells via the induction of lentivirus-mediated expression of doublesex and mab-3 related transcription factor 1 (DMRT1), GATA-binding protein 4 (GATA4), and NR5A1.[62] These results indicated that NR5A1 alone is sufficient to initialize the reprogramming process. DMRT1 and GATA4 are capable of stimulating the expression of LHCGR and decreasing the global DNA methylation level. DNA methylation is one of the major epigenetic factors that regulates gene expression during cell fate commitment and reprogramming. These three factors may cooperate with each other in modifying DNA methylation, upregulating the expression of steroidogenic genes, and promoting LH-mediated testosterone synthesis.

A similar study was also carried out in human cells. The combination of NR5A1, GATA4, and NGFI-B (also named nuclear receptor subfamily 4 group a member 1 [NR4A1]) was administered to induce human fibroblasts into Leydig-like cells.[63] In the reprogramming process, NR5A1 and GATA4 were particularly important for elevating the expression of steroidogenic genes. Unlike mouse fibroblasts, NR5A1 alone was not able to accomplish reprogramming of human cells. The team improved the protocol using an inducing cocktail containing forskolin, LY-374973, and purmorphamine in addition to NR5A1 transfection.[64] Adaption of the new protocol enabled the group to successfully reprogram human foreskin fibroblasts into functional Leydig-like cells. These cells were similar to human LCs in morphology, expression patterns of marker genes, and the ability to produce testosterone. Unfortunately, none of the studies described above assessed the testosterone-producing stability and survival rate of the cells generated after the induction conditions were removed, which could be important issues for future clinical applications.

Fibroblast reprogramming by clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR/Cas9) system

The CRISPR/Cas9 system provides powerful genetic manipulation tools for modifying DNA sequences at a given site. The tools were modified so that the nuclease activity of the Cas9 protein was disabled. Such modification enables the complex to recruit modifying enzymes or reporter proteins to DNA target sites without introducing irreversible changes in the DNA sequence. One of the most frequent uses of the modified tool (CRISPR activation) is to enable target expression of any gene or multiple genes through recruitment of transcriptional and/or epigenetic modifying effector domains. Guided by guide RNAs (gRNAs), the Cas9 protein fused with viral transcription factor (VP64), histone acetyltransferase p300 (P300), ten-eleven translocation methylcytosine dioxygenase 1 (TET1), the DNA methyltransferase (DNMT), or other activating factors was able to quickly change the original expression status of multiple targeted genes simultaneously.[65–67] This technology has become one of the most powerful means of cell reprogramming. Compared with the traditional expression method, the Cas9-based approach does not involve the introduction of exogenous genes but rather relies on the forced expression of endogenous genes. This tool has been used successfully in generating multiple cells, such as iPSCs,[68] skeletal myocytes,[69] oligodendrocyte progenitor-like cells,[70] neuronal cells,[71] and pancreatic β cells,[72] as well as Leydig-like cells.[73]

Huang *et al*.[73] applied this strategy for reprogramming human foreskin fibroblasts (HFFs) into functional Leydig-like cells. The team successfully reprogrammed the cells through activation of the expression of the endogenous transcription factors, DMRT1, GATA4, and NR5A1, using the CRISPR-dCas9 SAM system. After transplantation into the

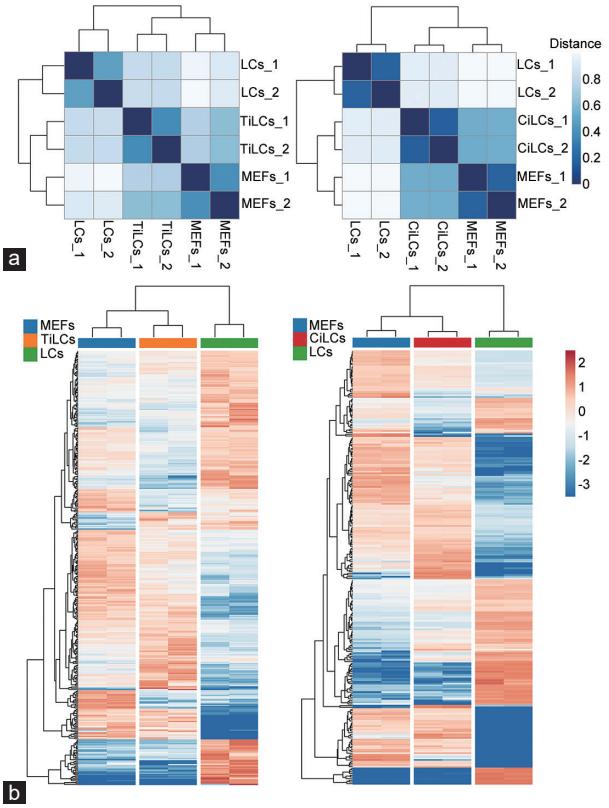


Figure 3: The similarity analysis of gene expression profiles and transcription factors between MEFs, Leydig-like cells, and LCs. (**a**) The similarity (Jaccard distance) of global gene expression profiles. The expression data of samples were extracted from 2 datasets GSE87020 and GSE145797 deposited in public databases. The data were log-transformed normalized and computed. The color difference of the distance represents the degree of similarity in expression profiles. The darker color means a higher similarity between samples. Left: comparisons among MEFs, LCs and TiLCs. Right: comparisons among MEFs, LCs and CiLCs. (**b**) Hierarchical clustering analysis of differentially expressed TFs. A total of 353 differentially expressed TFs among MEFs, LCs and Leydig-like cells were identified (fold change >1.5 and $P < 0.05$). The expression level of each transcriptional factor was normalized based on a global *z*-score transformation. The hierarchical tree on the left and on the top of the heatmap represents the clustering of TFs and the samples, respectively. Each column shows 2 cell replicates, and the color scales range from dark red to bright blue, corresponding to the up- or downregulation of TF expression, respectively. Left: comparisons among MEFs, LCs, and TiLCs. Right: comparisons among MEFs, LCs, and CiLCs. MEFs: mouse embryonic fibroblasts; TiLCs: transcription factor-based induced Leydig-like cells; CiLCs: compound-based induced Leydig-like cells; LCs: Leydig cells; TF: transcription factor.

fibrous capsule, the generated cells were able to survive *in vivo* and maintained their steroidogenic characteristics for more than 6 weeks. Meanwhile, treatment of the cells also partially restored the serum testosterone level of the castrated rats to that of the intact control.[74]

Fibroblast reprogramming by exogenous factors

As discussed above, stem cells from various sources have been successfully transdifferentiated into testosterone-producing Leydig-like cells by exogenous factors.[53–55] Conversion of human fibroblasts into neuron-like cells without overexpression of TFs has also been performed using a combination of small molecules.[75] This strategy offers a simpler and safer alternative for transdifferentiating stem cells or reprogramming differentiated cells for disease modeling

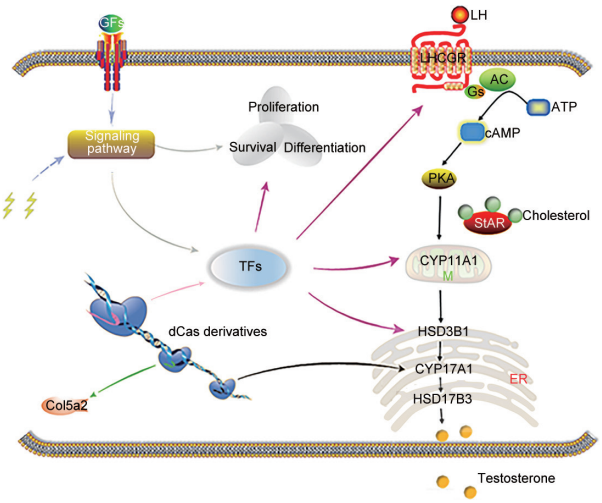


Figure 4: Diagram of Leydig-like cells generated from host cells using chemicals, growth factors, and CRISPR/Cas9 technologies. The CRISPR/Cas derivatives are capable of simultaneously activating or repressing targeted genes. Growth factors or chemicals can regulate the expression of genes and biological process by mediating signaling pathways. Growth factors or chemicals (agonists or antagonists) can regulate the biological function and gene expression by mediating signaling pathways. The CRISPR/Cas9 system, growth factor, and chemical promote synergistically the host cell reprogramming into steroid-producing Leydig-like cells. Lightning icon represents the agonists or antagonists of signaling pathways. CRISPR/Cas: clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9; Col5a2: collagen type V alpha 2 chain; GFs: growth factors; TFs: transcription factors; M: mitochondria; ER: endoplasmic reticulum; LH: luteinizing hormone; LHCGR: luteinizing hormone/choriogonadotropin receptor; ATP: adenosine triphosphate; Gs: G proteins; AC: adenylyl cyclase; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; StAR: steroidogenic acute regulatory protein.

and regenerative medicine.[76] By culturing fibroblasts with forskolin, 20α-hydroxycholesterol, LH, and SB431542 (a transforming growth factor beta [TGF-β] inhibitor) for 28 days, our group successfully reprogrammed mouse embryonic fibroblasts into Leydig-like cells.[77] The resulting cells were also tested for their steroidogenic function *in vivo* by implantation into the testes of EDS-treated rats. Moreover, these cells survived in the testis and produced testosterone with normal daily circadian rhythms for at least 21 days.

CHARACTERISTIC CONTROL FOR GENERATED LEYDIG-LIKE CELLS

Although the Leydig-like cells generated by various cell sources through different protocols can produce testosterone, they are not all the same. The overall quality of the cells may determine whether they are suitable for *in vivo* transplantation. In addition to testosterone production, other important characteristics include cell proliferation and the capacity to produce other major cell products in addition to testosterone, especially those that function as hormones or proteins that are able to trigger an immune response. Using the example below, we discuss how the overall cell quality of a reprogrammed cell population may be evaluated.

Characterization of the cells by transcriptome analysis

To evaluate the overall cell quality, the differences among wild-type LCs, induced cells, and donor cells should be compared using not only analyses of testosterone production or key steroidogenic genes or proteins but also global analysis of differences in overall gene expression patterns, transcription factors, and steroidogenic genes with transcriptome tools. For example, two Leydig-like cells reprogrammed

with mouse embryonic fibroblasts (MEFs) were generated with TFs and chemical compounds.[62,77] It is commonly accepted that genes with similar expression profiles are functionally related. In pursuit of authenticity, transcriptome data can be applied to more accurately define the status of cells during the differentiation from fibroblasts to Leydig-like cells. Based on this concept, the pairwise comparison of differentially expressed genes (DEGs) among reprogrammed Leydig-like cells, MEFs, and primary ALCs was conducted with 2 RNA-seq datasets that were uploaded to public databases (GSE87020 and GSE145797).

The results showed that the expression similarity scores of global gene profiles were significantly different between reprogrammed cells induced by transfection of TFs and those induced by molecular compounds, suggesting that these treatments affected not only the expression of steroidogenic genes but also the transition of the overall cellular state toward LCs (**Figure 3a**). Furthermore, like global gene profiles, the Leydig-like cells induced by both approaches were still closer to MEFs in terms of TF expression patterns (**Figure 3b**). Comparing the 2 inducing approaches, the TF-based (GATA4, NR5A1, and DMRT1) method was more effective in promoting the transition of fibroblasts into Leydig-like cells since the overall transcriptome characteristics of the resulting cells (TiLCs) were closer to those of wild-type LCs than those of the cells induced by the compound-based method (CiLCs), as shown in **Supplementary Table 1**.

Changes in the TF expression pattern reflect cell fate decisions.[78,79] Hierarchical clustering indicated that 353 TFs were differentially expressed during the fibroblast transdifferentiation process. Among these genes, 58 were differentially expressed during the transition from MEFs to TiLCs, and the directions of the expression changes were all toward the levels in wild-type LCs. On the other hand, during the transition from MEFs to CiLCs, 73 genes were differentially expressed. However, more than 1/3 (28 *vs* 73) of the genes were changed diametrically compared to those in wild-type LCs (**Supplementary Figure 1**). These observations suggest that the "compound-based method" has significantly less molecular targeting specificity and cell targeting specificity than the "TF-based method" in reprogramming fibroblasts.

Necessaries of "precise gene regulation" in fibroblast reprogramming

The methods used to reprogram and differentiate stem cells can be classified into 2 categories: precise gene regulation *vs* general induction. The former involves the precise activation of a few key TFs that will eventually change the fate of the cells. The latter strategy, on the contrary, usually needs an array of hormones, growth factors, and/or small signaling modifying compounds. Since this method reprograms cell fates by modifying the expression of TFs indirectly by affecting signaling molecules, it usually requires more time, and the cells go through multiple induction steps. Therefore, as discussed above, Leydig-like cells generated by the former method have a fate that better mimics real LCs.

To more precisely control endogenous gene expression, CRISPR-dCas9 system appears to be the best solution thus far.[80–82] For gene activation, for example, dCas9 can be fused to different transcriptional activation domains, such as VP64, v-rel avian reticuloendotheliosis viral oncogene homolog A (Rel-A), the Epstein–Barr virus R transactivator (RTA), or heat shock transcription factor 1 (HSF1), to force the expression of both endogenous coding and noncoding genes.[83–85] The simultaneous activation of different genes can also be easily achieved using multiple gRNAs targeted to the different promoter regions synergistically.[67,86,87] In addition to fusing transcriptional activators, dCas9 can be fused to epigenetic effectors for the activation of targeted genes. The human histone acetyltransferase P300 and the methylcytosine dioxygenase TET1 contain the most commonly used catalytic domains for dCas9-based DNA epigenetic modifications to date.[65,66]

For gene repression, several gene repressing modules, such as Kruppel-associated box protein (KRAB), lysine-specific demethylase 1 (LSD1), and histone deacetylases (HDACs), are reported to fuse with dCas9 to efficiently and specifically decrease gene expression.[88–92] The catalytic domain of DNA methyltransferases (DNMTs), site-specific DNA promoter methylation inducers, can also be used with dCas9 to induce targeted gene silencing.[93,94] At the RNA level, the Cas13-based system can mediate targeted mRNA expression and mRNA translation and mRNA decay, thereby regulating the yield of functional proteins.[95–98]

Chemical compound methods, however, reprogram or transdifferentiate somatic cells by modulating cellular transduction signals and chromatin-associated protein functions.[99–101] Compared to other methods, this approach has unique advantages, such as structural versatility, relatively inexpensive preparation in large quantities, and easy control in a time-dependent and concentration-dependent manner. Quite a few cell types, including neurons, astrocytes, neural stem cells, brown adipocytes, cardiomyocytes, endoderm progenitor cells, and pluripotent stem cells, have been fully generated from fibroblasts using chemical compound cocktails.[100] This is also true for Leydig-like cells. The factors considered for Leydig-like cell generation are related to 4 signaling molecules/pathways: growth factors, Hedgehog, TGF-β, and cAMP. A systematic diagram of the Leydig-like cells generated from host cells based on chemical, growth factor, and CRISPR/Cas9 technologies is presented in **Figure 4**.

GAPS AND CHALLENGES IN GENERATING OPTIMAL LEYDIG-LIKE CELLS

In the past decade, great progress has been made in the generation of Leydig-like cells. However, gaps and challenges remain in finding an effective and safe way to make cells with the high standards for clinical use.

The first challenge is to generate cells that only produce the desired steroids. Steroid hormones are a group of small molecules that are essential for human life. Although there are different forms, including cortisol, aldosterone, androgens, estrogens, and progestins, they share a basic structure and are made through similar pathways.[102] These characteristics make the cell-generating processes challenging since it is often difficult to obtain a cell that only produces the steroids desired. This challenge has not been fully recognized by researchers since most of the published studies only focus on whether the cells produce the steroids desired, not the whole steroidogenic profile. Overcoming this obstacle is important since implanting the cells for the purpose of supplementing one family of steroids may result in serious unexpected side effects if the cells also produce other undesired steroids. Therefore, it is essential to employ precisely analytic techniques that can monitor multiple steroids at the same time, such as liquid chromatography-tandem mass spectrometry (LC-MS/MS), instead of only relying on classic assay methods, such as the enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA).

Second, the targeted steroid synthesis needs precise regulation. The biosynthetic activity of steroids is strictly regulated by specific pituitary trophic hormones, such as adrenocorticotropic hormone (ACTH) for the adrenal gland and follicle-stimulating hormone (FSH) and LH for the gonads. These trophic hormones play important roles in both maintaining the steroidogenic machinery (trophic effects) and transporting cholesterol into the inner mitochondrial membrane (acute

effects). Both processes are essential for yielding a precisely controlled steroid output with physiological circadian rhythms. Steroidogenic cells from different tissues respond to their particular trophic hormones by expressing specific G protein-coupled cell membrane receptors. Therefore, it is critical for the generated cells to express the right receptors. Similarly, expressing more than one receptor may cause cells to respond to multiple trophic hormones and result in unexpected side effects.

Third, the selection of transplantation sites where the induced cells are capable of surviving and functioning in the long term represents another challenge in steroidogenic cell therapy. To date, the induced steroidogenic cells reported by previous studies have been transplanted in orthotropic ways, *i.e*., adrenal-like cells were implanted into the space between the kidney capsule and parenchyma, and Leydig-like cells were transplanted into the interstitium of the testis. These studies have suggested that the cells may still need the right niche to survive and function.[27,46,62,77,103] Clinically, transplanting the cells back into their home organ may not always be feasible, considering that it may not be possible or may have a significant risk of damaging the recipient organs, such as the kidney or gonads. Therefore, transplanting the steroidogenic cells into locations outside their home tissues may represent a better choice as long as these cells are capable of receiving trophic signaling from the pituitary and secreting the products back into the circulation. Some of the possible sites for implantation are subcutaneous areas under the armpit or in the abdomen. Subcutaneous transplantations of SLCs or partially differentiated LCs were tested in mice and rats.[104–106] Although the cells all appeared to function *in vivo* for the short time period tested, the long-term fates are still unclear. The challenge is to keep the cells surviving and functioning long term in an environment different from their physiological environment.

Last but not least, the issue of safety should always be of the highest priority. Currently, the most common cells used for generating Leydig-like cells are ESCs, iPSCs, ASCs, and differentiated fibroblasts.[18,28,35,36,49] Leydig-like cells made from these sources have the potential to proliferate or activate different immune responses once transplanted *in vivo*. These characteristics will determine whether the cells are suitable for eventual *in vivo* use since tumor formation and immune rejection are among the major safety considerations. On the other hand, transplantation of the cells to locations outside their home organs may also change the environment of the receiving organs. It is well known that ALCs are capable of producing inflammatory factors in addition to synthesizing androgens. The cells also respond to immunogenic factors (lipopolysaccharide).[107] If the reprogrammed Leydig-like cells function in a similar way, it is possible that transplantation of the cells may lead to significant changes in the environment of the receiving organs, including the imbalance of anti-inflammatory and proinflammatory factors, leading to functional impairment of the target tissues. All these risks must be considered and planned for before the cells can be tested in humans.

SUMMARY

For the purposes of LC developmental research and the development of cell-generating protocols for future regenerative medicine, functional Leydig-like cells have been differentiated from SLCs, ESCs, iPSCs, ASCs, and fibroblasts in the last two decades. Among the candidate cells used, fibroblasts appear to be among the best cell sources with the advantages of being easier to collect, expand, and reprogram. Although testosterone production is the gold standard for Leydig-like cell identification, broader parameters are required to better evaluate the overall quality of the produced cells, since even the cells with similar steroidogenic functions may have significantly different transcriptomes. The expression similarity of global genes and transcription factors is considered as an ideal indicator for assessing the overall similarity of the reprogram cells to real LCs. Considering cell differentiation only, Leydig-like cells made by the TF induction method are better than those made by the chemical compound induction method since the former are closer to real LCs. However, none of the protocols used thus far can generate cells that completely mimic LCs. The strategy for generating fully functional Leydig-like cells for clinical purposes is far from complete and still needs further optimization.

AUTHOR CONTRIBUTIONS

ZJS and HLC conceived and designed the manuscript. ZHL, ZJS, JDL, and SJL contributed to the data acquisition. ZHL, HLC, and ZJS drafted the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

ACKNOWLEDGMENTS

This work was partially supported by the National Natural Science Foundation of China (No. 91949123 and No. 81871155); the Natural Science Foundation of Guangdong Province (No. 2021A1515010947); the Forestry Science and Technology Innovation Project of Guangdong Province (No. 2021KJCX013); the Zhejiang Provincial Natural Science Foundation (No. LGF21H040001); and the Wenzhou City Public Welfare Science and Technology Project (No. ZY2019002 and No. ZY2019005).

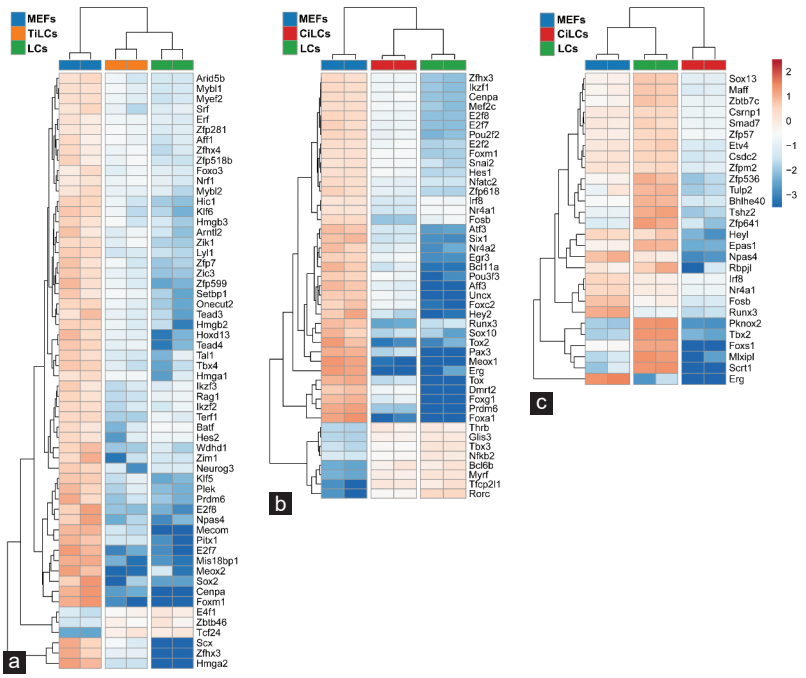
Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

REFERENCES

1 Wang Y, Chen F, Ye L, Zirkin B, Chen H. Steroidogenesis in Leydig cells: effects of aging and environmental factors. *Reproduction* 2017; 154: R111–22.
2 Midzak AS, Chen H, Papadopoulos V, Zirkin BR. Leydig cell aging and the mechanisms of reduced testosterone synthesis. *Mol Cell Endocrinol* 2009; 299: 23–31.
3 Ye L, Li X, Li L, Chen H, Ge RS. Insights into the development of the adult Leydig cell lineage from stem Leydig cells. *Front Physiol* 2017; 8: 430.
4 Chen H, Ge RS, Zirkin BR. Leydig cells: from stem cells to aging. *Mol Cell Endocrinol* 2009; 306: 9–16.
5 Basaria S. Male hypogonadism. *Lancet* 2014; 383: 1250–63.
6 Huhtaniemi I. Late-onset hypogonadism: current concepts and controversies of pathogenesis, diagnosis and treatment. *Asian J Androl* 2014; 16: 192–202.
7 Zirkin BR, Papadopoulos V. Leydig cells: formation, function, and regulation. *Biol Reprod* 2018; 99: 101–11.
8 Kaprara A, Huhtaniemi IT. The hypothalamus-pituitary-gonad axis: tales of mice and men. *Metabolism* 2018; 86: 3–17.
9 Chen H, Guo X, Xiao X, Ye L, Su Z. Identification and functional characterization of microRNAs in rat Leydig cells during development from the progenitor to the adult stage. *Mol Cell Endocrinol* 2019; 493: 110453.
10 Seftel AD, Kathrins M, Niederberger C. Critical Update of the 2010 Endocrine Society clinical practice guidelines for male hypogonadism: a systematic analysis. *Mayo Clin Proc* 2015; 90: 1104–15.
11 Snyder PJ, Bhasin S, Cunningham GR, Matsumoto AM, Stephens-Shields AJ, *et al*. Lessons from the testosterone trials. *Endocr Rev* 2018; 39: 369–86.
12 Yeap BB, Page ST, Grossmann M. Testosterone treatment in older men: clinical implications and unresolved questions from the testosterone trials. *Lancet Diabetes Endocrinol* 2018; 6: 659–72.
13 Petering RC, Brooks NA. Testosterone therapy: review of clinical applications. *Am Fam Physician* 2017; 96: 441–9.
14 U.S. Food and Drug Administration. FDA Drug Safety Communication: FDA Cautions About Using Testosterone Products for Low Testosterone due to Aging; Requires Labeling Change to Inform of Possible Increased Risk of Heart Attack and Stroke with Use. Available from: http://www.fda.gov/Drugs/DrugSafety/ucm436259.htm. [Last accessed on 2018 Feb 26].
15 Bassil N, Alkaade S, Morley JE. The benefits and risks of testosterone replacement therapy: a review. *Ther Clin Risk Manag* 2009; 5: 427–48.
16 Chen P, Zirkin BR, Chen H. Stem Leydig cells in the adult testis: characterization, regulation and potential applications. *Endocr Rev* 2020; 41: 22–32.

17 Payne AH, Hales DB. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocr Rev* 2004; 25: 947–70.
18 Shima Y. Development of fetal and adult Leydig cells. *Reprod Med Biol* 2019; 18: 323–30.
19 Griswold SL, Behringer RR. Fetal Leydig cell origin and development. *Sex Dev* 2009; 3: 1–15.
20 Guo JT, Nie XC, Giebler M, Mlcochova H, Wang YQ, *et al.* The dynamic transcriptional cell atlas of testis development during human puberty. *Cell Stem Cell* 2020; 26: 262–76.
21 Guo JT, Sosa E, Chitiashvili T, Nie XC, Rojas EJ, *et al.* Single-cell analysis of the developing human testis reveals somatic niche cell specification and fetal germline stem cell establishment. *Cell Stem Cell* 2021; 28: 764–78.
22 Svingen T, Koopman P. Building the mammalian testis: origins, differentiation, and assembly of the component cell populations. *Gene Dev* 2013; 27: 2409–26.
23 Jackson CM, Jackson H. Comparative protective actions of gonadotrophins and testosterone against the antispermatogenic action of ethane dimethanesulphonate. *J Reprod Fertil* 1984; 71: 393–401.
24 Guo J, Zhou H, Su Z, Chen B, Wang G, *et al.* Comparison of cell types in the rat Leydig cell lineage after ethane dimethanesulfonate treatment. *Reproduction* 2013; 145: 371–80.
25 Lo KC, Lei ZM, Rao CV, Beck J, Lamb DJ. *De novo* testosterone production in luteinizing hormone receptor knockout mice after transplantation of Leydig stem cells. *Endocrinology* 2004; 145: 4011–5.
26 Davidoff MS, Middendorff R, Enikolopov G, Riethmacher D, Holstein AF, *et al.* Progenitor cells of the testosterone-producing Leydig cells revealed. *J Cell Biol* 2004; 167: 935–44.
27 Jiang MH, Cai B, Tuo Y, Wang J, Zang ZJ, *et al.* Characterization of Nestin-positive stem Leydig cells as a potential source for the treatment of testicular Leydig cell dysfunction. *Cell Res* 2014; 24: 1466–85.
28 Chen P, Guan X, Zhao X, Chen F, Yang J, *et al.* Characterization and differentiation of CD51$^+$ stem Leydig cells in adult mouse testes. *Mol Cell Endocrinol* 2019; 493: 110449.
29 Zang ZJ, Wang J, Chen Z, Zhang Y, Gao Y, *et al.* Transplantation of CD51$^+$ stem Leydig cells: a new strategy for the treatment of testosterone deficiency. *Stem Cells* 2017; 35: 1222–32.
30 Zhang M, Wang J, Deng C, Jiang MH, Feng X, *et al.* Transplanted human p75-positive stem Leydig cells replace disrupted Leydig cells for testosterone production. *Cell Death Dis* 2017; 8: e3123.
31 Eliveld J, van den Berg EA, Chikhovskaya JV, van Daalen SK, de Winter-Korver CM, *et al.* Primary human testicular PDGFRα$^+$ cells are multipotent and can be differentiated into cells with Leydig cell characteristics *in vitro*. *Hum Reprod* 2019; 9: 1621–31.
32 Xia K, Ma Y, Feng X, Deng R, Ke Q, *et al.* Endosialin defines human stem Leydig cells with regenerative potential. *Hum Reprod* 2020; 35: 2197–212.
33 Ge RS, Dong QA, Sottas CM, Papadopoulos V, Zirkin BR, *et al.* In search of rat stem Leydig cells: identification, isolation, and lineage-specific development. *Proc Natl Acad Sci U S A* 2006; 103: 2719–24.
34 Odeh HM, Kleinguetl C, Ge R, Zirkin BR, Chen H. Regulation of the proliferation and differentiation of Leydig stem cells in the adult testis. *Biol Reprod* 2014; 90: 123.
35 Li X, Wang Z, Jiang Z, Guo J, Zhang Y, *et al.* Regulation of seminiferous tubule-associated stem Leydig cells in adult rat testes. *Proc Natl Acad Sci U S A* 2016; 113: 2666–71.
36 Guan X, Chen P, Zhao X, Hao X, Chen F, *et al.* Characterization of stem cells associated with seminiferous tubule of adult rat testis for their potential to form Leydig cells. *Stem Cell Res* 2019; 41: 101593.
37 Stanley E, Lin CY, Jin S, Liu J, Sottas CM, *et al.* Identification, proliferation, and differentiation of adult Leydig stem cells. *Endocrinology* 2012; 153: 5002–10.
38 Xia K, Chen H, Wang J, Feng X, Gao Y, *et al.* Restorative functions of autologous stem Leydig cell transplantation in a testosterone-deficient non-human primate model. *Theranostics* 2020; 10: 8705–20.
39 Yazawa T, Imamichi Y, Miyamoto K, Umezawa A, Taniguchi T. Differentiation of mesenchymal stem cells into gonad and adrenal steroidogenic cells. *World J Stem Cells* 2014; 6: 203–12.
40 Meinsohn MC, Smith OE, Bertolin K, Murphy BD. The orphan nuclear receptors steroidogenic factor-1 and liver receptor homolog-1: structure, regulation, and essential roles in mammalian reproduction. *Physiol Rev* 2019; 99: 1249–79.
41 Crawford PA, Sadovsky Y, Milbrandt J. Nuclear receptor steroidogenic factor 1 directs embryonic stem cells toward the steroidogenic lineage. *Mol Cell Biol* 1997; 17: 3997–4006.
42 Yazawa T, Mizutani T, Yamada K, Kawata H, Sekiguchi T, *et al.* Differentiation of adult stem cells derived from bone marrow stroma into Leydig or adrenocortical cells. *Endocrinology* 2006; 147: 4104–11.
43 Yazawa T, Inanoka Y, Mizutani T, Kuribayashi M, Umezawa A, *et al.* Liver receptor homolog-1 regulates the transcription of steroidogenic enzymes and induces the differentiation of mesenchymal stem cells into steroidogenic cells. *Endocrinology* 2009; 150: 3885–93.
44 Gondo S, Okabe T, Tanaka T, Morinaga H, Nomura M, *et al.* Adipose tissue-derived and bone marrow-derived mesenchymal cells develop into different lineage of steroidogenic cells by forced expression of steroidogenic factor 1. *Endocrinology* 2008; 149: 4717–25.
45 Tanaka T, Gondo S, Okabe T, Ohe K, Shirohzu H, *et al.* Steroidogenic factor 1/adrenal 4 binding protein transforms human bone marrow mesenchymal cells into steroidogenic cells. *J Mol Endocrinol* 2007; 39: 343–50.
46 Yang Y, Su Z, Xu W, Luo J, Liang R, *et al.* Directed mouse embryonic stem cells into Leydig-like cells rescue testosterone-deficient male rats *in vivo*. *Stem Cells Dev* 2015; 24: 459–70.
47 Yazawa T, Kawabe S, Inaoka Y, Okada R, Mizutani T, *et al.* Differentiation of mesenchymal stem cells and embryonic stem cells into steroidogenic cells using steroidogenic factor-1 and liver receptor homolog-1. *Mol Cell Endocrinol* 2011; 336: 127–32.
48 Sonoyama T, Sone M, Honda K, Taura D, Kojima K, *et al.* Differentiation of human embryonic stem cells and human induced pluripotent stem cells into steroid-producing cells. *Endocrinology* 2012; 153: 4336–45.
49 Li L, Li Y, Sottas C, Culty M, Fan J, *et al.* Directing differentiation of human induced pluripotent stem cells toward androgen-producing Leydig cells rather than adrenal cells. *Proc Natl Acad Sci U S A* 2019; 116: 23274–83.
50 Hah N, Murakami S, Nagari A, Danko CG, Kraus WL. Enhancer transcripts mark active estrogen receptor binding sites. *Genome Res* 2013; 23: 1210–23.
51 Yang HM, Do HJ, Kim DK, Park JK, Chang WK, *et al.* Transcriptional regulation of human Oct4 by steroidogenic factor-1. *J Cell Biochem* 2007; 101: 1198–209.
52 Jeyasuria P, Ikeda Y, Jamin SP, Zhao LP, De Rooij DG, *et al.* Cell-specific knockout of steroidogenic factor 1 reveals its essential roles in gonadal function. *Mol Endocrinol* 2004; 18: 1610–9.
53 Chen X, Li C, Chen Y, Xi H, Zhao S, *et al.* Differentiation of human induced pluripotent stem cells into Leydig-like cells with molecular compounds. *Cell Death Dis* 2019; 10: 220.
54 Chen Y, Li C, Ji W, Wang L, Chen X, *et al.* Differentiation of human adipose-derived stem cells into Leydig-like cells with molecular compounds. *J Cell Mol Med* 2019; 23: 5956–69.
55 Ji W, Chen Y, Wang L, Xu Z, Ahmed J, *et al.* Differentiation of human umbilical cord mesenchymal stem cells into Leydig-like cells with defined molecular compounds. *Hum Cell* 2020; 33: 318–29.
56 Gam R, Sung M, Pandurangan AP. Experimental and computational approaches to direct cell reprogramming: recent advancement and future challenges. *Cells* 2019; 8: 1189.
57 Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; 126: 663–76.
58 Inagawa K, Miyamoto K, Yamakawa H, Muraoka N, Sadahiro T, *et al.* Induction of cardiomyocyte-like cells in infarct hearts by gene transfer of Gata4, Mef2c, and Tbx5. *Circ Res* 2012; 111: 1147–56.
59 Gopalakrishnan S, Hor P, Ichida JK. New approaches for direct conversion of patient fibroblasts into neural cells. *Brain Res* 2017; 1656: 2–13.
60 Vallier L. Heps with Pep: direct reprogramming into human hepatocytes. *Cell Stem Cell* 2014; 14: 267–9.
61 Buganim Y, Itskovich E, Hu YC, Cheng AW, Ganz K, *et al.* Direct reprogramming of fibroblasts into embryonic sertoli-like cells by defined factors. *Cell Stem Cell* 2012; 11: 373–86.
62 Yang Y, Li Z, Wu X, Chen H, Xu W, *et al.* Direct reprogramming of mouse fibroblasts toward Leydig-like cells by defined factors. *Stem Cell Reports* 2017; 8: 39–53.
63 Hou YP, Zhang ZY, Xing XY, Zhou J, Sun J. Direct conversion of human fibroblasts into functional Leydig-like cells by SF-1, GATA4 and NGFI-B. *Am J Transl Res* 2018; 10: 175–83.
64 Zhou J, Hou Y, Zhang Z, Xing X, Zou X, *et al.* Conversion of human fibroblasts into functional Leydig-like cells by small molecules and a single factor. *Biochem Biophys Res Commun* 2019; 516: 1–7.
65 Hilton IB, D'Ippolito AM, Vockley CM, Thakore PI, Crawford GE, *et al.* Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nat Biotechnol* 2015; 33: 510–7.
66 Liu XS, Wu H, Ji X, Stelzer Y, Wu X, *et al.* Editing DNA methylation in the mammalian genome. *Cell* 2016; 167: 233–47.
67 Zhou H, Liu J, Zhou C, Gao N, Rao Z, *et al. In vivo* simultaneous transcriptional activation of multiple genes in the brain using CRISPR-dCas9-activator transgenic mice. *Nat Neurosci* 2018; 21: 440–6.
68 Weltner J, Balboa D, Katayama S, Bespalov M, Krjutskov K, *et al.* Human pluripotent reprogramming with CRISPR activators. *Nat Commun* 2018; 9: 2643.
69 Chakraborty S, Ji H, Kabadi AM, Gersbach CA, Christoforou N, *et al.* A CRISPR/Cas9-based system for reprogramming cell Lineage specification. *Stem Cell Reports* 2014; 3: 940–7.
70 Matjusaitis M, Wagstaff LJ, Martella A, Baranowski B, Blin C, *et al.* Reprogramming of fibroblasts to oligodendrocyte progenitor-like cells using CRISPR/Cas9-based synthetic transcription factors. *Stem Cell Reports* 2019; 13: 1053–67.
71 Black JB, Adler AF, Wang HG, D'Ippolito AM, Hutchinson HA, *et al.* Targeted epigenetic remodeling of endogenous loci by CRISPR/Cas9-based transcriptional activators directly converts fibroblasts to neuronal cells. *Cell Stem Cell* 2016; 19: 406–14.

72 Liao HK, Hatanaka F, Araoka T, Reddy P, Wu MZ, *et al*. *In vivo* target gene activation via CRISPR/Cas9-mediated trans-epigenetic modulation. *Cell* 2017; 171: 1495–507.
73 Huang H, Zou X, Zhong L, Hou Y, Zhou J, *et al*. CRISPR/dCas9-mediated activation of multiple endogenous target genes directly converts human foreskin fibroblasts into Leydig-like cells. *J Cell Mol Med* 2019; 23: 6072–84.
74 Huang H, Zhong L, Zhou J, Hou Y, Zhang Z, *et al*. Leydig-like cells derived from reprogrammed human foreskin fibroblasts by CRISPR/dCas9 increase the level of serum testosterone in castrated male rats. *J Cell Mol Med* 2020; 24: 3971–81.
75 Kim Y, Jeong J, Choi D. Small-molecule-mediated reprogramming: a silver lining for regenerative medicine. *Exp Mol Med* 2020; 52: 213–26.
76 Yang YM, Chen RG, Wu XM, Zhao YN, Fan YH, *et al*. Rapid and efficient conversion of human fibroblasts into functional neurons by small molecules. *Stem Cell Reports* 2019; 13: 862–76.
77 Yang Y, Zhou CX, Zhang TT, Li Q, Mei JX, *et al*. Conversion of fibroblast into functional Leydig-like cell using defined small molecules. *Stem Cell Reports* 2020; 15: 408–23.
78 Stadhouders R, Filion GJ, Graf T. Transcription factors and 3D genome conformation in cell-fate decisions. *Nature* 2019; 569: 345–54.
79 Iwafuchi-Doi M, Zaret KS. Cell fate control by pioneer transcription factors. *Development* 2016; 143: 1833–7.
80 Pickar-Oliver A, Gersbach CA. The next generation of CRISPR-Cas technologies and applications. *Nat Rev Mol Cell Biol* 2019; 20: 490–507.
81 Moon SB, Kim D, Ko JH, Kim YS. Recent advances in the CRISPR genome editing tool set. *Exp Mol Med* 2019; 51: 1–11.
82 Xu XB, Hulshoff MS, Tan XY, Zeisberg M, Zeisberg EM. CRISPR/Cas derivatives as novel gene modulating tools: possibilities and *in vivo* applications. *Int J Mol Sci* 2020; 21: 3038.
83 Chavez A, Tuttle M, Pruitt BW, Ewen-Campen B, Chari R, *et al*. Comparison of Cas9 activators in multiple species. *Nat Methods* 2016; 13: 563–7.
84 Chavez A, Scheiman J, Vora S, Pruitt BW, Tuttle M, *et al*. Highly efficient Cas9-mediated transcriptional programming. *Nat Methods* 2015; 12: 326–8.
85 Fang LJ, Hung SS, Yek J, El Wazan L, Nguyen T, *et al*. A simple cloning-free method to efficiently induce gene expression using CRISPR/Cas9. *Mol Ther Nucleic Acids* 2019; 14: 184–91.
86 Josipovic G, Tadic V, Klasic M, Zanki V, Beceheli I, *et al*. Antagonistic and synergistic epigenetic modulation using orthologous CRISPR/dCas9-based modular system. *Nucleic Acids Res* 2019; 47: 9637–57.
87 Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, *et al*. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature* 2015; 517: 583–8.
88 O'Geen H, Ren CH, Nicolet CM, Perez AA, Halmai J, *et al*. dCas9-based epigenome editing suggests acquisition of histone methylation is not sufficient for target gene repression. *Nucleic Acids Res* 2017; 45: 9901–16.
89 O'Geen H, Bates SL, Carter SS, Nisson KA, Halmai J, *et al*. Ezh2-dCas9 and KRAB-dCas9 enable engineering of epigenetic memory in a context-dependent manner. *Epigenetics Chromatin* 2019; 12: 26.
90 Thakore PI, D'Ippolito AM, Song LY, Safi A, Shivakumar NK, *et al*. Highly specific epigenome editing by CRISPR-Cas9 repressors for silencing of distal regulatory elements. *Nat Methods* 2015; 12: 1143–9.
91 Kearns NA, Pham H, Tabak B, Genga RM, Silverstein NJ, *et al*. Functional annotation of native enhancers with a Cas9-histone demethylase fusion. *Nat Methods* 2015; 12: 401–3.
92 Chen LF, Lin YT, Gallegos DA, Hazlett MF, Gomez-Schiavon M, *et al*. Enhancer histone acetylation modulates transcriptional bursting dynamics of neuronal activity-inducible genes. *Cell Rep* 2019; 26: 1174–88.
93 Schuijers J, Manteiga JC, Weintraub AS, Day DS, Zamudio AV, *et al*. Transcriptional dysregulation of MYC reveals common enhancer-docking mechanism. *Cell Rep* 2018; 23: 349–60.
94 Mkannez G, Gagne-Ouellet V, Nsaibia MJ, Boulanger MC, Rosa M, *et al*. DNA methylation of a PLPP3 MIR transposon-based enhancer promotes an osteogenic programme in calcific aortic valve disease. *Cardiovasc Res* 2018; 114: 1525–35.
95 Wilson C, Chen PJ, Miao Z, Liu DR. Programmable m(6)A modification of cellular RNAs with a Cas13-directed methyltransferase. *Nat Biotechnol* 2020; 38: 1431–40.
96 Zhao J, Li B, Ma JX, Jin WL, Ma XL. Photoactivatable RNA N^6-methyladenosine editing with CRISPR-Cas13. *Small* 2020; 16: e1907301.
97 Li JX, Chen ZJ, Chen F, Xie GY, Ling YY, *et al*. Targeted mRNA demethylation using an engineered dCas13b-ALKBH5 fusion protein. *Nucleic Acids Res* 2020; 48: 5684–94.
98 Rauch S, He C, Dickinson BC. Targeted m(6)A reader proteins to study epitranscriptomic regulation of single RNAs. *J Am Chem Soc* 2018; 140: 11974–81.
99 Li X, Zuo XH, Jing JZ, Ma YT, Wang JM, *et al*. Small-molecule-driven direct reprogramming of mouse fibroblasts into functional neurons. *Cell Stem Cell* 2015; 17: 195–203.
100 Takeda Y, Harada Y, Yoshikawa T, Dai P. Chemical compound-based direct reprogramming for future clinical applications. *Biosci Rep* 2018; 38: BSR20171650.
101 Xie X, Fu YB, Liu J. Chemical reprogramming and transdifferentiation. *Curr Opin Genet Dev* 2017; 46: 104–13.
102 Azhar S, Dong D, Shen WJ, Hu Z, Kraemer FB. The role of miRNAs in regulating adrenal and gonadal steroidogenesis. *J Mol Endocrinol* 2020; 64: R21–43.
103 Tanaka T, Aoyagi C, Mukai K, Nishimoto K, Kodama S, *et al*. Extension of survival in bilaterally adrenalectomized mice by implantation of SF-1/Ad4BP-induced steroidogenic cells. *Endocrinology* 2020; 161: bqaa007.
104 Arora H, Zuttion M, Nahar B, Lamb D, Hare JM, *et al*. Subcutaneous Leydig stem cell autograft: a promising strategy to increase serum testosterone. *Stem Cells Transl Med* 2019; 8: 58–65.
105 Feng X, Xia K, Ke Q, Deng R, Zhuang J, *et al*. Transplantation of encapsulated human Leydig-like cells: a novel option for the treatment of testosterone deficiency. *Mol Cell Endocrinol* 2020; 519: 111039.
106 Chen HL, Jin SY, Huang SS, Folmer J, Liu J, *et al*. Transplantation of alginate-encapsulated seminiferous tubules and interstitial tissue into adult rats: Leydig stem cell differentiation *in vivo*? *Mol Cell Endocrinol* 2016; 436: 250–8.
107 Cudicini C, Lejeune H, Gomez E, Bosmans E, Ballet F, *et al*. Human Leydig cells and Sertoli cells are producers of interleukins-1 and -6. *J Clin Endocrinol Metab* 1997; 82: 1426–33.



Supplementary Figure 1: Expression tendency analysis of the responding TFs during the differentiation of fibroblasts into Leydig-like cells. The TFs that changed significantly during the transdifferentiation process were identified and clustered. (**a**). In the TiLC group, 58 TFs changed their original expression status, and the final expression pattern of these factors was similar to that of LCs. In the CiLC group, 73 TFs changed their original expression status, 45 of which had similar expression patterns to LCs (**b**), while 28 had the opposite (**c**). TiLCs: transcription factor-based induced Leydig-like cells; CiLCs: compound-based induced Leydig-like cells; LCs: Leydig cells; TF: transcription factor.