Stem Cell Reports
Article
ISSCR
OPEN ACCESS

Conversion of Fibroblast into Functional Leydig-like Cell Using Defined Small Molecules

Yan Yang,[1,6] Chenxing Zhou,[1,6] Tiantian Zhang,[1] Quan Li,[1] Jiaxin Mei,[1] Jinlian Liang,[1] Ziyi Li,[1] Hanhao Li,[2] Qi Xiang,[1,3] Qihao Zhang,[1] Lei Zhang,[4] and Yadong Huang[1,2,5,*]

[1]Department of Cell Biology, Jinan University, Guangzhou 510632, China
[2]Department of Pharmacology, Jinan University, Guangzhou 510632, China
[3]Bioparmaceutical R&D Center of Jinan University, Guangzhou 510632, China
[4]Guangdong Provincial Institute of Biological Products and Materia Medica, Guangzhou 510440, China
[5]Guangdong Province Key Laboratory of Bioengineering Medicine of, Guangzhou 510632, China
[6]Co-first author
*Correspondence: tydhuang@jnu.edu.cn
https://doi.org/10.1016/j.stemcr.2020.07.002

SUMMARY

Recent studies have demonstrated that fibroblasts can be directly converted into functional Leydig cells by transcription factors. However, the transgenic approach used in these studies raises safety concerns for its future application. Here, we report that fibroblasts can be directly reprogrammed into Leydig-like cells by exposure to a combination of forskolin, 20α-hydroxycholesterol, luteinizing hormone, and SB431542. These chemical compound-induced Leydig-like cells (CiLCs) express steroidogenic genes and have a global gene expression profile similar to that of progenitor Leydig cells, although not identical. In addition, these cells can survive in testis and produce testosterone in a circadian rhythm. This induction strategy is applicable to reprogramming human periodontal ligament fibroblasts toward Leydig-like cells. These findings demonstrated fibroblasts can be directly converted into Leydig-like cells by pure chemical compounds. This strategy overcomes the limitations of conventional transgenic-based reprogramming and provides a simple, effective approach for Leydig cell-based therapy while simultaneously preserving the hypothalamic-pituitary-gonadal axis.

INTRODUCTION

Leydig cells are found in the interstitial space of the testis between the seminiferous tubules. These cells produce testosterone, which plays a critical role in the physiological functions of the male reproductive system throughout life (Abney, 1999; Chen et al., 2009; Zirkin and Chen, 2000). Testosterone synthesis in Leydig cells is tightly regulated by the hypothalamic-pituitary-gonadal (HPG) axis. In the HPG axis, the pituitary gland synthesizes and releases luteinizing hormone (LH) to regulate testosterone production by Leydig cells (Saez, 1994). LH binds its receptors (LH receptor gene, LHCGR) on the plasma membrane of Leydig cells and then activates adenylate cyclase to increase intracellular cyclic adenosine monophosphate (cAMP) production, which further results in rapid transport of cholesterol from the outer to the inner mitochondrial membrane in a manner mediated by steroidogenic acute regulatory protein (StAR). Subsequently, Leydig cells synthesize testosterone via a series of steroidogenic enzymes, such as cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1), 3β-hydroxysteroid dehydrogenase (HSD3B), cytochrome P450 17α-hydroxylase (CYP17A1), and 17β-hydroxysteroid dehydrogenase 3 (HSD17B3). The expression levels of these proteins are closely related to produce testosterone (LaVoie and King, 2009; Payne and Hales, 2004). In the testis, different stages in the development of adult Leydig cells (ALCs), including transitions from stem Leydig cells to progenitor Leydig cells (PLCs), from PLCs to ALCs, have been identified (Chen et al., 2017). The difference of PLCs and ALCs is the products of androgen. PLCs produce primarily androsterone, ALCs secrete mainly testosterone.

Male late-onset hypogonadism (LOH) is characterized by serum testosterone deficiency with advancing age (Nieschlag et al., 2005), which is associated with sexual dysfunction, central adiposity, mood disturbance, osteoporosis, amyotrophy, and other abnormalities (Howell et al., 2000). LOH affects approximately 30% of men aged 40–79 years, increasing in prevalence with age (Allan and McLachlan, 2004). Age-related declines in serum testosterone levels are typically not a response to reduced LH but rather a consequence of the decreasing responsiveness of Leydig cells to LH (Huhtaniemi and Forti, 2011). Currently, testosterone replacement therapy can be used to treat LOH and reverse many symptoms of low testosterone levels. However, exogenous testosterone supplementation has some adverse effects (gynecomastia, polycythemia, hypertension, acne, and hair loss) and potential risks (heart attack, stroke, and prostate cancer) (Abadilla and Dobs, 2012). This strategy is not appropriate for long-term therapy due to negative feedback loop of the HPG axis. Therefore, an alternative approach for testosterone supplementation in a physiological pattern is extremely desirable.

Leydig cell transplantation is an ideal alternative approach for long-term provision of physiological hormone patterns (Arora et al., 2019; Peak et al., 2016; Pfeil and Dobs, 2008). However, Leydig cells are terminally differentiated cells and do not proliferate *in vitro*. The scarcity of Leydig cells limits their use for Leydig cell transplantation (Chen et al., 2009; Haider, 2004). A previous study demonstrated for the first time that nuclear receptor subfamily 5 group A member 1 (NR5A1/SF-1) directly differentiates embryonic stem cells (ESCs) into steroidogenic cells (Crawford et al., 1997). Thereafter, several studies attempted to generate Leydig cells from mesenchymal stem cells (Gondo et al., 2008; Yazawa et al., 2006), and induced pluripotent stem cells (iPSCs) (Li et al., 2019a; Sonoyama et al., 2012) via ectopic expression of SF-1. However, iPSC- or ESC-derived cells may carry ethical concerns and a risk of tumor occurrence. In our previous study, we succeeded in directly reprogramming fibroblasts into Leydig cells by forced expression of the transcription factors, *Dmrt1*, *Gata4*, and *Nr5a1* (Yang et al., 2017). The induced Leydig-like cells expressed steroidogenic genes, and acquired the capability for androgen synthesis. Although this technique provided a promising strategy for obtaining functional Leydig cells, the risks associated with genomic insertion of exogenous DNA sequences invoked concerns about its safety for future applications.

Recently, an increasing number of studies have suggested that the potency and advantages of small molecules, which could overcome the limitations of genetic manipulation, in reprogramming cellular fate (Li et al., 2018; Ma et al., 2017). Small-molecule compounds can be easily manufactured, preserved and standardized. Zhou et al. (2019) reported a defined small-molecule cocktail that enabled the conversion of human fibroblasts into functional Leydig cells with only one transcription factor. The study suggested the feasibility of inducing the conversion of fibroblasts into Leydig cells by a chemical cocktail. However, in this study, forced expression of the transcription factor was needed. Thus, exploring a defined small-molecule cocktail to replace the transcription factor is necessary. Inspiringly, an increasing number of studies have shown the success of inducing the conversion of somatic cells into pluripotent stem cells (Hou et al., 2013), neurons (Li et al., 2015), cardiomyocytes (Cao et al., 2016), and pancreatic β cells (Li et al., 2014) via pure chemical compounds.

Therefore, in this study, we focused on generating Leydig cells with pure small-molecule compounds. We found a defined small-molecule cocktail that could replace transcription factors to directly convert fibroblasts into progenitor Leydig-like cells. During this conversion process, brief exposure to a chemical cocktail could facilitate the conversion of fibroblasts into functional progenitor Leydig-like cells without ectopic expression of genes. Our findings demonstrated the feasibility of using pure chemicals to generate functional Leydig-like cells, which may serve as an alternative and accessible source of cells for Leydig cell replacement therapies as well as *in vitro* disease modeling, toxicity testing, and drug development.

RESULTS

Small Molecules Induce Leydig Cell Fate without a Progenitor Stage

In our previous study, we demonstrated that *Nr5a1* is a pioneer transcription factor (Yang et al., 2017). *Nr5a1* can initiate fibroblast reprogramming toward a Leydig cell fate. Therefore, replacing the *Nr5a1* with defined small molecules represented an essential first step during the Leydig cell reprogramming process. To determine whether small molecules could activate the endogenous *Nr5a1*, we performed a preliminary screen, which identified 12 compounds (12C) that predominantly target epigenetic modifications (DAPT, XAV939, and 5-azacytidine), metabolic activities (LH, 20-hydroxycholesterol, and triiodothyronine [T3]), and signaling pathways involved in Leydig cell fate patterning (PDGFAA, activin B, forskolin, insulin growth factor 1 [IGF1], LiCl, and SB431542) (Figure 1A). To avoid contamination of gonad lineage cells among mouse embryonic fibroblasts (MEFs), we examined the preliminary cell population by immunofluorescence analysis. Approximately 99% of cells expressed the fibroblasts marker vimentin (Figure 1B). In addition, qPCR analysis did not reveal an increase in the levels of genes that are highly expressed in gonad lineage cells (Figure S1). These results indicated no detectable contamination of gonad lineage cells among the preliminary cells population, confirming the purity of the preliminary cell population for the subsequent experiments.

The combination of the 12C was added to the MEF culture medium for 7 days (Figure 1C). Testosterone production was analyzed, and the results suggested that 12C could convert fibroblasts into the testosterone-producing cells (Figure 1D). We next attempted to identify the critical molecule for Leydig cell conversion. To this end, we removed each factor separately from the cocktail. Removal of PDGFAA, activin B, DAPT, XAV939, IGF1, 5-azacytidine, LiCl, or T3 did not significantly change *Nr5a1* expression or testosterone production (Figures 1E and 1F), suggesting that these factors are dispensable during the reprograming process. However, removal of SB431542, the inhibitor of transforming growth factor β (TGF-β) type I receptor kinase (ALK-5), nearly abolished the induction of *Nr5a1* expression (Figure 1E), indicating that SB431542 plays an important role in Leydig cell fate transformation. The 12C combination without PDGFAA, activin B, DAPT, XAV939,

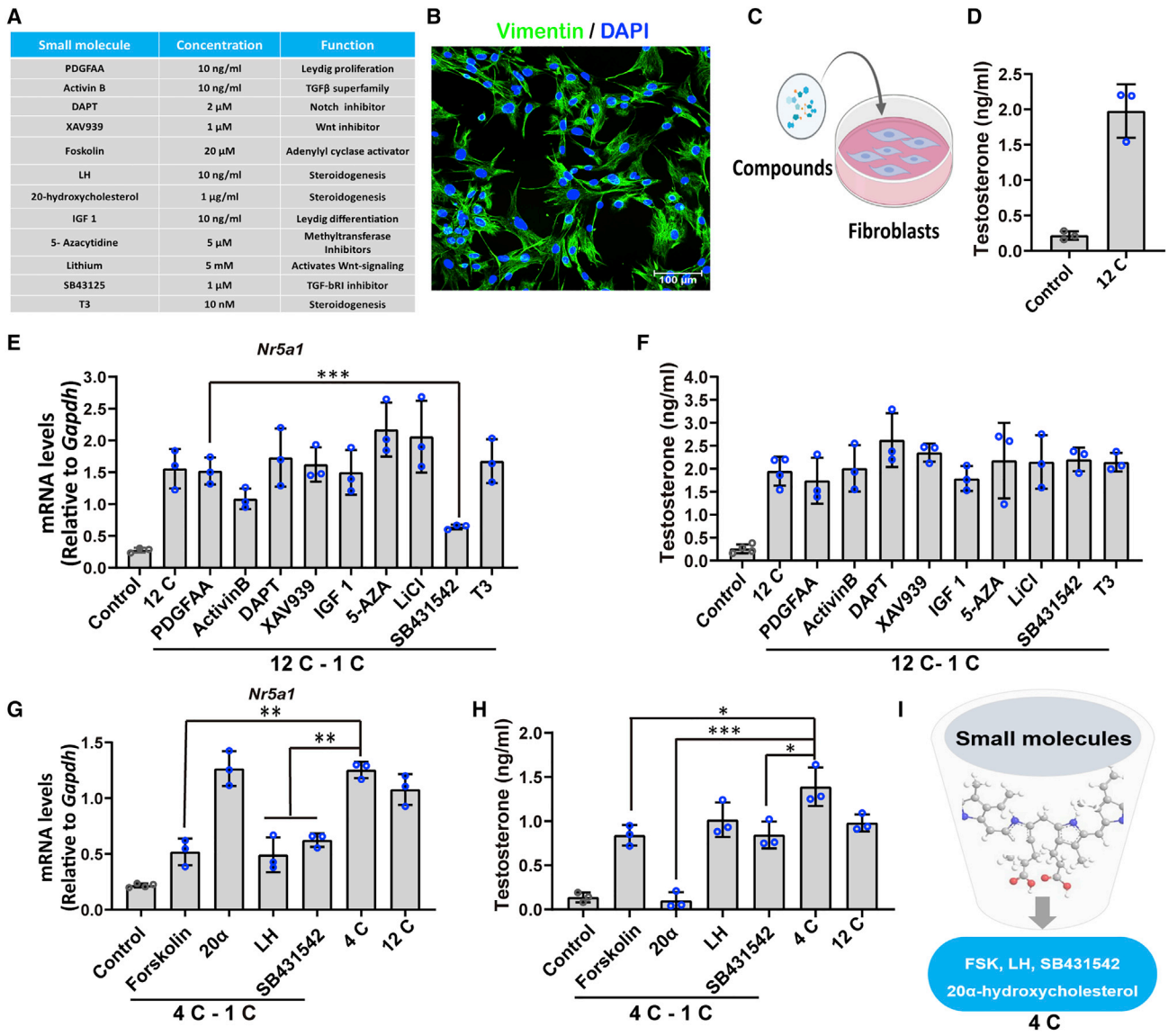


Figure 1. Identification of Leydig Cell Fate Inducing Small Molecules

(A) Candidate small-molecule compounds tested for their ability to produce Leydig-like cells.

(B) Immunostaining showing cultured cells expressing the fibroblast marker vimentin. Scale bar, 400 μm.

(C) Scheme of the preliminary approach.

(D) Testosterone production in MEFs treated with 12C after 1 week of induction.

(E) qRT-PCR analysis of the Leydig cell maker *Nr5a1* in MEFs treated with various combinations of 12 or 11 compounds for 1 week.

(F) Testosterone production in MEFs treated with various combinations of 12 or 11 compounds for 1 week.

(G) qRT-PCR analysis of the Leydig cell maker *Nr5a1* treated with various combinations of 4 or 3 compounds for 1 week.

(H) Testosterone production in MEFs treated with various combinations of 4 or 3 compounds for 1 week.

(I) Flowchart indicating the screening result.

The data were obtained from at least three independent experiments and are presented as mean ± SD values. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. 12C: the combination of 12C compounds. 4C: the combination of forskolin, 20α-hydroxycholesterol, LH, and SB431542. See also Figures S1 and S2.

IGF1, 5-azacytidine, LiCl and T3 was a pool of four compounds—forskolin, 20α-hydroxycholesterol, LH, and SB431542—and was referred to as 4C. The compounds in 4C were retained in the next round of screening. Individual removal of forskolin, LH, and SB431542 from 4C significantly decreased the expression of *Nr5a1* (Figure 1G), while

removal of 20α-hydroxycholesterol from 4C drastically suppressed testosterone synthesis (Figure 1H). This result suggested that forskolin, LH, and SB431542 were essential for *Nr5a1* expression and that 20α-hydroxycholesterol has an important role for in testosterone biosynthesis. In addition, MEFs were treated with forskolin, LH, SB431542, or 20α-hydroxycholesterol alone for 7 days. Although forskolin, LH, SB431542, or 20α-hydroxycholesterol treatment alone resulted in mild upregulation of *Nr5a1*, no significant difference was observed compared with the control. The expression levels of *Star*, *Cyp17a1*, *Hsd3b*, and *Hsd17b3* were significantly increased in the presence of LH, or SB431542 alone. 20α-Hydroxycholesterol significantly increased *Hsd3b* expression, which could increase steroidogenesis. The cooperative action of forskolin, LH, SB431542, and 20α-hydroxycholesterol significantly enhanced Leydig-related gene expression (Figure S2). Therefore, 4C was used for further study (Figure 1I).

MEFs treated with 4C (MEFs-4C) displayed elongated, bipolar Leydig-like extensions after 7 days, whereas MEFs not supplemented with small-molecule cocktail exhibited no morphological changes during the early stage of treatment. The confluency of cultured cells treated with 4C decreased, while that of MEFs increased (Figures 2A and 2B). We then analyzed the dynamic expression pattern of the Leydig cell fate transcription factors, *Nr5a1*, *Dmrt1*, and *Gata4* after 4C treatment. Interestingly, in the absence of exogenous transcription factors, the 4C was sufficient to upregulate the expression of the Leydig cell fate genes, *Nr5a1*, *Dmrt1*, and *Gata4* (Figures 2C–2E) and downregulate the expression of MEF marker genes (*Postn* and *Col5a2*) (Figures 2F and 2G). Moreover, almost no expression of the adrenal-specific steroidogenic genes *Cyp11b1*, *Cyp21a1*, and *Cyp11b2* was detected (Figures S3A–S3C).

We subsequently examined whether the conversion process involved transition through a defined pluripotent stage. As expected, almost no expression of the pluripotency markers *Oct4* and *Nanog* was detected throughout the reprogramming process (Figures S3D and S3E). In addition, the proliferation curve of MEFs-4C did not change significantly from 7 to 21 days (Figure 2H). These results suggested that the conversion process did not include a pluripotent stage.

Collectively, these findings demonstrated that the combination of forskolin, 20α-hydroxycholesterol, LH, and SB431542 initiated Leydig fates from fibroblasts rather than adrenal cells; and that this process did not include an intermediate pluripotent state.

MEFs-4C Exhibit Characteristics of Leydig Cells

To characterize the dynamic expression pattern of Leydig cell fate genes during conversion, the expression of Leydig-specific genes was assessed on days 7, 14, and 21. The expression of *Star*, *Cyp11a1*, *Hsd3b*, *Cyp17a1*, and *Hsd17b3* was significantly increased in MEFs-4C on day 7, increased gradually, and then remained stable from days 14 to 21 (Figures S4A–S4E). Correspondingly, culture supernatants were harvested for quantification of testosterone at the indicated time points. MEFs-4C began to secrete testosterone at day 4; and the yield gradually increased, plateauing on day 14 (Figure S4F).

On day 21, the induced cells acquired a short fusiform morphology resembling that of endogenous Leydig cells, while MEFs were arranged in a cobblestone pattern with polygonal shapes and large nuclei (Figure 3A). To identify the developmental stage of MEFs-4C, the expression of steroidogenic-related genes and the levels of intermediate products of testosterone were assessed. MEFs-4C expressed Leydig lineage biomarkers, such as *Nr5a1*, *Star*, *Cyp11a1*, *Hsd3b1*, *Cyp17a1*, and *Hsd17b3*. The expression level of some Leydig markers (*Star*, *Hsd3b*, and *Cyp17a1*) were close to primary PLCs (Figure 3B). Notably, MEFs-4C produced androsterone, and the level of androsterone by MEFs-4C was comparable with endogenous PLCs and ALCs (Figures 3C and 3D). However, the level of testosterone and dihydrotestosterone was lower in MEFs-4C than in ALCs. There was no significant difference between MEFs-4C and endogenous PLCs (Figures 3E and 3F). Subsequently, the expression of PLC lineage markers was further confirmed by immunocytochemistry. MEFs-4C strongly expressed the key steroidogenic enzymes StAR and CYP17A1. In contrast, MEFs were negative for the expression of those proteins (Figure 3G). The results of western blot showed that MEFs-4C expressed NR5A1, DMRT1, and GATA4 (Figure 3H). Taken together, these findings indicated that MEFs-4C expressed steroidogenic genes, and acquired androgen synthesis capabilities, which were much more similar—although not really equivalent—to endogenous PLCs than to MEFs.

The Steroidogenic Pathway of Testosterone Biosynthesis Is Present in MEFs-4C

Leydig cells are the only cells that respond to LH in the testis. LHCGR plays a central role in the maintenance of testicular testosterone production. After LH binds to LHCGR, the steroid substrate cholesterol is transported into Leydig cells via SRBI (a lipoprotein receptor). Subsequently, cholesterol is translocated into the mitochondria and is then used for testosterone synthesis via a series of steroidogenic enzymes (Figure 4A). We evaluated the expression of LHCGR and SRBI in MEFs-4C using western blotting. MEFs-4C expressed LHCGR and SRBI, but the expression of these proteins was almost undetectable in MEFs (Figure 4B). Oil red O staining showed that the cytoplasm of MEFs-4C and endogenous PLCs contained many lipid droplets, which are necessary for testosterone

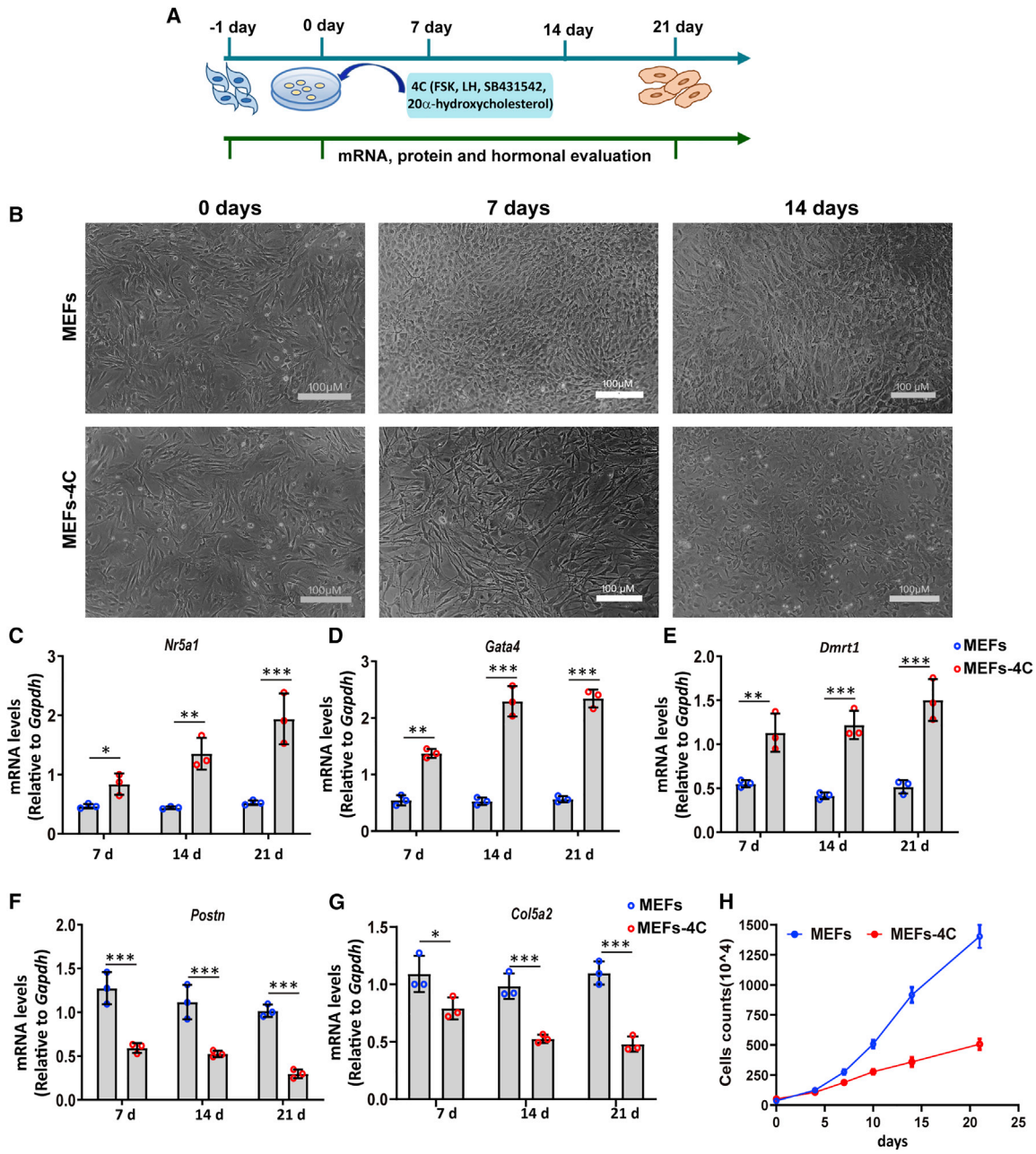


Figure 2. Conversion of MEFs into Leydig-like Cells by the Combination of Forskolin, 20α-Hydroxycholesterol, LH, and SB431542 (4C)

(A) Schematic of the experimental procedure.

(B) Cells morphology was transformed from a fibroblast morphology to a Leydig cell morphology during the induction process. Representative images from days 0, 7, and 14 are shown. Scale bars, 100 μm.

(C–E) qRT-PCR analysis of key Leydig transcription factor genes (C, *Nr5a1*; D, *Gata4*, and E, *Dmrt1*) after induction with 4C.

(F and G) qRT-PCR analysis of fibroblast genes (F, *Postn*; G, *Col5a2*) after induction with 4C.

(H) Cell proliferation curves during the induction process. Three wells were harvested for cell counting.

The data were obtained from three independent experiments and are presented as the mean ± SD values. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. MEFs-4C: MEFs treated with the combination of forskolin, 20α-hydroxycholesterol, LH, and SB431542. See also Figure S3.

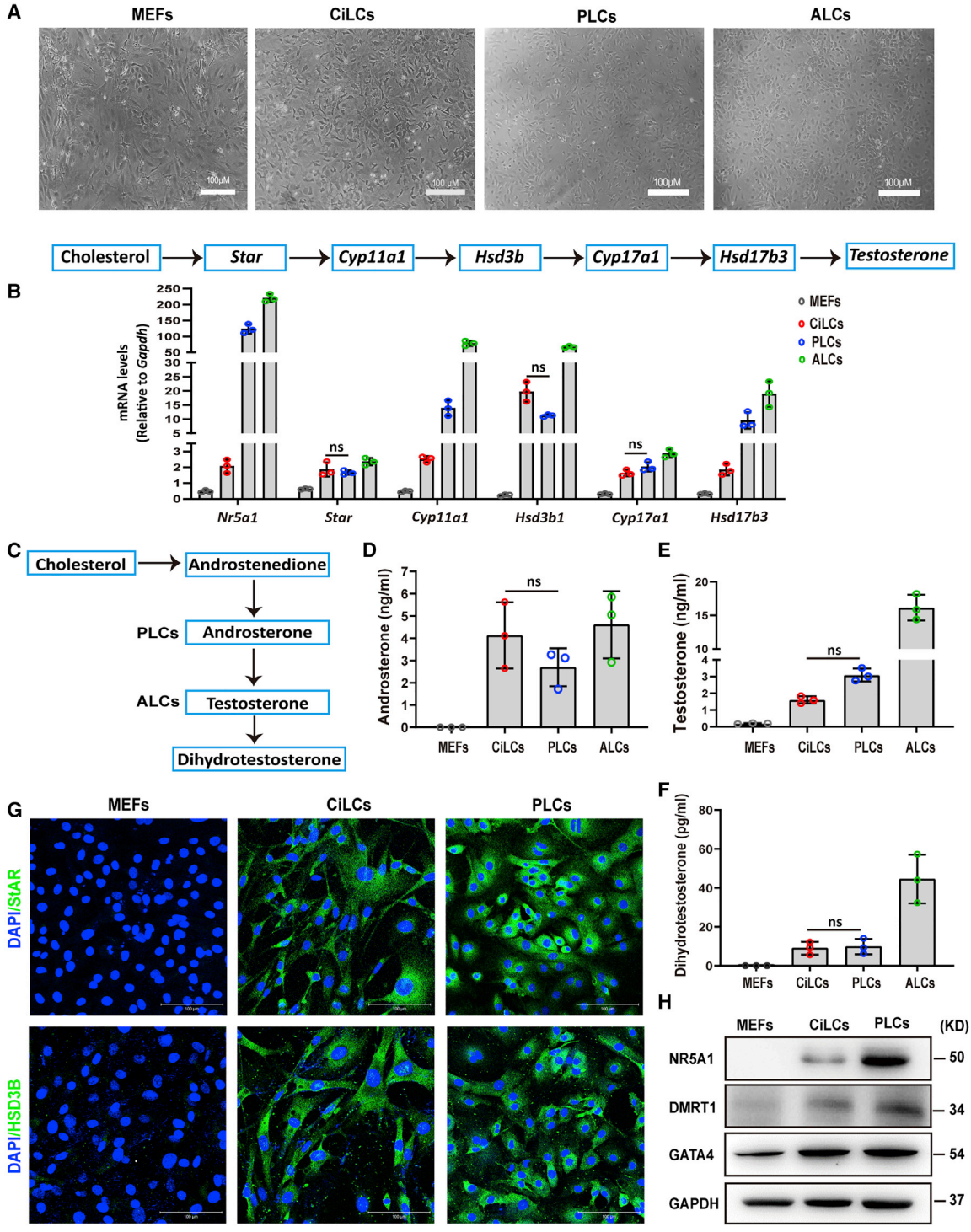


Figure 3. Characteristics of CiLCs

(A) Morphology of MEFs, MEFs-4C, PLCs, and ALCs. MEFs-4C: MEFs were treated with the combination of four small molecules (forskolin [FSK], 20α-hydroxycholesterol, LH, and SB431542) for 21 days. Scale bars, 100 μm.

(B) qRT-PCR analysis of key Leydig cell-related genes in MEFs, MEFs-4C, PLCs, and ALCs.

(C) Steroidogenic pathway in PLCs and ALCs.

(D–F) Production of androsterone (D), testosterone (E), and dihydrotestosterone (F) by MEFs-4C, PLCs, and ALCs.

(legend continued on next page)

production. In contrast, no lipid droplets were found in MEFs (Figure 4C). In addition, staining to evaluate HSD3B enzymatic activity indicated that MEFs-4C were HSD3B positive (deep purple color), confirming that MEFs-4C exhibit HSD3B enzymatic activity (Figure 4D). High-density lipoprotein (HDL) cholesterol is a major source of cholesterol for testosterone production in Leydig cells. To examine the potential role of SRBI, LHCGR, and HSD3B in cholesterol uptake and testosterone synthesis, testosterone production by MEFs-4C was examined after treatment with LH and HDL for 3 h (Figure 4E). MEFs-4C treatment with LH significantly increased testosterone production in a dose-dependent manner (Figure 4F).

Taken together, these results demonstrated that MEFs-4C expressed steroidogenic genes, acquired the capacity for testosterone synthesis. More importantly, the MEFs-4C expressed LHCGR, which made these cells responsive to LH signaling and producing testosterone. Therefore, we named these MEFs-4C as chemical-induced Leydig-like cells (CiLCs).

Transcriptome Analysis of CiLCs

To understand the global transcriptional changes, we investigated the gene expression profiles of MEFs, CiLCs, and PLCs. Hierarchical clustering analysis showed that the transcriptome profiles of CiLCs were more distant from those of MEFs than from those of PLCs, indicating a variation in tendency from fibroblasts to Leydig cells. Among the 17,749 genes analyzed, 1,711 genes were differentially expressed by more than 2-fold between MEFs and CiLCs, with 944 genes upregulated and 767 genes downregulated in CiLCs (Figure 5A). Scatterplot visualization showed that 4C downregulated the MEF-enriched genes, such as *Col5a2*, *Slit2*, *Mfap4*, *Fbln5*, *Itga11*, and *Tnc*, and activated Leydig cell-specific genes, including *Nr5a1*, *Hsd17b3*, *Cyp11a1*, *Gata4*, *Star*, and *Hsd3b* (Figure 5B). Gene ontology analysis suggested that the genes upregulated in the CiLCs were enriched in cell differentiation, cholesterol metabolic process, which is related to the steroidogenic function of PLCs (Figure 5C).

The heatmap and qRT-PCR further revealed that the expression of steroidogenic genes increased significantly, including *Nr5a1*, *Gata4*, *Dmrt1*, *Star*, *Cyp11a1*, *Hsd3b*, *Cyp17a1*, and *Hsd17b3*. In contrast, levels of the fibroblast markers *Col5a2*, *Postn*, *Fbln5*, *Itga11*, and *Mfap4* were markedly downregulated (Figures 5D–5F). Taken together, these data further verified that 4C can successfully initiate MEF reprogramming toward Leydig-like cell. The genome-wide gene expression profile of CiLCs resembled that of endogenous PLCs, although not identical to the real PLCs.

Intratesticular Transplantation of CiLCs Rescue Testosterone Deficiency in Male Rats

To assess the ability of CiLCs to survive and function *in vivo*, we transplanted CiLCs into the testes of ethylene dimethanesulfonate (EDS)-treated rats (a model of androgen deficiency). To trace the transplanted cells *in vivo*, CiLCs and MEFs were labeled with PKH26, a red fluorescent lipophilic dye, and the CiLCs were then implanted into the parenchyma of the recipient EDS-treated rats. Negative control rats received PBS alone or were implanted with MEFs. Serum was collected for quantitative testosterone determination on days 7, 14, and 21 after transplantation (Figure 6A). The serum testosterone level began to increase in CiLC-treated rats at 14 days. After 21 days, implantation of CiLCs dramatically promoted the recovery process, and the serum testosterone level in CiLC-transplanted group was restored to near the normal control level. However, the level of testosterone was not significantly different between the EDS-MEFs group and the EDS-PBS group (Figure 6B).

To determine whether CiLC-treated rats exhibited a diurnal testosterone rhythm, we collected blood samples every 6 h from 23:00 h on day 21 to 23:00 h on day 22 after cell transplantation. As expected, the serum testosterone level in the EDS-CiLC group displayed a pulsatile circadian rhythm similar to that of saline-injected normal rats, whereas this rhythm was not observed in the EDS-MEFs group or EDS-PBS group (Figure 6C).

The rats were euthanized, and their testes were removed and examined on day 22 after transplantation. The PKH26-labeled cells were localized exclusively to the testicular interstitium and expressed the Leydig cell marker CYP17A1. In contrast, MEFs did not express CYP17A1 (Figure 6D). Together, these findings indicated that CiLCs acquired key properties of real Leydig cells, they were capable of colonizing the interstitium and have the potential to restore the serum testosterone level while simultaneously preserving the HPG axis.

Reprogramming Human Periodontal Ligament Fibroblasts into Human CiLCs

To confirm whether our reprogramming strategy is suitable for the human fibroblast line, we treated HPLFs with 4C. Similar to MEFs, human periodontal ligament fibroblasts (HPLFs) treated with 4C expressed Leydig cell marker genes

(G) Immunostaining of the Leydig cell markers StAR and HSD3B in MEFs-4C and PLCs.
(H) Representative western blotting for the protein expression in MEFs-4C and PLCs.
The data were obtained from at least three independent experiments and were presented as mean ± SD values. ns, not significant. See also Figure S4.

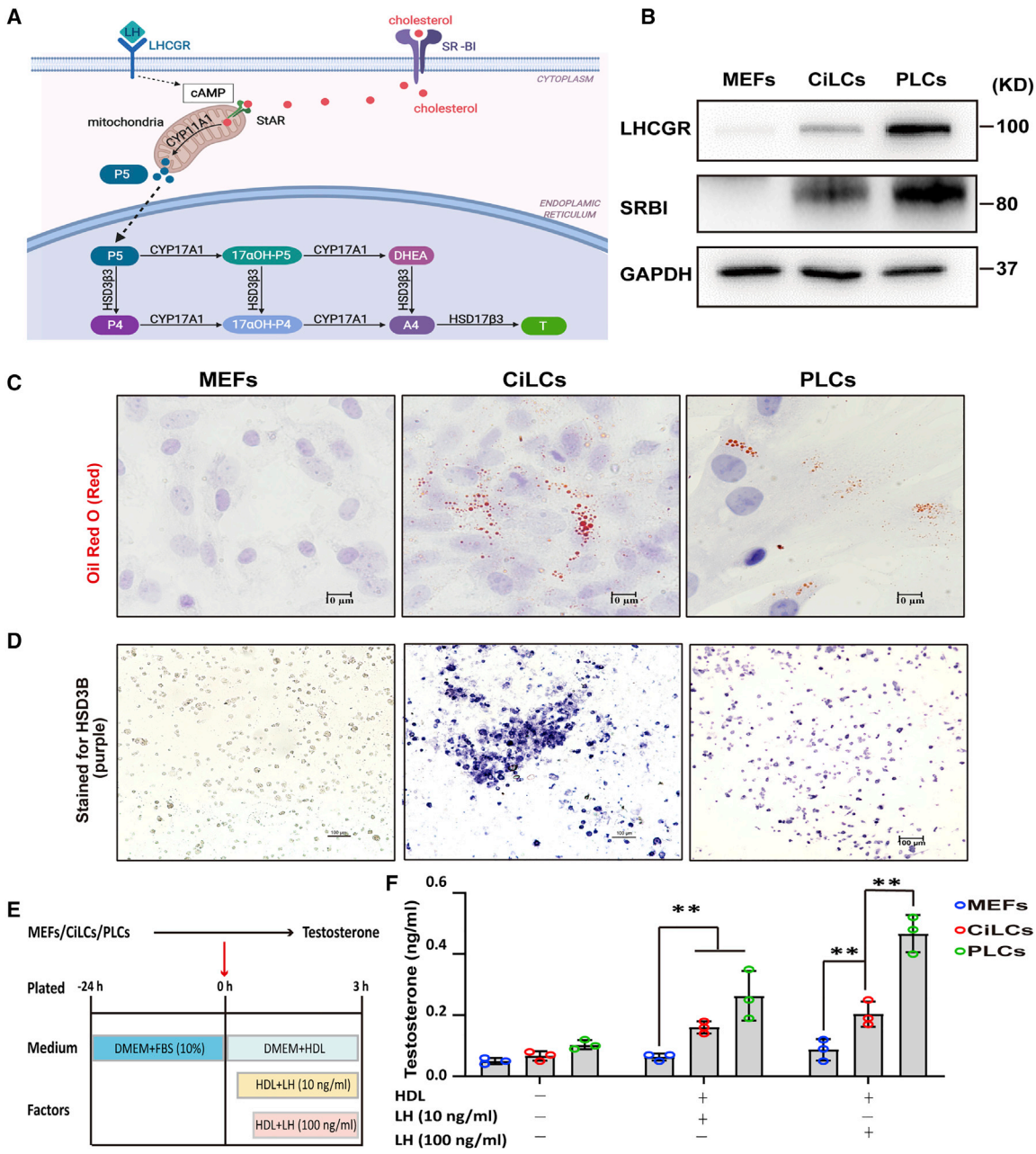


Figure 4. Testosterone Synthesis in Induced Leydig-like Cells

(A) Schematic illustration of the process by which cholesterol is converted to testosterone in Leydig cells.

(B) Representative western blotting for LHCGR and SRBI in MEFs, MEFs-4C, and PLCs.

(C) Cells stained with Oil red O (red). Scale bars, 10 μm.

(D) MEFs, MEFs-4C, and PLCs stained for HSD3B enzymatic activity (purple). Scale bars, 100 μm.

(E) Schematic of the experimental procedure.

(F) Testosterone production in cells incubated in medium containing LH (10 ng/ml and 100 ng/ml) and HDL for 3 h.

The data were obtained from three independent experiments and were presented as the mean ± SD values. **p < 0.01.

(Figure 7A). The immunofluorescence results revealed that human CiLCs (h-CiLCs) expressed HCG, NR5A1, STAR, CYP17A1, and HSD3B (Figure 7B). Similarly, induced h-CiLCs stopped proliferation after treatment with the small molecules (Figure 7C). In addition, h-CiLCs exhibited testosterone synthesis capability, whereas HPLFs did not

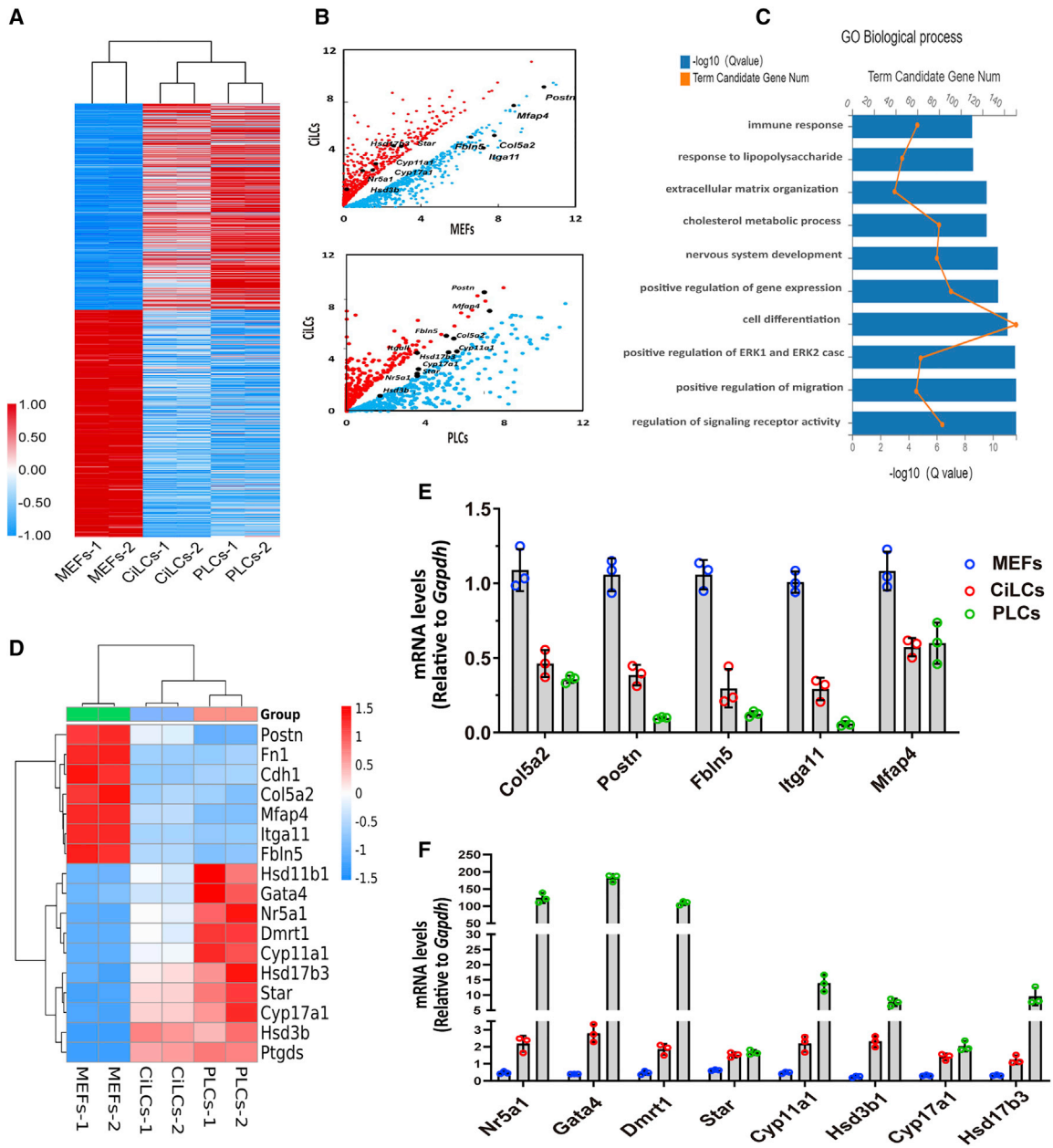


Figure 5. Transcriptome Analysis of CiLCs

(A) Heatmap of microarray data. Blue indicates decreased expression, and red indicates increased expression.

(B) Scatterplots showing that, in CiLCs, the majority of PLC markers were switched on, whereas fibroblast markers were silenced.

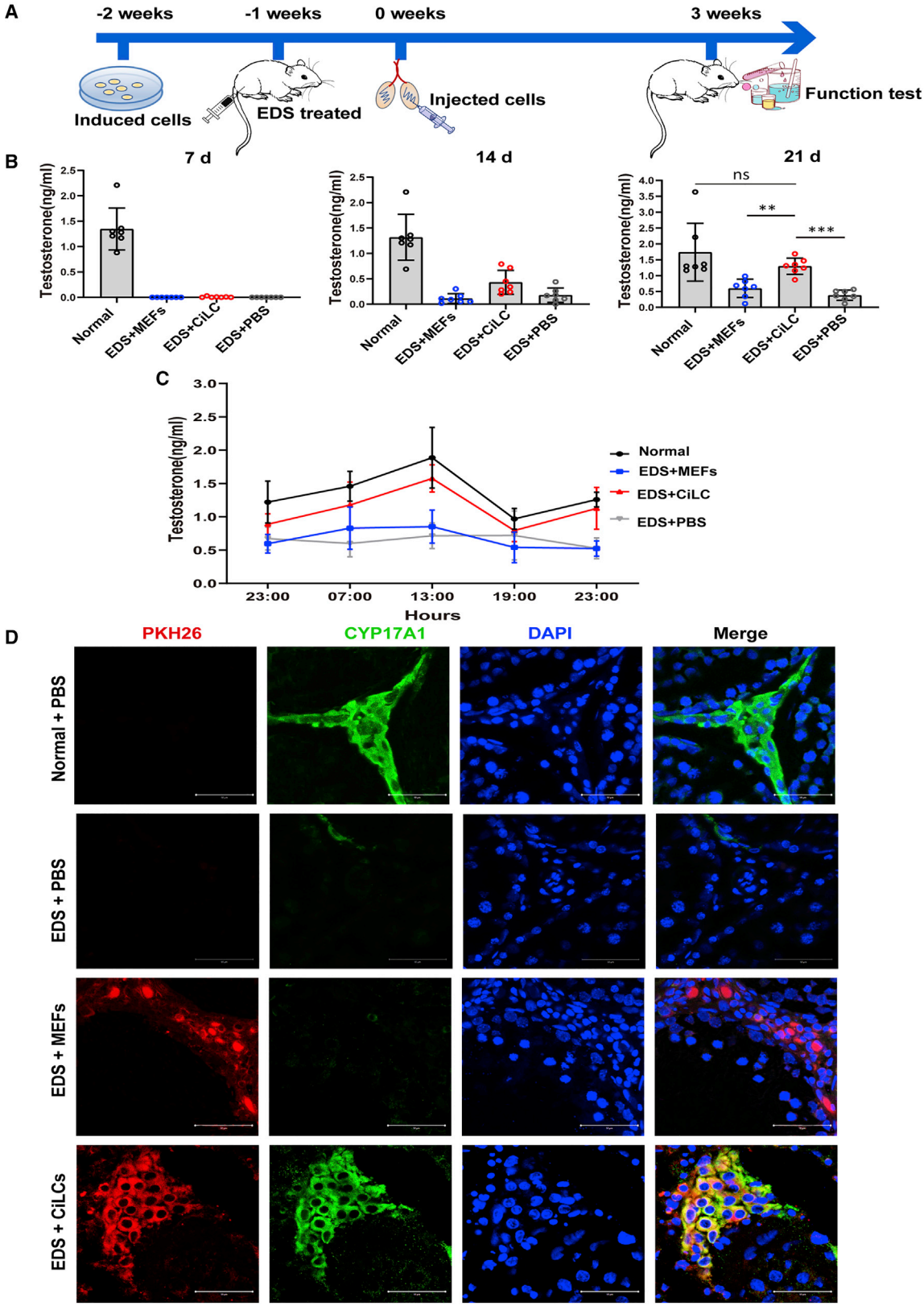
(C) Gene ontology (GO) term analyses of the top 10 GO categories in CiLCs compared with MEFs. The p values represent the Bonferroni-corrected EASE score.

(D) Heatmap image of gene expression of Leydig cell and fibroblast enriched genes in MEFs, CiLCs, and PLCs.

(E) Experimental verification of by qRT-PCR of fibroblast gene expression level in MEFs, CiLCs, and PLCs using qRT-PCR.

(F) Experimental verification of by qRT-PCR of Leydig gene expression level in MEFs, CiLCs, and PLCs using qRT-PCR. The data were obtained from three independent experiments and are presented as the mean ± SD.

CiLCs: MEFs were treated with the combination of four small molecules (FSK, 20α-hydroxycholesterol, LH, and SB431542) for 21 days.



(legend on next page)

produce testosterone (Figure 7D). Collectively, these results indicated that the combination of forskolin, 20α-hydroxycholesterol, LH, and SB431542 can induce the conversion of human adult fibroblasts into functional Leydig cells.

DISCUSSION

Nr5a1 has been considered a pioneer transcription factor of Leydig cell development and function (Ferraz-de-Souza et al., 2011; Hatano et al., 2016; Lalli et al., 2013; Martin and Tremblay, 2010). Previously, we demonstrated that fibroblasts can be converted into Leydig-like cells by *Dmrt1*, *Nr5a1*, and *Gata4* (Yang et al., 2017). Thereafter, several studies used *Nr5a1* overexpression to induce the conversion of fibroblast to steroidogenic cells (Li et al., 2019b; Zhou et al., 2019). However, in these studies, the viral vectors did not allow for control over protein expression and might induce oncogenesis upon integration into the host genome, raising safety concerns for the future applications of this technique. Accumulating studies have shown the potency and advantages of small-molecule-based approaches for cellular fate reprogramming, which could overcome the limitations of transcription factor-induced reprogramming, as this technique does not require genetic manipulation (Huang et al., 2018; Li et al., 2018; Nie et al., 2017; Tremblay, 2015).

In this study, we investigated whether treatment with small molecules could induce the conversion of fibroblasts into Leydig cells. To screen small molecules for reprogramming conversion into Leydig cells, we focused mainly on chemicals known to play important roles in the Leydig cell fate patterning, especially in epigenetic modulation and signaling pathways—for example, PDGFAA, activin B, DAPT, XAV939, forskolin, LH, 20α-hydroxycholesterol, IGF1, and 5-azacytidine (Chen et al., 2017; Li et al., 2016, 2017; Martin, 2016). *Nr5a1* expression and testosterone production are two basic characteristics of Leydig cells. And *Nr5a1* is considered an important contributor to Leydig cell fate transformation. Therefore, *Nr5a1* expression and testosterone production were used to determine whether these small molecules efficiently reprogrammed fibroblasts into Leydig-like cells. We found the combination of forskolin, 20α-hydroxycholesterol, LH, and SB431542 effectively induced fibroblast reprogramming into progenitor Leydig-like cells.

In our previous study, we demonstrated that forskolin induced mESC differentiating into Leydig-like cells *in vitro* (Yang et al., 2014). Forskolin activates cAMP/protein kinase A signaling and triggers the first step in testosterone synthesis (Golkowski et al., 2016; Shimizu-Albergine et al., 2012; Ye et al., 2017). In this study, removal of forskolin greatly decreased the *Nr5a1* expression and testosterone production; and removal of SB431542 nearly abolished the induction of *Nr5a1* expression. This lack of induction might occur because TGF-β negatively regulates steroidogenic gene expression (Wang et al., 2016), and TGF-β inhibits the differentiation of PLCs into the ALCs lineage in the testis (Li et al., 2017). In addition, the critical role of LH in steroidogenic function is well established (Riccetti et al., 2017). Without LH stimulation, steroidogenic enzyme activities in Leydig cells are reduced. In our study, forskolin, LH, and SB431542 were important in inducing *Nr5a1* expression. 20α-Hydroxycholesterol played a pivotal role in testosterone synthesis. Since 20α-hydroxycholesterol is a kind of steroid, to exclude the possibility that RIA detected 20α-hydroxycholesterol as testosterone, the testosterone levels in basal medium with and without 20α-hydroxycholesterol was measured by RIA. Testosterone could be detected in the positive control, whereas the testosterone levels in basal medium with and without 20α-hydroxycholesterol were very low (Figure S5).

In addition, MEFs were treated with forskolin, LH, SB431542, or 20α-hydroxycholesterol alone for 7 days, which resulted in mild upregulation of *Nr5a1* expression. However, no significant differences were observed compared with the control cells. The expression levels of *Star*, *Cyp17a1*, *Hsd3b*, and *Hsd17b3* were significantly increased in the presence of LH or SB431542 alone. In addition, 20α-hydroxycholesterol significantly increased the expression of *Hsd3b*. Therefore, the cooperative action of forskolin, LH, SB431542, and 20α-hydroxycholesterol

Figure 6. Intratesticular Transplantation of CiLCs Rescues Testosterone Deficiency in Male Rats

(A) Summary schematic showing the experimental procedure used for cell transplantation.

(B) Total serum testosterone levels on days 7, 14, and 21 after transplantation.

(C) Rats in the CiLC-transplanted group appeared to exhibit a diurnal rhythm of testosterone secretion. The serum testosterone concentration was measured at the indicated time points.

(D) Immunofluorescence staining showing the cells positive for PKH26 (red) and CYP17A1 (green) on day 21 after grafting. Nuclei were stained with DAPI (blue). Scale bars, 50 μm.

EDS + PBS: rats treated with EDS and injected with PBS; EDS + MEFs: rats treated with EDS and injected with MEFs; EDS + CiLCs: rats treated with EDS and injected with CiLCs.

All quantitative data are shown as the mean ± SD values, each group contained at least seven animals. $^{**}p < 0.01$ and $^{***}p < 0.001$. ns, not significant.

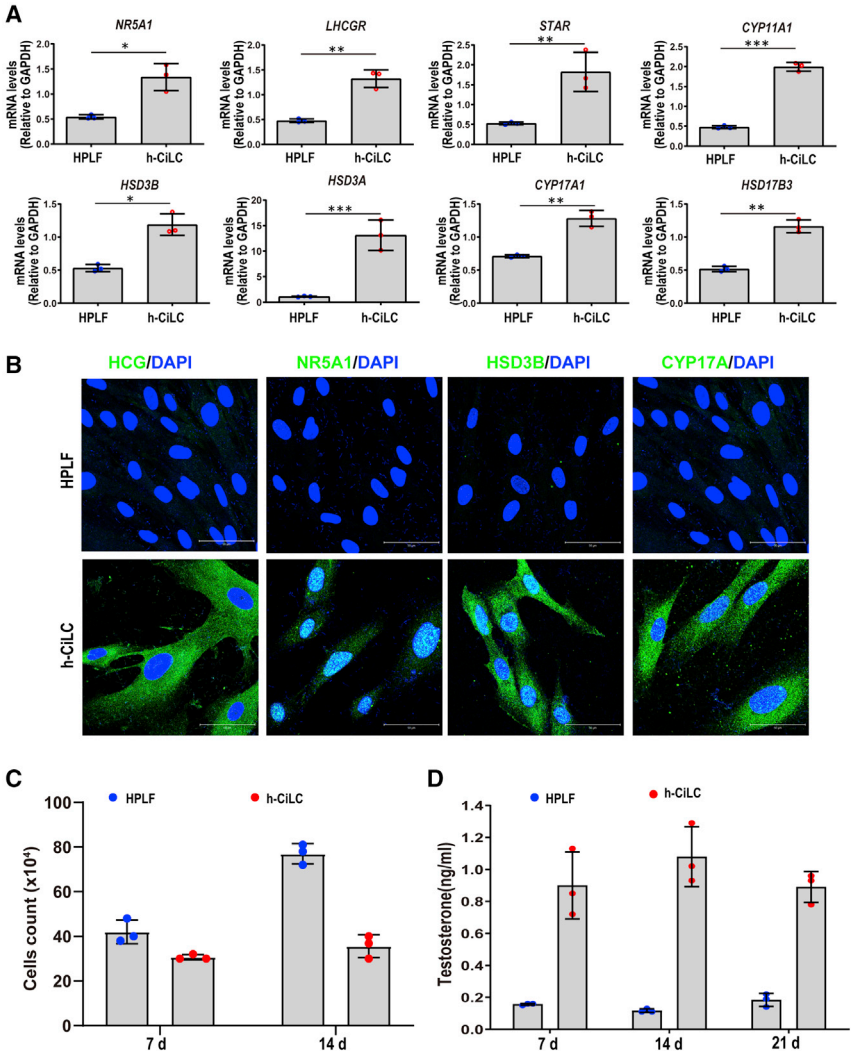


Figure 7. Small Molecules Facilitate the Conversion of Human Periodontal Ligament Fibroblasts into Leydig-like Cells (h-CiLCs)

(A) RT-PCR analysis of Leydig cell-specific markers on day 21 after treatment of human periodontal ligament fibroblasts (HPLFs) with the combination of FSK, 20α-hydroxycholesterol, LH, and SB431542.

(B) Immunostaining for the Leydig cell markers HCG, NR5A1, HSD3B, and CYP17A1 on day 21.

(C) Changes in the numbers of HPLFs and h-CiLCs.

(D) Analysis of testosterone production during culture.

The data were obtained from three independent experiments and were presented as the mean ± SD values. **p < 0.01 and ***p < 0.001.

is essential for promoting global Leydig-related gene expression. The combination of forskolin, 20α-hydroxycholesterol, LH, and SB431542 activated transcription factors to direct fibroblast reprogramming toward Leydig cell linage, significantly upregulated the expression of Leydig cell-related gene expression, and promoted production of testosterone by CiLCs.

CiLCs expressed steroidogenic genes, and acquired the ability to synthesize steroid hormones. Furthermore, the reprogramming process was direct and did not include an intermediate pluripotent state, and these cells ceased to proliferate, indicating that they lacked the ability to form high numbers of tumor-like spheroids. In addition, *Hsd17b3* and *Lhcgr* were upregulated in CiLCs. HSD17B3 specifically metabolizes androsterone to testosterone in steroidogenic pathway. The expression of LHCGR made these cells responsive to LH signaling, which is essential for the production of testosterone by CiLCs in a circadian rhythm. More importantly, CYP21B, which is involved in cortisol and aldosterone synthesis, was barely detectable, suggesting that these CiLCs were more similar to Leydig cells rather than to adrenal cells. Besides, this induction strategy is applicable to the induction of Leydig-like cells from HPLFs. In the future, we plan to study multiple fibroblast lines with variable genetic backgrounds.

The current standard of care for men with testosterone deficiency is lifelong exogenous testosterone therapy. However, exogenous testosterone therapy has several adverse effects, including infertility due to negative feedback effects on HPG axis (Samplaski and Nangia, 2015). The ideal treatment for testosterone deficiency should provide physiological testosterone levels, exhibit the appropriate circadian rhythm, and be modulated by the HPG axis. Leydig cell autograft is a promising therapeutic approach to increase serum testosterone while simultaneously preserving testicular function. However, isolating

and proliferating Leydig cells is difficult because of the lack of specific markers and Leydig cell population in adult testis, which limits application of Leydig cell transplantation. Our findings demonstrated that fibroblasts can be directly converted into CiLCs by pure chemical compounds. Although partial gene expression levels of CiLCs are still significantly lower than those in endogenous PLCs, the CiLCs are not really equivalent to the real PLCs. Importantly, the CiLCs indeed express steroidogenic genes and have a global gene expression profile resembling that of endogenous PLCs, and produce testosterone in a circadian rhythm. In addition, the expression level of Leydig cell-specific genes, *Star*, *Hsd3b*, and *Cyp17a1* in CiLCs showed no significant difference compared with PLCs. When CiLCs were transplanted into rats that were selectively depleted of endogenous ALCs, the transplanted cells could survive and function in the interstitium of the testis, resulting in the restoration of normal levels of serum testosterone while simultaneously preserving the HPG axis. This strategy would provide an alternative strategy allowing the treatment of male hypogonadism with an appropriate circadian rhythm.

In addition, although *Nr5a1*, *Dmrt1*, and *Gata4* expression in CiLCs was significantly lower than in PLCs after induction for 28 days, the expression of genes involved in steroidogenesis (*Star*, *Cyp11a1*, *Hsd3b*, and *Cyp17a1*) increased significantly, there was no significant difference between CiLCs and PLCs (Figure S5). However, CiLCs were difficult to maintain 28 days later *in vitro*. Further optimization of the *in vitro* culture system is needed to improve the viability of CiLCs. To determine whether CiLCs reverted back to the fibroblastic stage after prolonged cultivation. After 21 days of induction, the medium was replaced with basal medium without chemical compounds and CiLCs were cultured for another week. The Leydig cell-related genes were still detectable in CiLCs after the removal of small molecules (Figure S7) for 7 days, suggesting that CiLCs are stably converted.

In summary, this study demonstrated that a pure chemical cocktail is sufficient to reprogram fibroblasts into Leydig-like cells without transitioning through an intermediate state. In addition, this induction strategy was applicable to HPLFs. These induced cells were modulated by the HPG axis and produced testosterone in a diurnal rhythm. Unlike genetic manipulation, which may cause mutations, reprogramming fibroblasts into Leydig cells with small molecules likely represents an alternative source of Leydig cells independent of donor testis, which would be of great significance for both basic research and clinical application. In future, by optimizing the protocol, a safe and effective treatment can be developed for men with low testosterone who desire fertility preservation.

EXPERIMENTAL PROCEDURES

Preparation and Culture of MEFs

MEFs were prepared from embryos of wild-type C57BL/6 mice at embryonic days 12.5–13.5. The head and visceral tissues were carefully removed from the embryos, and the remaining tissues were minced with a pair of forceps and incubated in a solution containing 0.25% trypsin and 1 mM EDTA for 10–15 min at 37°C. After the trypsinization, MEF medium Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Natocor), 2 mM L-glutamine, and penicillin/streptomycin (all from Life Technologies)) were added and pipetted to dissociate the tissue fragments. Cells were collected by centrifugation (250 × g for 5 min) and resuspended in MEF medium. Cells were seeded in 90-mm dishes and cultured in an incubator at 37°C with 5% CO_2. After 4–6 h, nonadherent cells were discarded, and adherent MEFs were cultured to confluence, with replacement of the growth medium every 2 days. MEFs were used at passage 3.

Generation of CiLCs

Small molecules were dissolved according to the manufacturer's instructions and were then processed for use at the following final concentrations: PDGFAA (Proteintech), 10 ng/mL; activin B (Proteintech), 2 μM; DAPT (Selleck), 1 μM; XAV939 (Selleck), 20 μM; forskolin (Selleck), 10 ng/mL; LH (Sigma), 1 μg/mL; 20α-hydroxycholesterol (Sigma), 10 ng/mL; IGF1 (Proteintech) 50 ng/mL; 5-azacytidine (Selleck), 5 μM; LiCl (Sigma) 5 mM; SB431542 (Selleck) 1 μM, and T3 (Sigma), 10 mM. Passaged MEFs and HPLFs (purchased from ScienCell) were seeded at a density of 100,000 cells per well in six-well plates and cultured in medium (DMEM, 10% FBS) overnight. Then, the medium was replaced with induction medium (DMEM supplemented with 10% FBS) containing the following small molecules: PDGFAA, activin B, DAPT, XAV939, forskolin, LH, 20α-hydroxycholesterol, IGF1, 5-azacytidine, LiCl, SB431542, and T3. The induction medium containing small molecules was replaced every 2 days.

Isolation and Culture of Leydig Cells

PLCs were obtained from male mice at postnatal day 21. ALCs were obtained from adult male mice (8 weeks old). In brief, decapsulated testes were enzymatically dispersed with 0.25 mg/mL collagenase D (Roche) in DMEM for 10 min at 34°C. The dispersed cells were filtered through 100-μm nylon mesh, and the cells were separated by Percoll gradient (Sigma) centrifugation. After centrifugation at 23,500 × *g* for 45 min at 4°C, the fractions of PLCs with densities between 1.070 and 1.088 g/mL, and those of ALCs with densities of 1.070 g/mL and higher were collected. The isolated Leydig cells were directly seeded into six-well plates, and cultured at 34°C in DMEM containing 10% FBS. After 24 h days of culture, the cells were collected for PCR, sequencing, and other analyses. Meanwhile, cell culture supernatant was used for hormone quantification.

Animals

Sprague-Dawley rats (90 days old) were obtained from the Experimental Animal Center of Guangdong Province, China. All animals were housed under controlled temperature (24°C ± 1°C) and

relative humidity (50%–60%) conditions on a light:dark cycle of 12 h:12 h. Standard rodent chow and drinking water were freely accessible. All surgical and postoperative care procedures were approved by the Institutional Animal Care and Use Committee of Jinan University.

Cell Transplantation

Before transplantation, male Sprague-Dawley rats were administered a single intraperitoneal injection of EDS (75 mg/kg body weight) to eliminate their endogenous Leydig cells. Four days after EDS treatment, we evaluated whether CiLCs facilitated the recovery of Leydig cell dysfunction in rats. CiLCs (grown on 90-mm plates) and MEFs were gently dissociated, resuspended manually, and collected. Then, the cells were rinsed twice with PBS and stained with the red fluorescent dye PKH26 (Sigma) according to the manufacturer's instructions. The PKH26-labeled CiLCs (1×10^6 cells in 50 μL PBS) were injected into the testicular parenchyma of the recipient rats. As a control, EDS-treated rats were subjected to testicular injection of the same volume of PBS or MEFs. Sera from all animals were collected 0, 7, 14, and 21 days after transplantation and were examined by RIA. Testes from all animals were collected 21 days after transplantation for histological analysis.

Statistical Analyses

All experiments were repeated at least three times, and data are expressed as the mean ± one standard deviation around the mean (SD). Statistical analyses were performed with an unpaired Student's t test or a one-way ANOVA for comparisons among more than two groups. A two-tailed value of $p < 0.05$ was considered statistically significant.

ACCESSION NUMBERS

The microarray data have been deposited under accession number GEO: GSE145797.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.stemcr.2020.07.002.

AUTHOR CONTRIBUTIONS

Y.Y., C.Z., and Y.H. conceived and designed the experiments. Y.Y. and C.Z performed most of the experiments. T.Z., Q.L., J.L., and Z.L. provided support for animal experiments and western blotting. H.L., J.M., Z.C., Q.Z., L.Z., and Q.X. contributed technical assistance for animal experiments, the reagents, and analysis tools. Y.Y. and Y.H. analyzed the data and drafted the manuscript.

ACKNOWLEDGMENTS

This work received financial support from National Natural Science Foundation of China (81871150, 31801235), Natural Science Foundation of Guangdong Province (2018A030313473 and 2015A030310132), the Science and Technology Plan Project of Guangzhou (201803010044, 201704YG066, and 202002030168), the Major Scientific and Technological Special Project of Administration of Ocean and Fisheries of Guangdong Province (to Y.H., 2017), and China Postdoctoral Science Foundation funded project (2017M622914).

Received: February 25, 2020
Revised: July 1, 2020
Accepted: July 1, 2020
Published: July 30, 2020

REFERENCES

Abadilla, K.A., and Dobs, A. (2012). Topical testosterone supplementation for the treatment of male hypogonadism. Drugs *72*, 1591–1603.

Abney, T.O. (1999). The potential roles of estrogens in regulating Leydig cell development and function: a review. Steroids *64*, 610–617.

Allan, C.A., and McLachlan, R.I. (2004). Age-related changes in testosterone and the role of replacement therapy in older men. Clin. Endocrinol. (Oxf) *60*, 653–670.

Arora, H., Zuttion, M.S.S.R., Nahar, B., Lamb, D., Hare, J.M., and Ramasamy, R. (2019). Subcutaneous Leydig stem cell autograft: a promising strategy to increase serum testosterone. Stem Cell translational Med. *8*, 58–65.

Cao, N., Huang, Y., Zheng, J., Spencer, C.I., Zhang, Y., Fu, J.-D., Nie, B., Xie, M., Zhang, M., and Wang, H. (2016). Conversion of human fibroblasts into functional cardiomyocytes by small molecules. Science *352*, 1216–1220.

Chen, H., Ge, R.-S., and Zirkin, B. (2009). Leydig cells: from stem cells to aging. Mol. Cell Endocrinol *306*, 9–16.

Chen, H., Wang, Y., Ge, R., and Zirkin, B.R. (2017). Leydig cell stem cells: identification, proliferation and differentiation. Mol. Cell Endocrinol *445*, 65–73.

Crawford, P.A., Sadovsky, Y., and Milbrandt, J. (1997). Nuclear receptor steroidogenic factor 1 directs embryonic stem cells toward the steroidogenic lineage. Mol. Cell Biol *17*, 3997–4006.

Ferraz-de-Souza, B., Lin, L., and Achermann, J.C. (2011). Steroidogenic factor-1 (SF-1, NR5A1) and human disease. Mol. Cell Endocrinol *336*, 198–205.

Golkowski, M., Shimizu-Albergine, M., Suh, H.W., Beavo, J.A., and Ong, S.E. (2016). Studying mechanisms of cAMP and cyclic nucleotide phosphodiesterase signaling in Leydig cell function with phosphoproteomics. Cell Signal *28*, 764–778.

Gondo, S., Okabe, T., Tanaka, T., Morinaga, H., Nomura, M., Takayanagi, R., Nawata, H., and Yanase, T. (2008). Adipose tissue-derived and bone marrow-derived mesenchymal cells develop into different lineage of steroidogenic cells by forced expression of steroidogenic factor 1. Endocrinology *149*, 4717–4725.

Haider, S.G. (2004). Cell biology of Leydig cells in the testis. Int. Rev. Cytol. *233*, 181–241.

Hatano, M., Migita, T., Ohishi, T., Shima, Y., Ogawa, Y., Morohashi, K.I., Hasegawa, Y., and Shibasaki, F. (2016). SF-1 deficiency causes lipid accumulation in Leydig cells via suppression of STAR and CYP11A1. Endocrine *54*, 484–496.

Hou, P., Li, Y., Zhang, X., Liu, C., Guan, J., Li, H., Zhao, T., Ye, J., Yang, W., and Liu, K. (2013). Pluripotent stem cells induced from

mouse somatic cells by small-molecule compounds. Science *341*, 651–654.

Howell, S.J., Radford, J.A., Smets, E., and Shalet, S.M. (2000). Fatigue, sexual function and mood following treatment for haematological malignancy: the impact of mild Leydig cell dysfunction. Br. J. Cancer *82*, 789.

Huang, C., Tu, W., Fu, Y., Wang, J., and Xie, X. (2018). Chemical-induced cardiac reprogramming in vivo. Cell Res *28*, 686–689.

Huhtaniemi, I., and Forti, G. (2011). Male late-onset hypogonadism: pathogenesis, diagnosis and treatment. Nat. Rev. Urol. *8*, 335–344.

Lalli, E., Doghman, M., Latre de Late, P., El Wakil, A., and Mus-Veteau, I. (2013). Beyond steroidogenesis: novel target genes for SF-1 discovered by genomics. Mol. Cell Endocrinol *371*, 154–159.

LaVoie, H.A., and King, S.R. (2009). Transcriptional regulation of steroidogenic genes: STARD1, CYP11A1 and HSD3B. Exp. Biol. Med. *234*, 880–907.

Li, K., Zhu, S., Russ, H.A., Xu, S., Xu, T., Zhang, Y., Ma, T., Hebrok, M., and Ding, S. (2014). Small molecules facilitate the reprogramming of mouse fibroblasts into pancreatic lineages. Cell Stem Cell *14*, 228–236.

Li, L., Li, Y., Sottas, C., Culty, M., Fan, J., Hu, Y., Cheung, G., Chemes, H.E., and Papadopoulos, V. (2019a). Directing differentiation of human induced pluripotent stem cells toward androgen-producing Leydig cells rather than adrenal cells. Proc. Natl. Acad. Sci. U S A. *116*, 23274–23283.

Li, L., Li, Y., Sottas, C., Culty, M., Fan, J., Hu, Y., Cheung, G., Chemes, H.E., and Papadopoulos, V.J.P. (2019b). Directing differentiation of human induced pluripotent stem cells toward androgen-producing Leydig cells rather than adrenal cells*116*, 23274–23283.

Li, L., Wang, Y., Li, X., Liu, S., Wang, G., Lin, H., Zhu, Q., Guo, J., Chen, H., Ge, H.S., et al. (2017). Regulation of development of rat stem and progenitor Leydig cells by activin. Andrology *5*, 125–132.

Li, X., Wang, Z., Jiang, Z., Guo, J., Zhang, Y., Li, C., Chung, J., Folmer, J., Liu, J., Lian, Q., et al. (2016). Regulation of seminiferous tubule-associated stem Leydig cells in adult rat testes. Proc. Natl. Acad. Sci. U S A. *113*, 2666–2671.

Li, X., Xu, J., and Deng, H. (2018). Small molecule-induced cellular fate reprogramming: promising road leading to Rome. Curr. Opin. Genet. Dev. *52*, 29–35.

Li, X., Zuo, X., Jing, J., Ma, Y., Wang, J., Liu, D., Zhu, J., Du, X., Xiong, L., Du, Y., et al. (2015). Small-molecule-driven direct reprogramming of mouse fibroblasts into functional neurons. Cell Stem Cell *17*, 195–203.

Ma, X., Kong, L., and Zhu, S. (2017). Reprogramming cell fates by small molecules. Protein Cell *8*, 328–348.

Martin, L.J. (2016). Cell interactions and genetic regulation that contribute to testicular Leydig cell development and differentiation. Mol. Reprod. Dev. *83*, 470–487.

Martin, L.J., and Tremblay, J.J. (2010). Nuclear receptors in Leydig cell gene expression and function. Biol. Reprod. *83*, 3–14.

Nie, B., Nie, T., Hui, X., Gu, P., Mao, L., Li, K., Yuan, R., Zheng, J., Wang, H., Li, K., et al. (2017). Brown adipogenic reprogramming induced by a small molecule. Cell Rep *18*, 624–635.

Nieschlag, E., Swerdloff, R., Behre, H., Gooren, L., Kaufman, J.-M., Legros, J.-J., Lunenfeld, B., Morley, J.E., Schulman, C., and Wang, C. (2005). Investigation, treatment and monitoring of late-onset hypogonadism in males. The Aging Male *8*, 56–58.

Payne, A.H., and Hales, D.B. (2004). Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr. Rev. *25*, 947–970.

Peak, T.C., Haney, N.M., Wang, W., DeLay, K.J., and Hellstrom, W.J. (2016). Stem cell therapy for the treatment of Leydig cell dysfunction in primary hypogonadism. World J. Stem Cells *8*, 306–315.

Pfeil, E., and Dobs, A.S. (2008). Current and future testosterone delivery systems for treatment of the hypogonadal male. Expert Opin. Drug Deliv. *5*, 471–481.

Riccetti, L., De Pascali, F., Gilioli, L., Poti, F., Giva, L.B., Marino, M., Tagliavini, S., Trenti, T., Fanelli, F., Mezzullo, M., et al. (2017). Human LH and hCG stimulate differently the early signalling pathways but result in equal testosterone synthesis in mouse Leydig cells in vitro. Reprod. Biol. Endocrinol. *15*, 2.

Saez, J.M. (1994). Leydig cells: endocrine, paracrine, and autocrine regulation. Endocr. Rev. *15*, 574–626.

Samplaski, M.K., and Nangia, A.K. (2015). Adverse effects of common medications on male fertility. Nat. Rev. Urol. *12*, 401–413.

Shimizu-Albergine, M., Tsai, L.C., Patrucco, E., and Beavo, J.A. (2012). cAMP-specific phosphodiesterases 8A and 8B, essential regulators of Leydig cell steroidogenesis. Mol. Pharmacol. *81*, 556–566.

Sonoyama, T., Sone, M., Honda, K., Taura, D., Kojima, K., Inuzuka, M., Kanamoto, N., Tamura, N., and Nakao, K. (2012). Differentiation of human embryonic stem cells and human induced pluripotent stem cells into steroid-producing cells. Endocrinology *153*, 4336–4345.

Tremblay, J.J. (2015). Molecular regulation of steroidogenesis in endocrine Leydig cells. Steroids *103*, 3–10.

Wang, Y., Bilandzic, M., Ooi, G.T., Findlay, J.K., and Stenvers, K.L. (2016). Endogenous inhibins regulate steroidogenesis in mouse TM3 Leydig cells by altering SMAD2 signalling. Mol. Cell Endocrinol *436*, 68–77.

Yang, Y., Li, Z., Wu, X., Chen, H., Xu, W., Xiang, Q., Zhang, Q., Chen, J., Ge, R.S., Su, Z., et al. (2017). Direct reprogramming of mouse fibroblasts toward Leydig-like cells by defined factors. Stem Cell Reports *8*, 39–53.

Yang, Y., Su, Z., Xu, W., Luo, J., Liang, R., Xiang, Q., Zhang, Q., Ge, R.-s., and Huang, Y. (2014). Directed mouse embryonic stem cells into Leydig-like cells rescue testosterone-deficient male rats in vivo. Stem Cells Dev *24*, 459–470.

Yazawa, T., Mizutani, T., Yamada, K., Kawata, H., Sekiguchi, T., Yoshino, M., Kajitani, T., Shou, Z., Umezawa, A., and Miyamoto, K. (2006). Differentiation of adult stem cells derived from bone marrow stroma into Leydig or adrenocortical cells. Endocrinology *147*, 4104–4111.

Ye, L., Li, X., Li, L., Chen, H., and Ge, R.S. (2017). Insights into the development of the adult Leydig cell lineage from stem Leydig cells. Front Physiol. *8*, 430.

Zhou, J., Hou, Y., Zhang, Z., Xing, X., Zou, X., Zhong, L., Huang, H., Zhang, Z., and Sun, J. (2019). Conversion of human fibroblasts into functional Leydig-like cells by small molecules and a single factor. Biochem. Biophys. Res. Commun. *516*, 1–7.

Zirkin, B.R., and Chen, H. (2000). Regulation of Leydig cell steroidogenic function during aging. Biol. Reprod. *63*, 977–981.