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## Article

# Direct Reprogramming of Mouse Fibroblasts toward Leydig-like Cells by Defined Factors

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## SUMMARY

Leydig cells (LCs) play crucial roles in producing testosterone, and their dysfunction leads to male hypogonadism. LC transplantation is a promising alternative therapy for male hypogonadism. However, the source of LCs limits this strategy for clinical applications. Here, we report our success in reprogramming mice fibroblasts into LCs by expressing three transcriptional factors, *Dmrt1*, *Gata4*, and *Nr5a1*. The induced Leydig-like cells (iLCs) expressed steroidogenic genes, had a global gene expression profile similar to that of adult LCs, and acquired androgen synthesis capabilities. When iLCs were transplanted into rats or mice testes that were selectively depleted of endogenous LCs, the transplanted cells could survive and function in the interstitium of testis, resulting in the restoration of normal levels of serum testosterone. These findings demonstrate that the fibroblasts were able to be directly converted into iLCs by few defined factors, which may facilitate future applications in regenerative medicine.

## INTRODUCTION

Leydig cells (LCs), also known as interstitial cells of Leydig, distribute in clusters between the seminiferous tubules in the testicle and are the primary source of androgen in the male body (Chen et al., 2009; Zirkin and Chen, 2000). LCs are responsible for androgen production, and their steroidogenic activities are strictly regulated by the hypothalamus-pituitary-gonad axis. Testosterone, referred to as the male hormone, plays a critical role in maintaining muscle bulk, bone health, and sexual function (Midzak et al., 2009). Testosterone synthesis in adult LCs depends on the pulsatile secretion of luteinizing hormone (LH) by the pituitary grand (Chen et al., 2007). LH binds LH receptors (LHCGR) on the LC plasma membrane and then activates adenylate cyclase to increase intracellular cyclic AMP production, which further results in the rapid transport of cholesterol from the outer to the inner mitochondrial membrane, mediated by steroidogenic acute regulatory protein (StAR). Subsequently testosterone is synthesized from cholesterol through a multistep process known as steroidogenesis, catalyzed by the following four enzymes: the cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1), 3β-hydroxysteroid dehydrogenase (HSD3B), cytochrome P450 17α-hydroxylase (CYP17A1), and 17β-hydroxysteroid dehydrogenase 3 (HSD17B3). It is well documented that the expression levels of these proteins are closely related to the ability of the cells to produce testosterone (LaVoie and King, 2009).

Male hypogonadism is characterized by the low production of testosterone, which is associated with typical symptoms including mood disturbance, sexual dysfunction, decreased muscle mass and strength, and decreased bone mineral density (Howell et al., 2000). Hypogonadism affects approximately 30% of men aged 40–79 years and increases in prevalence with age (Allan and McLachlan, 2004). Male hypogonadism may be caused by multiple factors, but the most important factor is LC dysfunction (Young et al., 2000). Currently testosterone replacement therapy is applied to treat androgen-deficient males with primary LC failure and can achieve some alleviation of symptoms. However, the therapy is limited by the risks of side effects, such as reducing the rate of spermatogenesis and increasing cardiovascular and prostate complications (Bassil et al., 2009).

LC transplantation could be a better alternative in providing physiological patterns of hormone for a longer period of time (Pfeil and Dobs, 2008). However, the key issue that restricts the use of LC transplantation is the lack of seed cells because adult LCs do not proliferate, while immature LCs lose their characteristics during prolonged culture in vitro (Haider, 2004). Also, it is unrealistic to isolate LCs from the patient's testis. Several studies have attempted to generate LCs by differentiating stem cells of different sources: such as mesenchymal stem cells (Gondo et al., 2008; Yazawa et al., 2006), embryonic stem cells (ESCs) (Crawford et al., 1997; Jadhav and Jameson, 2011), and induced pluripotent stem cells (iPSCs)
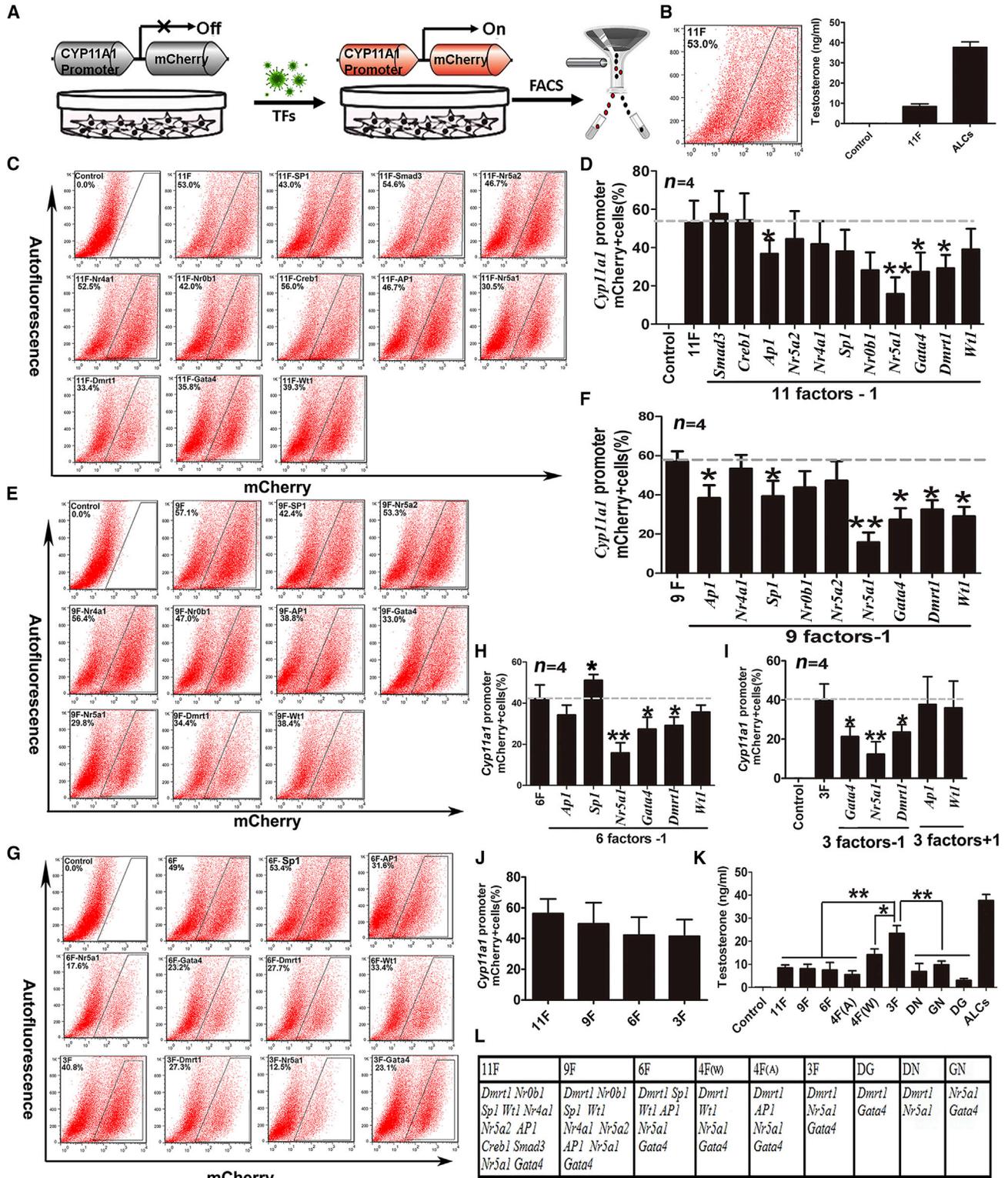
**Figure 1. Identifying a Minimal Cocktail of Transcription Factors to Reprogram MEFs toward Leydig-like Cells**

(A) Schematic representation of the strategy to test Leydig cell induction factors.

(B) Representative flow cytometry plot analyses (left) and testosterone production (right) of mCherry⁺ cells transfected with 11 factors.

(Sonoyama et al., 2012). Our previous study reported that ESC-derived Leydig-like cell has the potential to increase serum testosterone level (Yang et al., 2014). However, iPSC/ESC-derived cells may have ethical concerns and the risk of tumor occurrence. Furthermore, producing iPSCs/ESCs to treat individual patients would be time-consuming and costly (Vassena et al., 2015). Therefore, exploring an alternative source of LCs would be of great significance for both basic research and clinical applications.

Recently, direct lineage reprogramming has emerged as a promising approach for rapid and direct manipulation of cell fates, and also has lower teratoma risks associated with pluripotent stem cells (Rouaux and Arlotta, 2013; Vierbuchen and Wernig, 2011). Fibroblasts are frequently used for reprogramming studies as they can be obtained from multiple sources with less invasive procedures. They can be easily expanded in vitro, and provide a source of patient-specific cells to avoid possible immunological rejections. Attempts have been made to directly convert fibroblasts into a wide array of functional cell types (Efe et al., 2011; Marro et al., 2011; Selvaraj et al., 2010), such as cardiomyocytes, neurons, neural progenitors, blood progenitors, and hepatocyte-like cells. The generated cells were applied in autologous cell therapy (Xu et al., 2015). Here, we established an approach to directly convert fibroblasts into functional LCs (induced Leydig-like cells, iLCs) via the forced expression of defined transcriptional factors and also used the cells to increase serum testosterone levels of testosterone-deficient rodents.

## RESULTS

### Screening for Leydig Cell Reprogramming Factors

To quickly analyze the reprogram efficiency, we established an assay system in which the induction of Leydig-like cells from fibroblasts could be analyzed easily and quantitatively by a reporter-based fluorescence-activated cell sorting (FACS) procedure. To accomplish this, we infected mouse embryonic fibroblasts (MEFs) with a vector carrying the mCherry gene driven by *Cyp11a1* promoter (*Cyp11a1* promoter-mCherry-MEFs). *Cyp11a1* encodes the cholesterol side-chain cleavage enzyme (P450scc) that has been shown to be selectively expressed in gonadal steroidogenic cells (Hu et al., 1999). This tool allowed steroidogenic cells to be separated from fibroblasts by FACS, since mCherry could only be expressed in *Cyp11a1*-positive (*Cyp11a1*^{+}) cells (Figure 1A).

We used MatInspector software (Cartharius et al., 2005) to select potential LC reprogramming factors, based on the enrichment of their binding sites in the promoters of several known markers of LCs (Figure S1A). Among them, we screened 11 transcription factors (TFs), including *Dmrt1*, *Nr0b1*, *Sp1*, *Wt1*, *Nr4a1*, *Nr5a2*, *Ap1*, *Creb1*, *Smad3*, *Nr5a1*, and *Gata4*, which might have potential to regulate LC steroidogenic gene expression. Their coding regions were individually cloned into lentiviral vectors (Figures S1B and S1C) and transfected into *Cyp11a1* promoter-mCherry-MEFs with a same amount of lentivirus mixture expressing all 11 factors. The mCherry-positive (mCherry^{+}) cells were then quantitatively analyzed by FACS 4 days after the transfection (Figure 1A). In total, approximately 53.01% of cells became mCherry[+] and these mCherry^{+} cells were capable of synthesizing testosterone (8.46 ng/mL) (Figure 1B). These results indicated that the forced expression of 11 transcriptional factors could reprogram fibroblasts into the testosterone-producing cells that expressed a fluorescent marker driven by the promoter of an LC marker gene, *Cyp11a1*. Therefore, the system was feasible for screening essential TFs.

We next set out to narrow down the number of TFs required for the generation of induced LCs by removing individual factors serially from the pool of 11 factors (11F). Removing each of *Ap1*, *Dmrt1*, *Gata4*, or *Nr5a1* significantly decreased the reprogram efficiency while removing each of *Nr5a2*, *Nr4a1*, *Sp1*, *Nr0b1*, or *Wt1* had the potential to decrease the percentage of mCherry^{+} cells compared with the 11F group, since the differences did not reach

(C, E, and G) Representative flow cytometry plot for analyses of mCherry^{+} cells 4 days after infection of MEFs with lentiviruses expressing the indicated combinations of factors, and the effects of removing individual factors from the 11F (C), 9F (E), 6F (G), or 3F (G) pool on mCherry^{+} cells. Cells infected with empty vector lentivirus were used a control. The numbers in each plot indicate the percentage of mCherry^{+} cells.

(D, F, H, and I) Summary of flow cytometry analyses. Dashed lines indicate the percentage of mCherry^{+} cells induced by 11F (D), 9F (F), 6F (H), and 3F (I). *p < 0.05 and **p < 0.01 compared with dashed lines.

(J) Summary of results for mCherry^{+} cells after 11F, 9F, 6F, or 3F infection.

(K) Testosterone production in MEFs transduced with the different combinations of factors after 1 week of transfection. *p < 0.05 and **p < 0.01.

(L) Summary of each pool and abbreviations. 11F, 11 factors; 9F, nine factors; 6F, six factors; 3F, three factors; D, *Dmrt1*; G, *Gata4*; N, *Nr5a1*; ALCs, adult LCs.

All quantitative data are presented as mean ± SD (n = 4 independent experiments).

See also Figure S1.

statistical significance (p > 0.05). Removing *Smad3* and *Creb1*, however, did not change the proportion of mCherry+ cells, which suggested they were dispensable in the reprogram (Figures 1C and 1D). The 11F minus *Smad3* and *Creb1* was therefore named the nine-factor pools (9F). The 9F were retained to conduct the next round of screening. Lacking *Nr5a2*, *Nr4a1*, and *Nr0b1* in the 9F did not significantly change the proportion of mCherry+ cells compared with that of 9F control; therefore, these three genes were determined to be nonessential (Figures 1E and 1F). Subsequently, we conducted a third round of screening by withdrawing single factors from the six-factor pools (6F) remaining. The results indicated that removing *Sp1* significantly increased the proportion of mCherry+ cells (Figures 1G and 1H), which suggests that it is nonessential in this setting. Moreover, removing each of *Ap1* or *Wt1* could slightly decrease the average efficiency, but the effects were insignificant. Consistent with rounds 1 and 2, removing each of *Dmrt1*, *Gata4*, or *Nr5a1* from 6F significantly decreased the reprogram efficiency from 40% to 27.7%, 23.2%, and 17.6%, respectively (Figures 1G and 1H), suggesting they are essential in reprogramming. Adding *Ap1* or *Wt1* back to the 3F (*Dmrt1*, *Gata4* and *Nr5a1*) did not increase the number of mCherry+ cells further, confirming that they are also not essential in reprogramming (Figure 1I). Interestingly, although the *Cyp11a1*+ cells did not change significantly from 11F, 9F, and 6F to 3F (Figure 1J), the amount of testosterone produced by the reprogrammed cells with the three optimal factors (*Dmrt1*, *Gata4*, and *Nr5a1*) reached 23.4 ng/mL, which was 3.1-, 2.87-, and 2.76-fold higher than that of the cells induced by 6F, 9F, and 11F, respectively (Figure 1K), suggesting that factors outside the 3F may have negative effects on the expressions of other steroidogenic genes that are also required for testosterone production. Although adding back of *Ap1* or *Wt1* to 3F did not affect *Cyp11a1*+ cell numbers (Figure 1I), they indeed, however, inhibited testosterone production significantly (Figure 1K), suggesting that these two factors may have negative effects on the expressions of other steroidogenic genes other than *Cyp11a1*. Therefore, *Dmrt1*, *Gata4*, and *Nr5a1* represent the minimal and optimal set of TFs (DGN) to convert fibroblasts into steroidogenic Leydig-like cells.

### Mechanism by which *Dmrt1, Gata4,* or *Nr5a1* Converts Mouse Embryonic Fibroblasts into Leydig-like Cells

To elucidate the mechanism by which *Dmrt1*, *Gata4*, or *Nr5a1* converts fibroblasts into iLCs, we first transfected individual factors into MEFs and measured the expression levels of several steroidogenic marker genes. We found that the mRNA expression of *Star*, *Cyp11a1*, *Hsd3b*, *Cyp17a1*, *Hsd17b3*, and *Lhcgr* were all upregulated significantly in MEFs induced by *Nr5a1* compared with those of mock MEFs. In contrast, *Dmrt1* and *Gata4* had little effect on steroidogenic genes except for *Lhcgr* (Figure 2A). These observations were also confirmed by western blotting analysis (Figure 2B).

As DNA methylation is one of the major epigenetic factors that regulates gene expression during cell-fate commitment and reprogramming, the DNA methylation levels of MEFs induced by single factor or the combinations were assessed. The results demonstrated that *Dmrt1*, *Gata4*, or *Nr5a1* alone could decrease the global DNA methylation levels of MEFs, and the combination of the three could significantly downregulate the methylation level further from 4.05% to 1.26% (Figure 2C). Analysis of the promoter-specific methylations on individual genes after the reprogramming indicated that the methylations of steroidogenic genes may also be reduced. For example, the percentage of methylated CpGs in the medium CpG density regions of *Cyp17a1* and *Lhcgr* promoters was 79.3% and 41.9% in MEFs and that in the 10-day MEFs-DGN was 62.5% and 24.2% (p < 0.0001) (Figure 2D), suggesting that methylated *Cyp17a1* and *Lhcgr* promoters were partly demethylated after reprogramming.

Subsequently, we used LH to stimulate the Leydig-like cells induced by each of *Dmrt1*, *Gata4*, *Nr5a1*, or the combination (DGN) and compared their testosterone productions. Cells induced by each of *Dmrt1*, *Gata4*, *Nr5a1*, or DGN produced testosterone at levels of 0.21, 0.19, 12.89, and 17.84 ng/mL, respectively (Figure 2E). These results, in combination with the global DNA methylation studies, suggested that *Dmrt1*, *Gata4*, and *Nr5a1* may cooperate with each other in modifying DNA methylations, upregulating the expression of steroidogenic enzymes, and promoting LH-mediated testosterone synthesis.

### Induced Leydig-like Cells Exhibit Adult Leydig Cell Characterizations

After transduction by the DGN factors, the cells were cultured and then sorted by FACS at day 4 after transfection (Figures 3A and 3B). The sorted cells were spindle shaped (Figure S2A) and continued to develop and mature in LC medium. The expression levels of steroidogenic genes were evaluated by RT-PCR at day 10 after transfection. The results showed that these examined genes were switched on in reprogrammed cells (Figure 3C). Staining of HSD3B enzymatic activity indicated that all iLCs were HSD3B positive (deep purple color), which confirmed that FACS-sorted cells also expressed HSD3B enzyme (Figure 3D).

We next examined the expression of key steroidogenic proteins by immunocytochemistry and western blot. The results showed that all LC steroidogenic enzymes in the testosterone synthetic pathway in LCs, including StAR,
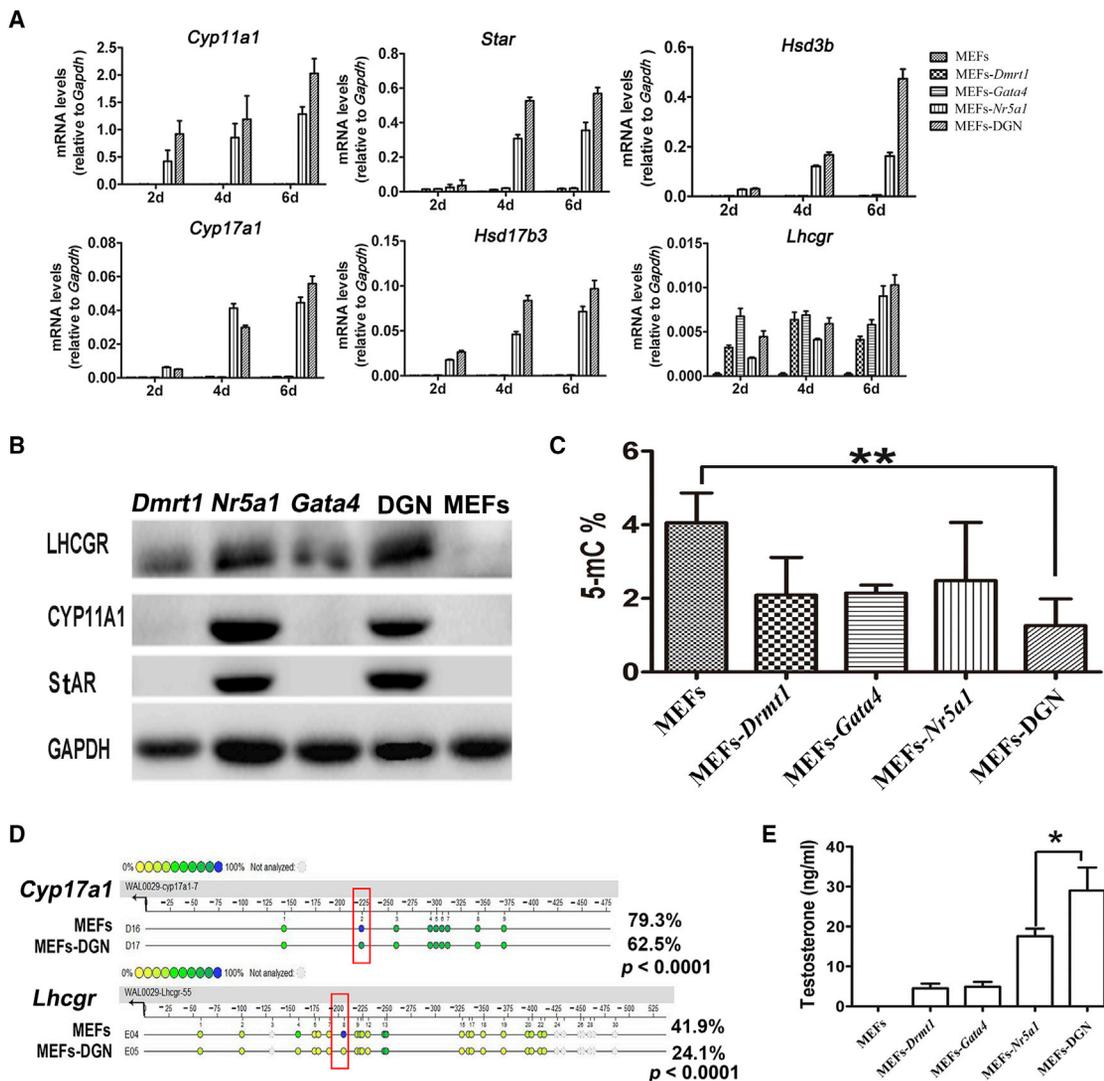
**Figure 2. Conversion of MEFs into Leydig-like Cells by *Dmrt1*, *Gata4*, and *Nr5a1***

(A) Time course of mRNA expression after infection. The copy number of the mRNA of each gene was normalized with *Gapdh*.

(B) Representative western blotting for the protein expression at day 10 after transfection.

(C) The global DNA methylation status was assessed by percentage of DNA 5-methylcytosine (5-mC%) at day 10 after transfection.

(D) Bisulfite sequencing analysis of *Cyp17a1* and *Lhcgr* promoter methylation status. Methylation levels of *Cyp17a1* and *Lhcgr* promoter from 0 to −500 bp were analyzed in MEFs and MEFs-DGN at day 10 after transfection. Yellow circles indicate unmethylated CpG dinucleotides; blue circles indicate methylated CpGs. Green circles indicate 50% methylated CpGs. Red boxed areas indicate the different loci of methylated CpGs.

(E) Testosterone production in MEFs-*Dmrt1*, MEFs-*Gata4*, MEFs-*Nr5a1*, and MEFs-DGN. At day 10 the cell medium was changed and cells were treated with LH for 24 hr, after which the hormones secreted into the medium were measured.

All quantitative data were obtained from three independent experiments and are presented as mean ± SD; **$p < 0.01$ and *$p < 0.05$.

CYP11A1, HSD3B, CYP17A1, and HSD17B3 as well as LHCGR, were expressed at day 10 after transfection (Figures 3E and 3F). Mock-induced MEFs did not express these steroidogenic enzymes (Figure S2B). Expressions of other testicular cell marker genes, such as *Dhh*, *Sox9*, *Ptgds*, *Shbg*, *Gdnf*, and *Erbb4*, were also analyzed in MEFs, iLCs, adult LCs (ALCs), and primary Sertoli cells (Figure S3). The data confirmed that the MEFs induced by DGN combination reprogrammed the cells directly toward the LC lineage. Furthermore, the concentration of testosterone in the supernatant of culture medium indicated that the iLCs began to secrete testosterone by day 4 and the yield
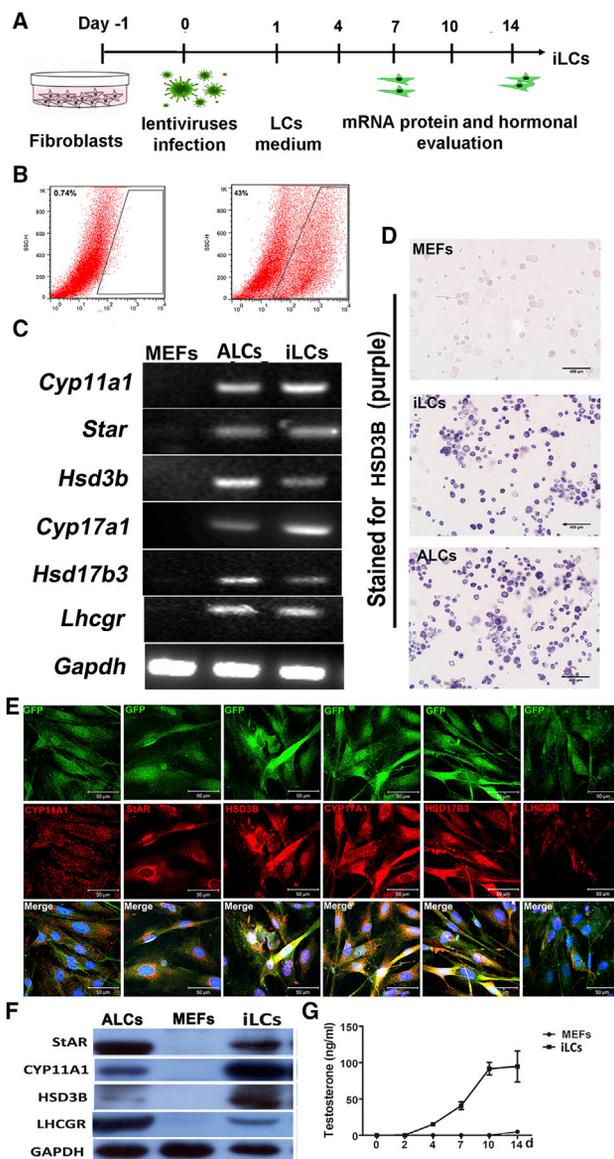
**Figure 3. Characteristics of Induced Leydig-like Cells**

(A) Schema of the experimental procedures.

(B) Representative FACS plots of MEFs at 4 days after infection with DGN.

(C) RT-PCR results for the detection of LC steroidogenic gene expression in iLCs, ALCs, and MEFs at 10 days after infection with DGN.

(D) MEFs, iLCs, and ALCs stained for HSD3B enzyme (purple). Scale bars, 400 μm.

(E) Immunofluorescent staining confirmed that the expression of the Leydig steroidogenic markers at day 10 after infection. Nuclei were stained with DAPI (blue). Scale bars, 50 μm.

(F) Representative western blotting for protein expression of Leydig steroidogenic markers in iLCs at day 10 after infection.

(G) Analysis of testosterone production during culture. All quantitative data were obtained from three independent experiments and are presented as mean ± SD.

See also Figures S2–S4.

gradually increased, peaking at day 10 after transduction (Figure 3G). This result indicated that these reprogramming cells gained the ability to produce testosterone rather quickly after transduction, although the optimal productions need time to develop/mature.

**Transcriptome Analysis**

To understand the global transcriptional changes, we investigated the gene expression profiles of MEFs transfected with null vector, iLCs, and ALCs. Hierarchical clustering analysis demonstrated that the transcriptome profiles of the iLCs more closely resembled those of adult LCs than MEFs. Among the 26,423 genes analyzed, 3,008 genes were differentially expressed by more than 2-fold between MEFs and iLCs, with 1,359 genes upregulated and 1,649 genes downregulated in the iLCs (Figure 4A). Scatterplots showed that the genes upregulated in the iLCs were significantly enriched in LCs compared with MEFs; conversely, the downregulated genes were enriched in fibroblasts (Figure 4B). Gene ontology (GO) analysis suggested that the genes upregulated in the iLCs were enriched in DNA replication, homologous recombination, and steroid biosynthesis, which is related to the steroidogenic function of LCs (Figure 4C).

The heatmap and qRT-PCR further revealed that the expression of steroidogenic genes increased significantly, including *Cyp11a1*, *Star*, *Hsd3b*, *Cyp17a1*, *Hsd17b3*, and *Lhcgr*. In contrast, expression levels of the fibroblast markers *Thy1*, *Col5a2*, *Col6a2*, *S100a4*, *Postn*, *Tnc*, and *Slit2* were significantly downregulated by at least 5-fold (Figures 4D–4F). These findings indicated that treatment with DGN did not merely activate the expression of a few steroidogenic specific genes of LCs, but also led to a loss of fibroblast molecular hallmarks. Taken together, these results suggest successful cell reprogramming.

**DGN Bypassed Cell-Cycle Dependency during MEF Reprogramming into iLCs**

We next asked whether iLCs originated from dedifferentiating fibroblasts or arose from direct reprogramming of the cells. We examined the reprogramming kinetics by analyzing the expression of LC-specific genes using qRT-PCR during 14 days of culture. Androgen synthesis-related genes were upregulated from 2 to 10 days, and the iLCs subsequently exhibited persistent, stable expression of these genes for 14 days (Figure 5A). We subsequently examined whether the conversion to iLCs involves passing through a defined pluripotent stage. As expected, no expression of the pluripotency markers *Oct4*, *Sox2*, or *Nanog* was detected at day 2 after transfection (Figure 5B). In addition, these iLCs did not show any significant changes in cell proliferation (Figure 5C). Reprogramming of the cells resulted in
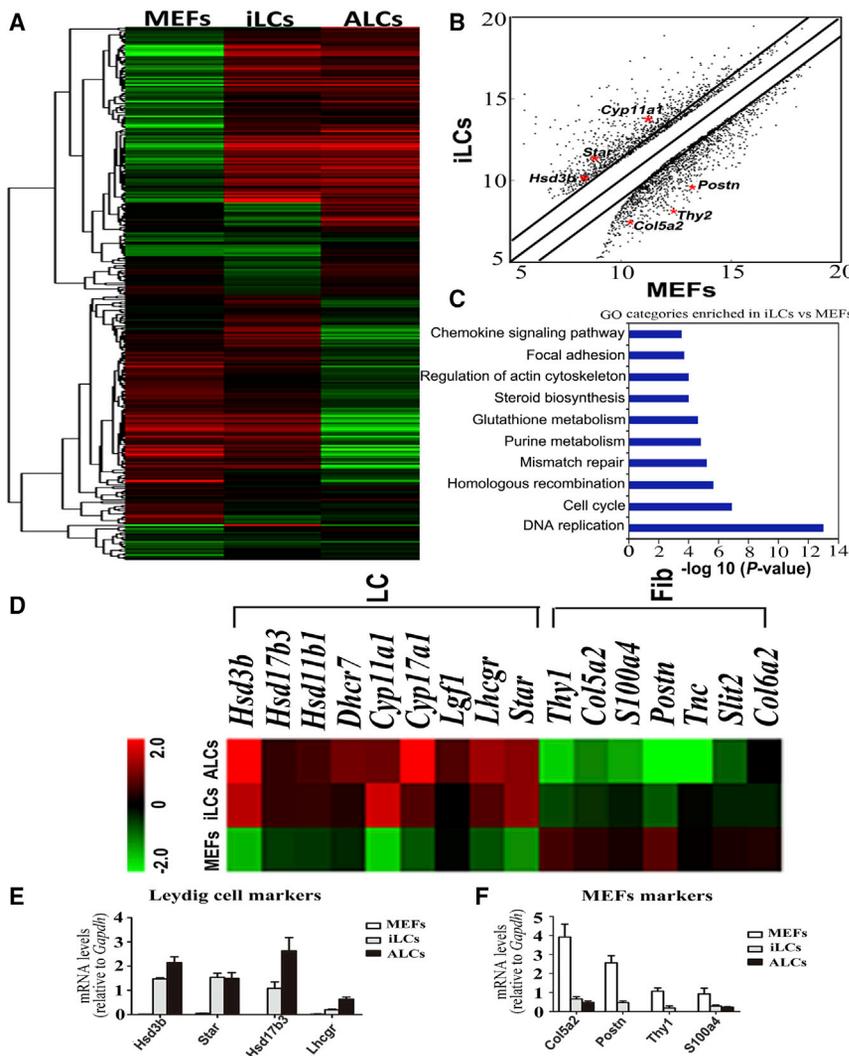
**Figure 4. Gene Expression Profile**

(A) Heatmap image of microarray data. Green indicates decreased expression and red indicates increased expression.

(B) Scatterplots show that in iLCs the majority of ALCs markers were switched on, whereas fibroblasts markers were silenced.

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

(D) Heatmap image of gene expression of Leydig cell (LC) and fibroblast (Fib) enriched genes in MEFs, iLCs, and ALCs. The scale is −2 to +2 in $\log_{10}$.

(E and F) mRNA expression levels in MEFs, iLCs, and ALCs were determined by qRT-PCR. All quantitative data were obtained from three independent experiments and are presented as mean ± SD.

loss of cell-dividing activity from day 2, which is consistent with loss of the expression of cell-cycle-related genes, including cyclin A (*Ccna2*), cyclin B1 (*Ccnb1*), cyclin B2 (*Ccnb2*), cyclin E1 (*Ccne1*), and cyclin E2 (*Ccne2*) (Figure 5D). The cell cycle was also analyzed using propidium iodide staining at 48 hr after transfection. As showed in Figures 5E and 5F, all of the iLCs entered into the G0/G1 phase at day 2 after transfection. More detailed analysis by 5-bromodeoxyuridine (BrdU) incorporation showed that only 0.4% of the cells were positive for BrdU incorporation, while MEFs scored 18.5% (Figures 5G and 5H), suggesting that iLCs did not pass through a mitotic cell state.

### Reprogramming Mice Adult Fibroblasts into iLCs

To further examine whether DGN can also convert fibroblasts from adult animals, we applied the same experimental procedures on adult mouse tail-tip dermal fibroblasts (TTFs). Like the MEFs, the TTFs transfected with

DGN also expressed essential proteins for testosterone synthesis (Figure S4A). Interestingly, the expression levels of the steroidogenic genes were not consistent between the two induced cells, with *Star*, *Hsd3b*, and *Cyp17a1* being significantly lower and *Lhcgr* being significantly higher in TTF-induced cells, with *Cyp11a1* and *Hsd17b3* being the same (Figure S4B). Meanwhile the fibroblast genes, such as *Col5a2*, *Thy1*, *Vimn*, *Postn*, and *S1004a*, were also downregulated significantly in TTFs-DGN when compared with the mock-induced TTFs (Figure S4C). Similarly, the cells stopped proliferation after the reprogram (Figure S4D). Moreover, TTFs-DGN were also able to synthesize and secrete testosterone. However, the mean testosterone yield was 19.8 ng/mL, which was lower than that of MEFs-DGN (28.5 ng/mL) (Figure S4E). Collectively, these results indicate that the fibroblasts from both fetal and adult sources (MEFs or TTFs) could be reprogrammed to functional Leydig-like cells by DGN.
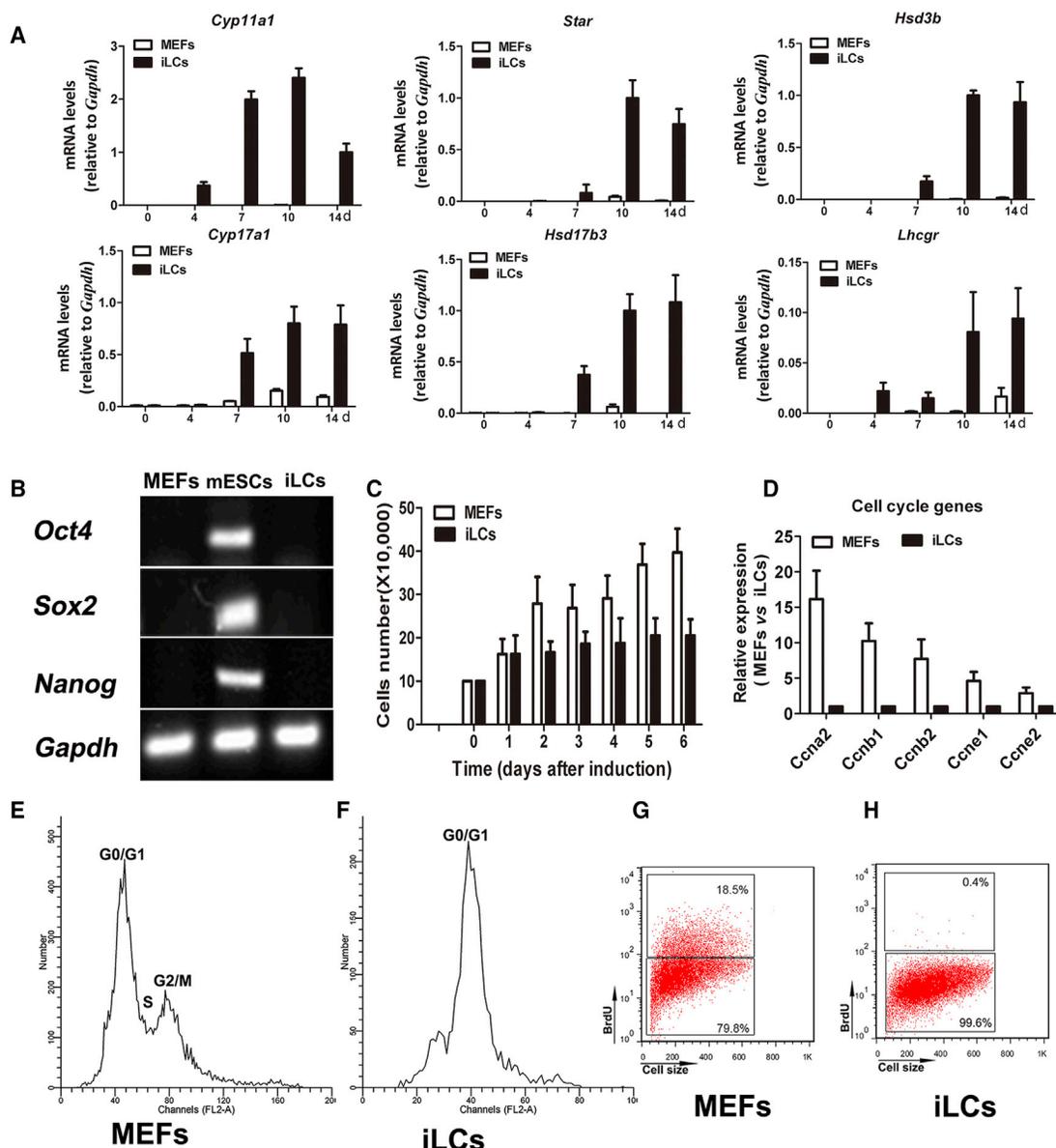
**Figure 5. The Generation of iLCs Is Not Mediated through Mitotic Cells**

(A) Time course of mRNA expression in iLCs and MEFs.

(B) RT-PCR analysis of an ESC-specific marker at day 2 after infection.

(C) Cell proliferation curves. $1 \times 10^4$ MEFs or iLCs were seeded per well. Three wells were harvested every 24 hr to count the number of cells.

(D) Phalanx array expression profiles of five cell-cycle genes at day 10 after infection.

(E and F) Propidium iodide staining analysis of MEFs and iLCs at day 2 after infection.

(G and H) BrdU incorporation in MEFs and iLCs at day 2 after infection.

All quantitative data were obtained from three independent experiments and are presented as mean ± SD.

## Intratesticular Transplantation of iLCs and Restoration of Serum Testosterone Level

To determine whether iLCs can survive and function in vivo, we transplanted these cells into the testis of rats treated with ethylene dimethanesulfonate (EDS) (an androgen deficiency model) (Figure 6A). Consistent with our earlier experiment results (Yang et al., 2014), H&E staining showed that EDS could specifically eliminate the LCs in adult rat testes (Figure S5A) and led to a dramatic decline in serum testosterone levels 7 days after EDS treatment (Figure 6B). In the EDS-treated model animals, serum testosterone began to increase by 14 days, and by 28 days

serum testosterone was recovered to about 25% of control. Implantation of MEF cells did not change the recovery process significantly. However, implantation of iLCs dramatically improved the recovery process, and by 28 days the serum testosterone concentration reached the normal control level (Figure 6C).

Testosterone plays an important role in maintaining normal body and reproductive organ weights. At 14 days after exposure to EDS, the body and testes weights of animals were dramatically decreased. By day 28 after transplantation, the body and testes weights significantly increased in the iLC-treated group compared with the model and rats receiving MEFs (Figure 6D).

As MEFs and iLCs expressed GFP, we used fluorescent tracer techniques to investigate whether iLCs possess the characteristic potential of migrating into interstitial regions of the testis. Meanwhile, histological evaluations were performed on testes sections at 14 and 28 days after transplantation to confirm whether the GFP-positive cells expressed LC steroidogenic markers. The results showed that iLCs were localized exclusively in the interstitium of the testes and expressed the LC steroidogenic markers CYP11A1 and CYP17A1 (Figures S5B and S5C). Importantly, these iLCs could survive at least 28 days in vivo (Figure 6E), which demonstrated that they successfully integrated into the host niche. Although the animals that received MEF transplantation also showed that MEFs were capable of localizing in the interstitium of the testes, these cells did not express the steroidogenic enzymes CYP11A1 and CYP17A1.

To further confirm the function of iLCs, we also transplanted iLCs into EDS-treated mice testes, a partial androgen deficiency model, as previously described (Jiang et al., 2014). Adult mice were treated with EDS (400 mg/kg) and then iLCs were injected into the parenchyma at the cranial pole of the testis 1 day later (Figure 7A). In the EDS-treated control group, the expression of LC marker genes *Hsd3b* and *Hsd17b3* as well as the concentration of testosterone were dramatically reduced by 81.5% 6 days after EDS treatment. Consistent with the observations in rats, the transplanted iLCs were also localized exclusively in the interstitium of the testis and expressed the LC-specific markers CYP11A1 and CYP17A1 at day 12 (Figure 7B). Moreover, they exhibited significantly increased mRNA expression levels of *Hsd3b* and *Hsd17b3*, while serum testosterone concentrations and expression levels of StAR were significantly increased and restored to normal control levels 12 days after iLC transplantation (Figures 7C–7G), which is a much faster rate than is seen in rats. The difference could be due to the fact that EDS treatment cannot completely eliminate endogenous LCs. However, the results demonstrated that, just as in rats, the transplanted iLCs in mice have acquired key properties

of ALCs as they are capable of colonizing the interstitium and have the potential to restore the serum testosterone level when transplanted into the testes where local LCs are partially depleted.

## DISCUSSION

In this study, we first identified a cocktail of transcriptional factors (*Dmrt1*, *Gata4*, and *Nr5a1*) from 11 candidates and were able to convert fibroblasts into iLCs directly by the three factors. Remarkably, the steroidogenic genes appeared in reprogrammed cells, iLCs, at day 2, in contrast to cells differentiated from iPSCs, which typically exhibit this behavior from days 10 to 20 (Takahashi et al., 2007b). In addition, these iLCs quickly exited the cell cycle as early as day 2 after transduction, which suggested that the transformation of iLCs from fibroblasts was direct and bypassed a proliferative intermediate, and did not depend on the generation of iPSCs. In comparison with previous methods of staged differentiation to derive tissue-specific somatic cells from ESCs (Yang et al., 2014) or iPSCs (Sonoyama et al., 2012), the direct reprogramming methods described here could eliminate the possibility of teratoma formation and are devoid of ethical and legal concerns.

It is well known that *Nr5a1* is an essential factor for LC development and survival (Shima et al., 2011). Overexpression of *Nr5a1* has been shown to be capable of promoting ESCs into Leydig-like cells (Karpova et al., 2015). In this study, *Nr5a1* also seemed to be sufficient, by itself, to induce Leydig-like features, but the additional expression of *Dmrt1* and *Gata4* could significantly increase the efficiency of iLCs and testosterone production. Moreover, for the cocktail containing the three factors *Dmrt1*, *Gata4*, and *Nr5a1*, removal of any one could reduce the induction efficiency and testosterone production dramatically. These results suggested that *Dmrt1*, *Gata4*, and *Nr5a1* showed a positive synergistic regulation of LC steroidogenic gene expression. *Dmrt1* is essential in triggering testis-determining process in fetal development in mammals, maintaining mammalian testis determination, and competing regulatory networks to maintain testis identity long after the fetal choice between male and female is made (Ieda et al., 2010; Padua et al., 2015). *Nr5a1* and *Gata4*, on the other hand, played pivotal roles in LC development and functions, and also were key regulators of luteal steroidogenesis and required for the normal response of luteal cells to LH (Convissar et al., 2015; Kyrönlahti et al., 2011).

In the EDS-treated rat model, the implantation of iLCs induced by either DGN or *Nr5a1* alone was able to increase the serum testosterone concentration by day 28 after transplantation. However, the serum testosterone concentration in MEFs transfected with *Nr5a1* alone was significantly
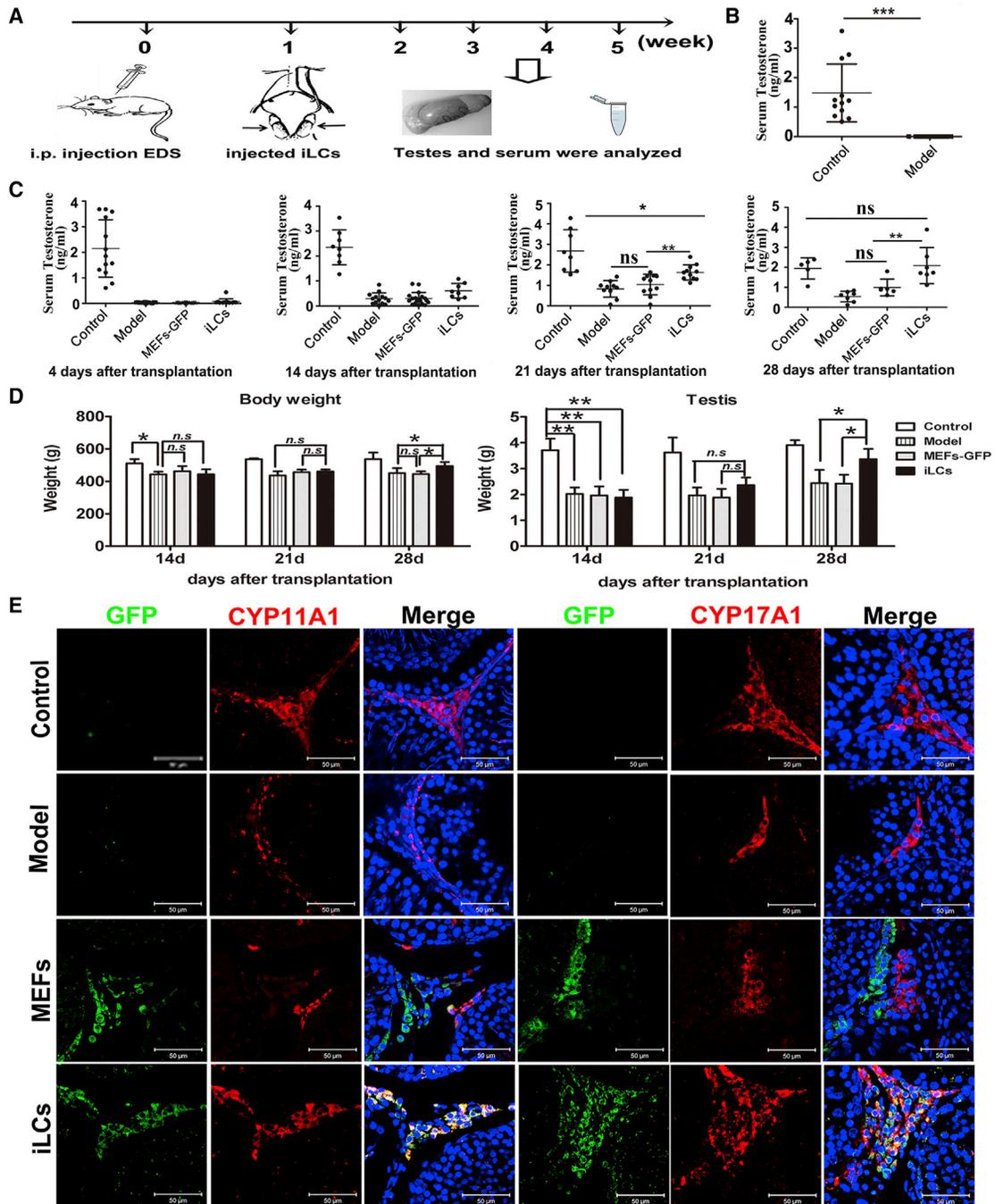
**Figure 6. Transplanted iLCs Were Capable of Producing Testosterone in the Testes of Adult Rats in Which Adult Leydig Cells Were Depleted by EDS**

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

(B) Total serum testosterone levels at 7 days after EDS treatment.

(C) Total serum testosterone levels were assayed after transplantation.

(D) The body weights and weights of the testes were assayed after transplantation.

(E) Immunofluorescent staining detection of CYP11A1 (red) and CYP17A1 (red) in the testes at 4 weeks after grafting. Nuclei were stained with DAPI (blue). Scale bars, 50 μm.

*(legend continued on next page)*

lower than that of MEFs transfected with DGN or the normal control (Figure S6). The results of the experiment in vitro indicated that *Dmrt1* and *Gata4* could activate the expression of *Lhcgr*, which is the critical step of androgen synthesis. These results suggested that *Dmrt1*, *Gata4*, and *Nr5a1* cooperated to promote programming and steroidogenesis.

To study the mechanisms by which DGN convert fibroblasts into iLCs, we investigated the DNA methylation levels of MEFs induced by *Dmrt1*, *Gata4*, and *Nr5a1* alone or in combination. Because the rearrangement of DNA

methylation patterns is essential for successful somatic cell reprogramming (Huang and Fan, 2010; Messerschmidt et al., 2014), demethylation is required for silencing transduced retroviral promoters and activating endogenous genes (Hu et al., 2014; Wang et al., 2014). As expected, the endogenous MEF genome showed higher levels of DNA methylation than that of the iLC genome. *Dmrt1*, *Gata4*, and *Nr5a1* alone could decrease the global DNA methylation levels, and the combination of the three factors could decrease it further. Importantly, DGN combination was also able to decrease the methylation level of

steroidogenic genes (*Cyp17a1* and *Lhcgr*) promoter. The mechanism involved might be that these factors opened the chromatin structure in the Leydig loci and induced epigenetic resetting of the fibroblast genome, allowing other Leydig-specific TFs to reach the target sites and leading to a full activation of the LC program. Thus, the roles of *Dmrt1* and *Gata4* in iLC reprogramming could be increasing not just the expression of *Lhcgr* but also the levels of DNA demethylation. The precise regulatory mechanisms of DGN in gene methylation deserve further investigation.

Besides MEFs, we also tested the effectiveness of the cocktail in reprogramming fibroblast from adult animals (TTFs). Interestingly, although the expected reprogramming indeed happened in TTF-derived cells, which showed LC characteristics, the gene expression levels and steroidogenic capabilities did not reach to the same levels as that of MEF-derived iLCs. Indeed, cells from adult animals (TTFs) might have less plasticity in reprogramming than the fetal cells (MEFs) (Pang et al., 2011). It is possible that TTFs possess higher levels of DNA methylation and are prone to undergo more complex processes than MEFs during reprogramming. It has been documented that age-associated hypermethylation in differentiated tissues often overlaps with promoters of genes that encode TFs necessary for differentiation or transdifferentiation, while these genes are deregulated in younger cells, which affects plasticity (Jung and Pfeifer, 2015; Zampieri et al., 2015).

To evaluate the therapeutic function of iLCs in vivo, we used two types of animal models, androgen deficiency and partial androgen deficiency syndrome, with complete loss of LCs in EDS-treated rat testis and partial loss of LCs in EDS-treated mice testis. Compared with other organs, testis is immunologically privileged (Meinhardt and Hedger, 2011). It has been reported that allogeneic and xenogeneic pancreatic islets survived transplantation in the testis with little immunosuppression (Bellgrau and Selawry, 1990). Our preliminary study also demonstrated that mouse ESC-derived Leydig-like cells were able to be transplanted into the testes of rats and survive. Therefore, we transplanted iLCs into the testes of rats and mice. The results showed that iLCs were capable of restoring the serum testosterone concentrations to the normal control levels in both rat and mouse models. Interestingly, it took more than 3 weeks to fully recover serum testosterone in rat, compared with only took 2 weeks in mice. In addition to the difference in the severity of endogenous LC loss, it is also possible that allogeneic cells are more adaptive to the niche of mouse testis (Selawry and Whittington, 1984). Nevertheless, these results indicated that allogeneic and xenogeneic iLCs could survive in the testes in both species and that this characteristic may be beneficial for clinical use.

In summary, by screening multiple transcriptional factors, we have found three transcriptional factors: *Nr5a1*, *Dmrt1*, and *Gata4*, which are essential in inducing the transition of fibroblasts (MEFs or TTFs) into functional Leydig-like cells. The gene expression profile and steroidogenic characteristics of iLCs were closely matched to those of ALCs. Importantly, these iLCs have the powerful capacity to restore the serum testosterone level when transplanted into the testes of rats or mice with testosterone deficiency. Our finding provides a potential approach to produce Leydig-like cells with steroidogenic function for cell therapy of male androgen-deficient diseases in humans.

## EXPERIMENTAL PROCEDURES

### Animals

C57BL/6 mice and Sprague-Dawley rats (at 8 weeks of age) were obtained from the experimental animal center of Guangdong Province, China. All animals were kept under conditions with controlled temperature ($24°C \pm 1°C$), relative humidity (50%–60%), and a light/dark cycle of 12:12 hr. The standard rodent diet and drinking water were freely accessible. All surgical procedures and postoperative care were approved by the Institutional Animal Care and Use Committee of Jinan University.

### Cell Culture and Derivation of iLCs

MEFs and TTFs were isolated and cultured as previously described (Qian et al., 2013; Takahashi et al., 2007a). In brief, MEFs were prepared from 13.5 days post coitum embryos. 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 min at 37°C. After the trypsinization, MEFs medium DMEM containing 10% fetal bovine serum (FBS, Life Technologies), 2 mM L-glutamine, and penicillin/streptomycin (all from Life Technologies) were added and pipetted to dissociate the tissue fragments. The triturated cells were collected by centrifugation ($250 \times g$ for 5 min) and resuspended in MEFs medium. The cells were plated on 10-cm tissue culture dishes and grown in medium for 2–3 days at 37°C under 5% $CO_2$ before freezing. TTFs were prepared from adult male mice. After washing with PBS, a lengthwise incision was made in the harvested tail by an injection needle. The superficial dermis was then peeled and the remaining tail was minced into 1-cm pieces with scissors. Two pieces were placed per well of gelatin-coated 6-well plates, and 2 mL DMEM containing 10% FBS and penicillin/streptomycin was added and incubated at 37°C for 5 days, during which time the fibroblasts migrated out of the tails. The tissues of tails were removed with sterile forceps and discarded. The medium was replaced with 2 mL fresh medium, and the cells cultured until they reached confluence. The medium was aspirated and washed twice with 2 mL of PBS, then 0.3 mL of 0.25% trypsin/1 mM EDTA was added before incubation at 37°C for 3 min. Two milliliters of DMEM containing 10% FBS was then added, and the cells suspended and transferred to a 15-mL tube for centrifugation at $250 \times g$ for 5 min. The supernatant was

discarded and the cells resuspended with 10 mL of medium before being plated on a 100-mm tissue culture dish and passaged to new 100-mm dishes at 1:4 dilution. These cells usually become confluent within 3–4 days. For the generation of iLC cells, MEFs and TTFs were used within three passages to avoid replicative senescence.

To derive iLCs, we originally passaged MEFs and TTFs onto gelatin-coated tissue culture plates. Cells were incubated with lentivirus for 12 hr, after which infected MEFs were switched to Leydig reprogramming media containing DMEM with 10% embryonic stem cell-qualified FBS, 1% Glutamax, 1% nonessential amino acids (all from Life Technologies), 50 ng/mL insulin-like growth factor, and 10 ng/mL platelet-derived growth factor AA (R&D Systems) at 37°C under 5% $CO_2$, and culture medium was changed every 2 days.

## Cell Transplantation

Before transplantation, male Sprague-Dawley rats were administered a single intraperitoneal injection of EDS, which was synthesized as previously described (Jackson and Jackson, 1984; Tahtaoui et al., 2004) and dissolved in DMSO (Sigma-Aldrich) at a dose of 100 mg/kg body weight. Mice were treated at a dose of 400 mg/kg. Transplantation was then performed as previously described (Ge et al., 2006) with some modifications. In brief, iLC (grown on 100-mm plates) at day 4 were washed twice with PBS and incubated with 0.25% trypsin/1 mM EDTA for 2 min at 37°C. Cells were gently dissociated, resuspended manually, and collected in a 15-mL Falcon tube. Cells were rinsed twice with PBS following centrifugation at 200 × g for 5 min. Finally each pellet was resuspended in PBS for transplantation. Cells were loaded into a 1-mL syringe for injection into the testis of adult Sprague-Dawley male rats or mice that had been treated with EDS. Approximately $2 \times 10^6$ cells in a 50-μL volume of PBS were injected into the parenchyma of recipient testes 7 days after the rats received EDS, and $10^6$ cells in a 20-μL volume of PBS were injected into the parenchyma of recipient testes 24 hr after the mice received EDS. The control animals for the experimental group were EDS-treated rats that had received a testicular injection of the PBS vehicle. Testes from all animals were examined at 7 and 14 days after transplantation (days 14 and 28 after EDS).

## Statistical Analyses

All experiments were repeated at least three times unless otherwise specified, and data were expressed as the mean ± 1 SD around the mean. Statistical analyses were performed with an unpaired Student's t test or one-way ANOVA for 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: GSE87020.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and three tables and can be found

with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.11.010.

## AUTHOR CONTRIBUTIONS

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

## ACKNOWLEDGMENTS

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