Inhibition of glycogen synthase kinase-3 promotes efficient derivation of pluripotent stem cells from neonatal mouse testis

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BACKGROUND: Several studies have demonstrated the derivation of multi- or pluripotent stem cells from testicular cells of both newborn and adult mice by a spontaneous conversion process, when these cells are cultured in vitro for an extended time. To obtain a better and robust derivation, we have attempted to identify small molecules (SMs) that induce reprogramming of testicular cells in culture into germ-line-derived pluripotent stem cells (gPSCs).

METHODS: We tested several SMs based on previous reports that have shown enhancement of establishment of induced pluripotent stem cells or embryonic stem cells (ESCs) on mouse NMRI (outbred strain) and C57BL/6 (inbred strain) testicular cells. After appearance of ESC-like colonies at Day 6, they were passaged on mitotically arrested mouse embryonic fibroblasts in mouse ESC medium in the absence or presence of SMs up to Day 14. The generated cells were characterized using a variety of experimental approaches.

RESULTS: The application of several SMs involved in pluripotent reprogramming led to the discovery that CHIR99021 (CHIR), a glycogen synthase kinase-3 (GSK-3) inhibitor, promotes efficient derivation of gPSCs from neonatal mouse NMRI and C57BL/6 testes. The pluripotency of the generated cell lines has been confirmed by in vitro spontaneous and direct differentiation toward cardiac and neural lineages, and formation of chimeras after injection of gPSCs into blastocysts. We have shown that the generated gPSCs could be maintained and expanded under chemically defined serum and feeder-free conditions by inhibition of both the extracellular signal-regulated kinases (Erk1/2) and GSK-3.

CONCLUSIONS: To our knowledge, this is the first report of a simple and efficient protocol to reprogram gPSCs from testicular cells solely by inhibition of GSK-3 in two strains of mice with different genetic backgrounds. Additionally, this brings us closer to eliminating the need for genetic modification in pluripotent reprogramming. Future studies will determine whether the inhibition of GSK-3 could affect the generation of naïve gPSCs lines in other mammals.

Key words: Mouse testis / germline-derived pluripotent stem cells / CHIR99021 / glycogen synthase kinase-3 / extracellular signal-regulated kinases

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Introduction

Embryonic and induced pluripotent stem cells (ESCs and iPSCs) have extensive capacity for proliferation, self-renewal and differentiation. Therefore, they provide a pluripotent source for a variety of applications including developmental biology, drug screening, toxicology, disease research and cell-based therapy. However, standard methods for derivation of ESCs, or patient- or disease-specific iPSC lines, result in limitations in their possible biomedical applications because of ethical and immunological concerns. Some key advances have been achieved for the production of safe iPSCs including the use of non-integrating viruses (such as adenovirus or episomal plasmid transfection), which are known to be stable in mammalian cells for extended periods without integrating into the genome (Stadtfeld et al., 2008; Zhou and Freed, 2009; Nishimura et al., 2010), treatment of cells with cell-penetrating recombinant reprogramming factor proteins (Kim et al., 2009), transposon-based systems (Woltjen et al., 2009) and the delivery of reprogramming factors in plasmids (Okita et al., 2008; Yu et al., 2009; Si-Tayeb et al., 2010), repeated transfection with modified mRNA encoding reprogramming factors (Warren et al., 2010) and micro RNAs (Lin et al., 2011; Tian et al., 2011). However problems exist, such as low reprogramming efficiency, genomic alterations and immunogenicity (for review see Seifinejad et al., 2010; Stadtfeld and Hochdelinger, 2010; Gonzalez et al., 2011). Recently, it was reported that iPSCs could elicit an immune reaction in mice (Zhao et al., 2011). It has been demonstrated that the transplantation of ESCs led to teratomas, whereas most of the iPSCs were not able to form teratomas, or produced teratomas that were attacked or rejected by the immune systems of the host syngeneic mice. This immunogenicity of iPSC derivatives may relate to epigenetic differences between iPSCs and ESCs, and/or mutations in the coding sequences of iPSCs (Marchetto et al., 2009; Ji et al., 2010; Kim et al., 2010; Polo et al., 2010; Gore et al., 2011; Lister et al., 2011) could give rise to the ectopic expression of minor antigens.

In order to reach the final goal of clinical applications, it is necessary to develop technologies to overcome iPSC limitations or search for other sources of PSCs that have minimal ethical and immunological concerns. Although recent reports (Conrad et al., 2008; Golestanian et al., 2009; Kossack et al., 2009; Mizrak et al., 2010) claimed the derivation of multipotent or ESC-like cells from adult human testicular tissues by exposing human testicular cells to specific ESC conditions in vitro, the pluripotency of these cells has been questioned (Ko et al., 2010; Tapia et al., 2011).

Studies have demonstrated that both newborn and adult male murine testicular cells undergo a 'self-reprogramming' or spontaneous conversion process into multipotent stem cells or pluripotent embryonic stem (ES)-like cells in the absence of genetic manipulation when these cells are cultured in vitro for an extended time (Kanatsu-Shinohara et al., 2004; Guan et al., 2006; Seandel et al., 2007; Izadyar et al., 2008; Kanatsu-Shinohara et al., 2008; Ko et al., 2009). These mice ESC-like spermatogonia-derived stem cells were termed multipotent germline stem cells, multipotent adult germline stem cells, multipotent adult spermatogonia-derived stem cells or germine-derived pluripotent stem cells (gPSCs) according to different reports. The gPSCs resembled naive mouse ESCs (mESCs) in terms of morphology, continual passing without a decline in colony-forming ability or change in karyotype (de Rooij and Mizrak, 2008), spontaneous differentiation into derivatives of the three germ layers, their contribution to chimeric after injection into blastocysts (Kanatsu-Shinohara et al., 2004; Guan et al., 2006; Seandel et al., 2007) or germ-line transmission (Ko et al., 2009). gPSCs can be propagated in the medium that contains serum and leukemia inhibitory factor (LIF), and have been induced to directly differentiate into functional hepatocytes, cardiomyocytes, neurons and glial cells in vitro (Baba et al., 2007; Guan et al., 2007; Streekfuss-Bomeke et al., 2009; Fagoonee et al., 2010). Thus, gPSCs could differentiate into the required cell type and be transplanted back to the autologous patient without ethical or immunological problems, or they can be genetically corrected for using in regenerative medical therapies (Tapia et al., 2011).

However, gPSCs have been generated with very low efficiency and after several weeks following testicular culture. Small molecules (SMs) may offer one possible solution to this challenge (for review see Feng et al., 2009; Li and Ding, 2010; Efe and Ding, 2011; Lysiotis et al., 2011; Yuan et al., 2011). SMs can reversibly perturb specific functions of a single protein (or multiple proteins) with exquisite temporal control in the absence of genetic modification(s). Recently, several reports have demonstrated the efficient and reproducible generation of ESCs from mouse strains previously considered to be refractory and non-permissive to ESC establishment, such as BALB/c, C57BL/6, DBA/2, NMRI, FVB/N, NOD (Buehr and Smith, 2003; Umehara et al., 2007; Hanna et al., 2009; Nichols et al., 2009a; Sato et al., 2009; Gertsenstein et al., 2010; Kiyonari et al., 2010; Wray et al., 2010; Hassan et al., 2011) and from rat (Buehr et al., 2008; Li et al., 2008; Kawamata and Ochiya, 2010; Leitch et al., 2010) and iPSCs production (Feng et al., 2009; Nichols et al., 2009b; Li and Ding, 2010; Wray et al., 2010; Efe and Ding, 2011; Hassan et al., 2011; Lysiotis et al., 2011; Yuan et al., 2011) by treatment with SM(s).

In this study, we introduce a simple and efficient protocol for the generation of gPSCs from neonatal mouse testis in conventional mESC medium supplemented with SMs and without genetic manipulation. Thus far, no report has shown the reprogramming of somatic cells using just one SM. Additionally, we have demonstrated that gPSCs could propagate and maintain their pluripotency under feeder-free defined conditions in a chemically defined N2B27 supplemented medium, as previously reported for mESCs (Ying et al., 2008). Our method helps to overcome the problems of gPSC establishment and clarifies a new signaling pathway involved in the generation of gPSCs from different mouse strains.

Materials and Methods

Isolation of testicular cells and primary culture condition

All animal care was in accordance with the approval of the Royan Institutional Review Board and Institutional Ethical Committee. Testicular cells were isolated from neonatal male mice (3 day old, NMRI and C57BL/6 strains, Pasteur Institute, Tehran, Iran). Embryonic day (E) 12.5–13.5 fetuses (NMRI strain) were used to produce mouse embryonic fibroblasts (MEFs) as feeder cells.

Isolation of testicular cells was performed as previously described (Izadyar et al., 2008) with slight modification. Briefly, the bilateral testes of 5–10 neonatal mice were collected in phosphate-buffered saline (PBS), placed on ice and then transferred to the laboratory within 15 min of decapsulation. Seminiferous tubules were placed in a dish that...
contained knockout Dulbecco’s modified Eagle’s medium (Ko-DMEM, Invitrogen) and separated from one another by pipetting for 1 min. The separated tubules were collected by centrifugation at 500g for 2 min, then suspended in Ko-DMEM supplemented with collagenase, dispase and hyaluronidase (each at 1 mg/ml; all enzymes purchased from Sigma-Aldrich) for 10 min with incubation at 37°C (first enzyme digestion step). After a second centrifugation (500g for 2 min), in which the majority of interstitial cells and blood vessels were removed, a second digestion step was performed in Ko-DMEM by adding fresh enzymes to the seminiferous cord fragments. After filtration through a 70-μm nylon filter, isolated cells were replated (2.5 × 10^5 cells per gelatin-coated center-well plate, 2 cm², Falcon) at 37°C in an atmosphere of 5% CO₂ in air. In Ko-DMEM supplemented with L-glutamine (2 mM, Invitrogen), non-essential amino acids (NEAAs, 1%, Invitrogen), penicillin (100 U/ml, Invitrogen), streptomycin (100 mg/ml, Invitrogen), β-mercaptoethanol (0.1 mM, Sigma-Aldrich) and 2% fetal calf serum (FCS, HyClone).

One day after plating, most testicular cells were attached to the gelatin-coated surface. The medium was replaced with the same medium supplemented with 1% FCS, glial-derived neurotrophic factor (GDNF, 20 ng/ml, Sigma-Aldrich), mouse epidermal growth factor (EGF, 20 ng/ml, Sigma-Aldrich), human basic fibroblast growth factor (bFGF, 10 ng/ml; Royan Institute) and mouse LIF (1000 U/ml; Millipore) in the absence of SM. The culture medium was changed every 2–3 days. The mESC medium contained the same components in addition to 15% FCS and MEF in the mESC medium in the absence or presence of SMs. Mouse ESCs were used as a positive control. For immunofluorescence staining, undifferentiated germ line-derived ESC-like colonies were fixed in 4% paraformaldehyde in PBS, pH 7.4 (Invitrogen), for 20 min. Cells were washed twice with 0.1% Tween-20 in PBS to remove residual fixative and permeabilized with 0.2% Triton X-100 in PBS for 20 min prior to blocking in 10% normal goat serum in PBS for 60 min followed by incubation with primary antibody solution overnight at 4°C, or for 1 h at 37°C. The primary antibodies used in this study were Oct-4 (1:50; Santa Cruz Biotechnology, SC-5279), Nanog (1:100; Santa Cruz Biotechnology, SC-30329) and stage-specific embryonic antigen-1 (SSEA-1; 1:50, R&D, MAB2155) for determination of undifferentiated state, and Gata4 (1:200; Santa Cruz, SC-1237), Mef2c (1:200; Abcam, ab64644), α-Mhc (1:200; Abcam, Ab15), Tuji (1:500; Sigma-Aldrich, T-8660), Map2 (1:200; Sigma-Aldrich) Tbx3 (1:200; Santa Cruz, SC-48782) and FoxA2 (1:100; Abcam, ab60721) for differentiated cells. Then, cells were washed twice with 0.1% Tween-20 in PBS for 5 min and incubated with the appropriate secondary antibody in PBS. Fluorescence-conjugated secondary antibodies, goat anti-mouse immunoglobulin (IgG fluorescein isothiocyanate (FITC, 1:200; Sigma, F9006), mouse anti-goat IgG phycoerythrin (1:200, Santa Cruz, SC-3725), rabbit anti-mouse IgG Texas red (1:400; Jackson Lab, 315-075-003), rabbit anti-Goat IgG FITC (1:200; Sigma-Aldrich, F7367) and goat anti-rabbit IgG FITC (1:200; Sigma-Aldrich, F1262) were used, as appropriate, for 1 h at 37°C. After two washes with PBS + 0.1% Tween 20 for 5 min, cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) and analyzed with a fluorescent microscope (Olympus). Immunostaining without primary antibodies was also used as a negative control for the cells (Supplementary data, Figs S1–S3).

Alkaline phosphatase and immunofluorescence staining

Alkaline phosphatase (AP) staining was performed using a kit (Sigma-Aldrich) according to the manufacturer’s recommendations. Mouse ESCs were used as a positive control. For immunofluorescence staining, undifferentiated germ line-derived ESC-like colonies were fixed in 4% paraformaldehyde in PBS, pH 7.4 (Invitrogen), for 20 min. Cells were washed twice with 0.1% Tween-20 in PBS to remove residual fixative and

RNA isolation, reverse transcription and quantitative RT–PCR

Total RNA was isolated using TRIzol® reagent (Invitrogen) according to the manufacturer's protocol. To remove genomic DNA contamination, all RNA samples were subjected to DNase I (EN0521, Fermentas) treatment.

cDNA synthesis was performed using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (K1632, Fermentas), 0.2 μg random hexamer primer and 1 μg total RNA per reaction, according to the manufacturer’s instructions. For every reaction set, one RNA sample was used without the addition of reverse transcriptase to provide a no RT control (RT– reaction) as a negative control in the subsequent PCR.

Gene expression was assessed by quantitative RT–PCR for candidate genes in a Rotor-Gene 6000 (Corbett Life Science) using the following program, stage 1: 95°C for 10 min, stage 2: 95°C for 10 s, 60°C for 20 s and 72°C for 30 s, for 40 cycles. At the end of the run, a melting profile was determined to demonstrate the synthesis of a single PCR product. The primers were designed using the primer design software, Perprimer (Marshall, 2004). Primer sequences, expected fragment size and Gene Bank accession numbers are listed in Supplementary data, Table S1.

The PCR mix in each well included 10 μl of SYBR®Premix Ex Taq™ II (RR081Q, Takara Bio., Inc.), 6 μl dH₂O, 1 μl each of the forward and reverse primers (5 pmol/μl), 2 μl of single-strand cDNA (15 ng/μl) in a final reaction volume of 20 μl. Each experiment included at least three biological replicates and each replicate was analyzed in duplicate.

For relative quantification, a standard curve was generated in every individual run using a serial dilution of the pool of cDNA samples (50, 10, 2, 0.4 and 0.08 ng) with high expression values.

Data were analyzed using the relative standard curve method. The output data from Rotor-Gene 6000 Analysis software (version 1.7; Corbett Life Science) were transferred to Microsoft Excel for analysis. For each unknown sample, the relative amount was calculated by normalization of each target gene to the geometric mean of two reference genes, B2m and beta Tubulin, and then calibrated against a control group (day 0 testicular cells).
Spontaneous and direct differentiation of gPSCs into cardiomyocytes, endodermal and neuronal cells in vitro

For demonstrating the pluripotency of ESC-like lines, spontaneous differentiation was done by embryoid body (EB) formation in low attachment bacterial plates (Grainer, Germany; 628–102) for 6 days; EBs were further plated for an additional 5 days on gelatin-coated tissue culture plates in the mESC medium without LIF.

For direct cardiomyocyte differentiation (Moghadasali et al., 2007; Farokhzad et al., 2009), 800 gPSCs were cultured for 2 days in 20 μl of ESC medium that contained 10⁻⁴ M ascorbic acid (vitamin C; Sigma-Aldrich, A4403) in the absence of LIF in hanging drops to produce EBs. Subsequently, EBs were cultured as suspensions in bacterial dishes for 5 additional days. On Day 7, EBs were plated separately in 1% gelatin-coated wells of a 24-well tissue culture plate for an additional 14 days to allow adherence and development of beating cardiomyocytes. In order to confirm the functionality of gPSCs-derived cardiomyocytes, the effect of treatment with diltiazem, a calcium channel blocker, on cells was evaluated. Diltiazem (10⁻⁵ M; Sigma-Aldrich, D2521) was added to beating EBs for 2–3 min. Subsequently, the number of beats per minute was counted and compared with the number of beats per minute before treatment.

In order to promote directed differentiation of gPSCs into a neural fate (Moghadasali et al., 2007; Farokhzad et al., 2009), EBs were produced by culturing 10⁵ gPSCs in a 3 ml medium in non-adhesive bacterial dishes that contained ESC medium without LIF. Subsequently, EBs were cultured in suspension in the presence of retinoic acid (1 μM; Sigma-Aldrich, R2625) in the same medium. At Day 6, the EBs were plated onto poly-l-lysine-coated dishes in ESC medium supplemented with 5% FCS for 5 days, for further differentiation of precursor cells into mature neurons.

To induce endodermal cell differentiation, 4 day EBs were plated in gelatin-coated dishes and were treated with Activin A (50 ng/ml; R&D, 338-AC) in the mESC medium without LIF and serum supplemented with 1% N2 supplement (Invitrogen) and 2% B27 supplement (Invitrogen) for a further 6 days. The medium was changed every 2 days.

Production of chimeric mice

To examine the differentiation potential of ES-like cells in vivo, chimeras were generated. Briefly, 3.5 day post coitus blastocysts were collected from superovulated female BALB/c mice and placed in the M2 medium. Then, 10–15 single-cell ESC-like colonies were injected into each blastocyst. Approximately 10 injected blastocysts were transferred into the uterine horns of pseudo-pregnant BALB/c × C57BL/6 F1 mice. Chimeras were identified by coat color.

Culture of gPSCs under defined conditions

For expansion of gPSCs under defined conditions, MEF was replaced by gelatin (0.1%; Sigma-Aldrich), and N2B27-supplemented medium was used instead of conventional mESC medium. Serum-free N2B27-supplemented medium (100 ml) contained the following: 45 ml DMEM/Ham’s F12 (DMEM/F12, Invitrogen), 45 ml neurobasal (Invitrogen), 1 ml N2 supplement, 2 ml B27 supplement, 2 mM l-glutamine, 1% NEAAs, penicillin (100 U/ml), streptomycin (100 mg/ml), 0.1 mM β-mercaptoethanol, 1000 U/ml mouse LIF, 5 mg/ml bovine serum albumin (Sigma-Aldrich), an inhibitor of the Erk1/2 cascade (Ying et al., 2008), PD0325901 (PD, 1 μM; Stemgent) and CHIR (3 μM).

Statistical analysis

The experiment of gPSC generation was repeated more than 10 times. All other experiments were conducted in at least three independent cultures. Real-time data were expressed as mean ± SD and analyzed with one-way analysis of variance followed by the post hoc Tukey honest significant difference test for multiple comparisons. P-values < 0.05 were considered significant. The data for the number of ESC-like colonies at Day 6 and treatment with diltiazem were analyzed by Student’s t-test.

Results

Screening of SMs to generate gPSCs from testicular cells

In initial attempts to derive gPSCs, we obtained testes from neonatal NMRI mice and produced cell suspensions by enzymatic digestion. We then plated isolated cells on gelatin-coated dishes in the Ko-DMEM medium supplemented with 2% FCS for 1 day. The experimental scheme for establishment of gPSCs used in the current study is illustrated in Fig. 1A.

Then, we explored SM to induce the derivation of mESC-like colonies in Ko-DMEM medium supplemented with GDNF, EGF, bFGF, LIF, FCS (1%) and in the absence (control) or presence of SMs for 5 days. At Day 6 post-digestion mESC-like colonies appeared in the CHIR group, with a packed spindle- to round-shaped morphology with smooth borders. In the experiments including the CHIR group, all colonies were mESC like. After plating 250,000 testicular cells, 164.1 ± 29.9 ESC-like colonies were observed at Day 6. Therefore, the percentage of ESC-like colony formation in the CHIR group was 0.065%. The remaining SMs or their combinations did not result in the formation of mESC-like colonies after 6 days or later under these conditions but produced cell aggregates which were not expanded (Fig. 1B and Supplementary data, Fig. S4).

Subsequently, the mESC-like colonies were transferred onto fresh feeder layers of mitotically arrested MEF in the mESC medium in the presence of CHIR (Fig. 1B). Then, some colonies were picked up from the plate by a thin Pasteur pipette under stereomicroscope and replated on fresh MEF in the mESC culture medium for 2 additional days (Day 8); growth factors were eliminated. We found this procedure influenced the generation of gPSCs in comparison with trypsinization of whole ESC-like colonies at Day 6. At Day 8, cells were dissociated into single cells with trypsin/EDTA and replate into 12-well plates, including MEF. Following an additional 6 days of culture, typical mESC-like colonies could be identified, which we considered them to be passage one. The mESC-like colonies expanded as dome shaped and compact colonies, with high nuclear–cytoplasmic ratios. Under the phase contrast microscope it was difficult to distinguish the individual cells in the mESC-like colonies, although nuclei could be recognized in some cells and contained one to three dark prominent nucleoli (Fig. 1B). Daily observation showed that the colonies continued to increase in number and size by proliferation without differentiation. After this step, CHIR was omitted from the ESC medium. Cell lines were then propagated by enzymatic subculture every 2–3 days with a doubling rate comparable to mESCs. The mESC medium was changed daily. We designated these to be putative gPSC colonies.
Figure 1 The process of establishing mouse gPSCs. (A) Schematic of the derivation of gPSCs. Three-day derived testicular cells were plated on gelatin-coated plates in the Ko-DMEM medium supplemented with 2% FCS. The next day, the medium was replaced by a cocktail of growth factors GEFL (= GDNF + EGF + bFGF + LIF) plus FCS (1%), in the absence (control) or presence of SM. At Day 6, the ESC-like colonies were isolated mechanically and replated on a mitotically arrested MEFs feeder layer in the mESC medium. After 2 days, colonies were passaged by trypsin/EDTA treatment and plated on a fresh MEF feeder layer in the same conditions. Typical mESC-like colonies were observed after 4–6 days. These colonies were passaged on Day 14 in the absence of SM (passage 1, P1). (B) Phase contrast microscopy of cultured cells during generation and passaging of gPSCs. In the absence of SM (control) we only observed fibroblast-like aggregates which did not produce gPSCs after passaging in these conditions. However, in the presence of CHIR99021 (CHIR), a GSK-3 inhibitor, mESC-like colonies, with packed spindle to round morphology and smooth borders, appeared at Day 6. After passaging, typical mESC-like colonies were observed at Day 14. These colonies could be passaged every 2–3 days. The results were also similar regardless of genetic background in both outbred NMRI and inbred C57BL/6 strains. The lines were referred to as RNh/gPSC and RBn/gPSC, which were derived from neonatal male NMRI and C57BL/6 strains, respectively (R: ‘Royan’ which means ‘embryo’ in Persian; 'N' and 'B', generated from NMRI and C57BL/6 strains; and ‘n’ for ‘neonatal’).
The same protocol was used to evaluate the universality of inhibition by GSK3 of the production of gPSC lines from another mouse strain, C57BL/6. Results were similar to those of the NMRI strain (Fig. 1B). These results clearly demonstrated that mouse gPSC generation was promoted by the inhibition of GSK-3 only, in two strains of mice with different genetic backgrounds.

Figure 2 Characterization of established, undifferentiated gPSCs. (A) The expression of mESC-specific markers, AP and immunofluorescence staining for Oct4, Nanog and SSEA1 in the gPSC lines established by CHIR after at least 20 passages. (B) Real-time RT–PCR analysis of pluripotency markers (Oct4 and Nanog) and the germ cell-specific marker (Dazl) in the gPSCs and mESC lines. Testicular cells (d0) and MEF were used as controls. The number of biological replicates was at least three. Data are mean ± SD. One-way ANOVA and Tukey test were used. *P < 0.05.
The established lines were named RNn/gPSC and RBn/gPSC (R: ‘Royan’ which means ‘embryo’ in Persian; ‘N and ‘B’, derived from NMRI and C57BL/6 strains; and ‘n’ for ‘neonatal’).

The established cell lines were successfully cryopreserved and thawed with no loss in proliferation or differentiation capacities.

Characterization of undifferentiated gPSCs

The gPSC lines were passaged 1:3 – 1:6 for more than 20 times following trypsin digestion, with an estimated doubling time of 48 h. These cells maintained their undifferentiated state in multiple passages and showed clonal growth from single cells. To examine the pluripotency of generated gPSC lines, they were evaluated for mESC markers (Fig. 2). AP was highly expressed in gPSCs and the cells were strongly positive for pluripotency markers Oct4, Nanog and SSEA-1 (Fig. 2A). The expression of pluripotent markers, Oct4, Nanog and AP was not detected by immunofluorescence staining in non-SM-treated control cells (fibroblast-like aggregates; Supplementary data, Fig. S5).

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To further confirm the pluripotency of our gPSCs, we tested the capacity of C57BL/6-derived gPSCs to contribute to chimeric mice. RBn/gPSCs were injected into host 3.5 day old blastocysts of the BALB/c strain. We transferred 155 injected blastocysts into the uteri of 19 pseudo-pregnant female mice. From 10 pregnant foster mothers, we obtained 32 live pups. The pups were of a similar size to a normal birth pup, with no abnormalities. Nine out of 32 pups were chimeric and four out of nine chimeric pups were male and highly chimeric but they did not show germ-line transmission (Fig. 3C).

Next, we investigated directed differentiation of the generated RNn/gPSC line into cardiomyocytes and neuronal cells (Fig. 4A – C). The cells that differentiated into cardiomyocytes demonstrated beating (Fig. 4A). Furthermore, negative chronotropic effects on cardiomyocytes were observed after diltiazem treatment (Fig. 4B). Immunofluorescent staining revealed the expression of muscle markers that included α-Mhc, Mef2c, Gata4 and Tbx5 (Fig. 4C). RT–PCR of differentiating cardiomyocytes showed the expression of cardiac-specific genes, including Mlc2a, Nkx2.5, Mlc-2v, cTNT and α-Acetin (data not shown). Endodermal cells expressed FoxA2 after activin A treatment (Fig. 4D). Neurons were also generated by directed differentiation of gPSCs and were positive for Tuj1 and Map2 (Fig. 4E).

Therefore, with the above in vitro and in vivo differentiation assays, our results have revealed that the gPSC lines produced by treatment with CHIR are pluripotent.

Expansion of gPSCs in chemically defined conditions, supplemented with PD + CHIR

To examine the expansion of gPSCs in defined conditions, we cultured gPSCs under feeder-free conditions in a chemically defined N2B27-supplemented medium with inhibition of both GSK-3 and Erk1/2 by CHIR and PD, respectively (Ying et al., 2008). gPSCs were propagated in these culture conditions with no feeder cell layer or serum for at least 20 passages. gPSCs were differentiated in the presence of CHIR only. However, we found that PD + CHIR along with LIF replaced the requirement for feeder and serum, and supported robust long-term gPSC propagation. The cells showed a morphology which was typical of mESCs, expression of pluripotency markers (Fig. 5) and differentiation by EB formation (Supplementary data, Fig. S6).

Discussion

Here, we report that testicular cells isolated from neonatal mice can be efficiently reprogrammed into PSCs under relatively simple mESC culture conditions. The rate of conversion of testicular cells into
Figure 4  Directed differentiation of gPSCs in vitro. (A) Directed differentiation of RNn/gPSCs into beating cardiomyocytes. (B) The differentiated cardiomyocytes responded to diltiazem, a negative chronotropic drug. Data are mean ± SD. The Students t-test was used. *P < 0.05. (C) The differentiated cardiomyocytes expressed αMhc, Mef2c, Gata4 and Tbx5 as detected by immunofluorescence staining. Nuclei were stained with DAPI (blue). (D) Differentiated cells expressing FoxA2 after treatment with Activin A. Nuclei were stained with DAPI (blue). (E) Neuronal cells as detected by phase contrast microscopy and immunofluorescence staining for Tuj1 and Map2. Nuclei were stained with DAPI (blue).
gPSCs is 0.065% of the initially plated testicular cells (an average 164 colonies per 250,000 seeded testicular cells).

These cells were reprogrammed without genetic modification and without enrichment for spermatogonial stem cells in culture. Specifically, application of CHIR reduced the time required to reprogram testicular cells and increased the fraction of cells that became gPSCs. The resultant cells shared many features with ESCs. All cell lines were passaged at least 20 times and maintained ESC morphology, as indicated by light microscopy. All cell lines showed strong positive staining for AP activity. Additionally, they expressed SSEA1 and the pluripotency markers Oct4 and Nanog. The germ cell-specific gene Dazl showed down-regulation. The cell lines produced EB-like structures in suspension with the apparent potential to differentiate into derivatives of the three germ layers in vitro. Additionally, they could be

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**Figure 5** Proliferation and characterization of gPSCs in chemically defined conditions. gPSCs were expanded under a chemically defined N2B27-supplemented medium by the inhibition of both the mitogen-activated protein kinase (Erk1/2) and GSK-3 (PD0325901 + CHIR) in the presence of LIF. gPSCs expressed the pluripotency markers Oct4, Nanog and SSEA1, and differentiated in vitro by EB formation (Fig. 3B and Supplementary data, Fig. S6).
stimulated to differentiate into cardiomyocytes and neurons, and contributed to chimera development. The new cell lines therefore appear to be pluripotent. However, their potential for teratoma formation in vivo and their contribution to forming tissues of all three germ layers in the chimeric mice remains to be demonstrated.

In previous studies (Kanatsu-Shinohara et al., 2004; Guan et al., 2006; Seandel et al., 2007; Izadyar et al., 2008; Kanatsu-Shinohara et al., 2008; Ko et al., 2009) the generation of gPSCs was a rare and stochastic process, and was uncontrollable. Reprogramming of testicular cells in our study by CHIR occurred over 6–14 days; this phenomenon has been shown consistently to occur within 2–4 weeks of purified germline stem cell culture (Ko et al., 2010). Additionally, our gPSCs maintained expansion and pluripotency in feeder and serum-free conditions in the presence of CHIR + PD in combination with LIF for an extended period (at least 10 passages) and shared typical features of mESCs. It has been reported that CHIR + PD in combination with LIF could replace serum and allow cultivation of mESCs in a chemically defined medium (Ying et al., 2008).

Recently, it has been demonstrated that GSK-3 inhibitors (CHIR or BIO) have an impressive effect on mESC establishment using strains which were previously considered refractory and non-permissive (Buehr and Smith, 2003; Umehara et al., 2007; Hanna et al., 2009; Nichols et al., 2009a; Sato et al., 2009; Gertsenstein et al., 2010; Kiyonari et al., 2010; Wray et al., 2010; Hassani et al., 2011). Additionally, it has been reported that CHIR, in combination with PD, A-83-01 and LIF, produced naïve ESC-like rat iPSCs (Li et al., 2009a) or rat ESCs (Buehr et al., 2008; Li et al., 2008; Kawamata and Ochiya, 2010) capable of contributing to chimerism. Moreover, it has been demonstrated that mouse and rat embryonic germ cells can be established with high efficiency using CHIR in combination with PD0325901 and cytokine LIF by culturing primordial germ cells from E8.5 to E12.5 mice and E10 rats (Leitch et al., 2010). It was also demonstrated that human pluripotent naïve cells were derived from human ESCs by ectopic expression of OCT4 and simultaneous treatment with LIF, inhibitors of GSK-3 and ERK1/2, and forskolin, a protein kinase A pathway agonist that can induce KLF2 and KLF4 expression (Hanna et al., 2010). Activation of the WNT pathway by a Wnt ligand or GSK-3 inhibitor (CHIR) has also been demonstrated to increase the induction of pluripotency reprogramming of somatic cells (Lluis et al., 2008; Marson et al., 2008). CHIR can also replace SOX2 in the pluripotent reprogramming of both MEF and human neonatal keratinocytes which were overexpressed with OCT4 and KLF4 in combination with parnate, a lysine-specific histone demethylase 1 inhibitor (Li et al., 2009b). Activation of Wnt/β-catenin signaling can stimulate ESC self-renewal and support short-term pluripotency in humans and mice (Sato et al., 2004). However, these results are somewhat controversial because self-renewal of the ESCs in Wnt-3a-supplemented medium has not been demonstrated over multiple passages (Dravid et al., 2005; Bakre et al., 2007). Additionally, although PD and CHIR are sufficient to generate mESCs from blastocysts, CHIR alone is not sufficient in this process (Hassani et al., 2011).

Although the exact mechanism(s) of GSK-3 inhibition in gPSC isolation remains unclear, it has been reported that β-catenin is the primary GSK-3 substrate regulating the differentiation of mESCs. Stable β-catenin can interact with DNA-binding Tcf factors in the nucleus, where the complex activates transcription of target genes (Lluis et al., 2011). Recently, the Doble group found that alternative β-catenin-mediated signaling, through independent T-cell factor/lymphoid enhancer (Tcf/Lef) factors, can reinforce the pluripotent status of mESCs and impair their efficient differentiation (Kelly et al., 2011). These data are consistent with new findings (Wray et al., 2011) and can be explained by β-catenin-mediated repression of Tcf3 targets, including Oct4 and modulation of Oct4 target genes (Tam et al., 2008). The interaction between β-catenin and Oct4 (Kelly et al., 2011) could reflect the recruitment of β-catenin by Tcf3 to promoter sites co-occupied by Oct4 (Wray et al., 2011).

Therefore, GSK-3 inhibition may act primarily through the stabilization of intracellular β-catenin, and by converting Tcf3 complexes from repressors to activators or by displacing Tcf3 with other Tcf factors through which β-catenin activates the pluripotency network (Wray et al., 2011).

Taken together, we have shown for the first time that gPSCs can be established, with some efficiency, from neonatal mouse testes in vitro in the presence of CHIR, and gPSCs could be propagated under defined conditions in the presence of chemicals. Notably, these cells are not genetically modified, as required for the derivation of iPSCs, and there are no ethical concerns associated with ESC derivation. The ability to derive and expand gPSCs in vitro may facilitate the development of novel therapeutic strategies to produce immune-matched differentiated cells for patient-specific treatment. Additionally, these pluripotent cells offer a source of patient-specific stem cells appropriate for the study of genetic diseases in different cell lineages in vitro.

Future studies will show whether CHIR or other inhibitors of GSK-3 (Meijer et al., 2004) could affect the isolation of gPSCs lines with ground-state properties—a basal proliferative state that is free of epigenetic restriction and has minimal requirements for extrinsic stimuli—from other mammals, such as rats, and in biopsies of human testes. Furthermore, this signaling pathway, GSK-3 inhibition may help in discovering the mechanisms involved in germ cell-related testicular teratoma formation and improve our understanding of human testicular cancer.

Supplementary data
Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors’ roles
S.-F.M. and F.A. were involved in collection and/or assembly of data, data analysis and interpretation and manuscript writing; A.S., H.S. and N.A. were involved in data analysis and interpretation; A.F., S.-N.H. and H.F. were involved in collection and/or assembly of data, data analysis and interpretation; H.B. was involved in conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

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Conflict of interest
None declared.

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