# In search of rat stem Leydig cells: Identification, isolation, and lineage-specific development

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**Leydig cells (LCs) are thought to differentiate from spindle-shaped precursor cells that exhibit some aspects of differentiated function, including 3β-hydroxysteroid dehydrogenase (3βHSD) activity. The precursor cells ultimately derive from undifferentiated stem LCs (SLCs), which are postulated to be present in testes before the onset of precursor cell differentiation. We searched for cells in the neonatal rat testis with the abilities to: (*i*) proliferate and expand indefinitely *in vitro* (self renew); (*ii*) differentiate (i.e., 3βHSD and ultimately synthesize testosterone); and (*iii*) when transplanted into host rat testes, colonize the interstitium and subsequently differentiate *in vivo*. At 1 week postpartum, spindle-shaped cells were seen in the testicular interstitium that differed from the precursor cells in that they were 3βHSD-negative, luteinizing hormone (LH) receptor (LHR)-negative, and platelet-derived growth factor receptor α (PDGFRα)-positive. These cells were purified from the testes of 1-week-old rats. The cells contained proteins known to be involved in LC development, including GATA4, c-kit receptor, and leukemia inhibitory factor receptor. The putative SLCs expanded over the course of 6 months while remaining undifferentiated. When treated in media that contained thyroid hormone, insulin-like growth factor I, and LH, 40% of the putative SLCs came to express 3βHSD and to synthesize testosterone. When transplanted into host rat testes from which LCs had been eliminated, the putative SLCs colonized the interstitium and subsequently expressed 3βHSD, demonstrating their ability to differentiate *in vivo*. We conclude that these cells are likely to be the sought-after SLCs.**

c-kit | leukemia inhibitory factor | platelet-derived growth factor receptor α | puberty | steroidogenesis

Leydig cells (LCs) are the primary source of testosterone in the male, and their differentiation in the testes during puberty is a signature event in the development of the male body plan. It is hypothesized, but far from proven, that LCs first arise from undifferentiated stem cells [stem LCs (SLCs)] (1–3). It has been suggested that, in rats, the putative SLCs are present in the testis at birth, and that by 11 days postpartum, at least some of their progeny express LC-specific genes and thus become committed to the LC lineage (4, 5).

The committed cells subsequently undergo phased transitions through progenitor and immature stages and ultimately to terminally differentiated adult LC stage (6). In particular, progenitor LCs (PLCs) form during days 12–28 postpartum (presumably from SLCs). The PLCs proliferate and also exhibit some aspects of differentiated function, including 3β-hydroxysteroid dehydrogenase (3βHSD) activity (7). Luteinizing hormone (LH) receptors (LHRs) first appear as the PLCs differentiate, suggesting that SLCs are likely to be independent of LH control (8). The development of the steroidogenic capacity of PLCs requires stimulation by LH (9). The mitotic activity of PLCs gradually is reduced, and the cells enlarge in transition to another intermediate, the immature LC (ILC), during days 28–56 postpartum. ILCs undergo a final division and transform into testosterone-secreting adult LCs (ALCs) by day 56 (10).

Analysis of purified LCs by DNA array profiling has shown that the genes for platelet-derived growth factor receptor α (PDGFRα) and c-kit attain their highest expression levels at the PLC stage and subsequently decrease with the differentiation of PLCs into ALCs (6). PDGFRα also appears *in situ* during the first week postpartum in the interstitial spindle-shaped cells that are putative SLCs (11), when neither 3βHSD nor LHR is detected in these cells (1, 4). Analyses of PLCs, ILCs, and ALCs make it evident that testicular PDGFRα expression is restricted to the LC lineage (6, 12). In mice, the knockout of PDGF-A prevents LC development, revealing a potentially critical function of PDGFRα signaling in SLCs (12).

The goals of the present study were to determine whether putative SLCs could be identified in the neonatal rat testis *in situ* and, if so, to isolate and characterize these cells and to demonstrate their involvement in lineage-specific development of the adult LCs. Putative SLCs were purified from the testes of 1-week-old rats by selection of spindle-shaped interstitial cells that were 3βHSD- and LHR-negative and immunoreactive for PDGFRα. These cells were found to divide *in vitro* in the presence of factors known to stimulate stem cell renewal and to express 3βHSD and produce androgen when stimulated by a differentiation-inducing medium (DIM). Moreover, transplantation of the putative SLCs into host testes from which LCs were eliminated resulted in their colonizing the testes and then differentiating.

## Results

**Identification of SLCs *in Situ*.** Fig. 1 shows cells that were double-immunolabeled for 3βHSD and BrdUrd in sections of postnatal day 7 (Fig. 1*A*) and 14 (Fig. 1*B*) rat testes. Fig. 1*A* shows that, on day 7, spindle-shaped cells, such as the BrdUrd-positive cell indicated by the arrow, were seen in the peritubular layer of testis sections that were 3βHSD-negative, as would be expected of undifferentiated cells. Round-shaped and clustered 3βHSD-positive cells also were seen (Fig. 1*A*; indicated by *), presumed to be fetal LCs (FLCs). By postnatal day 14, the spindle-shaped cells in the peritubular layer had become 3βHSD-positive (Fig. 1*B*, arrow) and consequently were designated PLCs. Fig. 1 *B*1 and *B*2 show spindle-shaped cells in testes on day 14 that were 3βHSD-positive and, respectively, BrdUrd-negative and -positive. Fig. 1*C* (upper-right-hand graph) shows the transition in 3βHSD immunolabeling of the spindle-shaped cells, from 100% unstained on day 7 to a cohort (17 ± 4%) that were 3βHSD-positive, designated as PLCs, on day 14. The percentages of BrdUrd-positive spindle-shaped cells that were

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Abbreviations: LC, Leydig cell; SLC, stem LC; PLC, progenitor LC; ALC, adult LC; 3βHSD, 3β-hydroxysteroid dehydrogenase; LH, luteinizing hormone; LHR, LH receptor; ILC, immature LC; FLC, fetal LC; PDGF, platelet-derived growth factor; PDGFRα, PDGF receptor α; PDGF-BB, PDGF β homodimer; DIM, differentiation-inducing medium; EDS, ethanedimethanesulfonate; LIF, leukemia inhibitory factor; LIFR, LIF receptor; IGF-I, insulin-like growth factor I.

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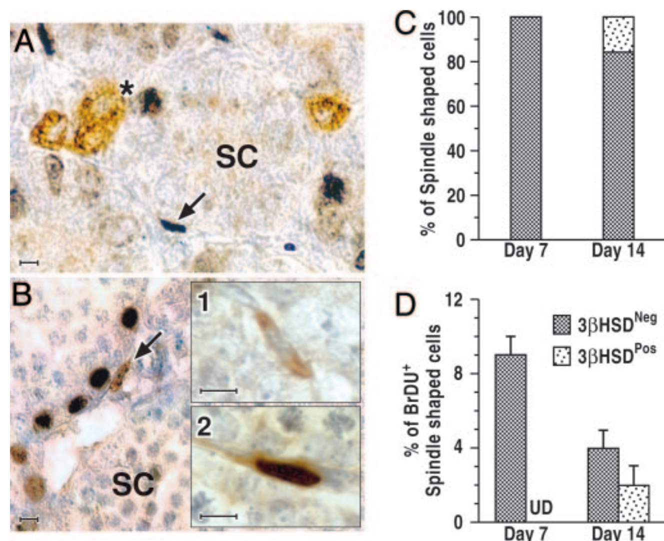


**Fig. 1.** Double immunolabeling of testicular cells for 3$\beta$HSD and BrdUrd in sections of testes from day 7 (*A*) and day 14 (*B*) rats. A cluster of 3$\beta$HSD-positive cells, presumed to be FLCs, is immunolabeled (brown staining, indicated by *) on day 7 (*A*). At this age, spindle-shaped interstitial cells, presumed to be SLCs (e.g., arrow), were often BrdUrd-labeled (dark blue). One week later (day 14, *B*), spindle-shaped PLC (e.g., brown stained, indicated by arrow) are seen. (*B*1 and *B*2) $\beta$HSD-positive spindle-shaped cells that were either negative (*B*1) or positive (*B*2) for BrdUrd staining. (*C* and *D*) Graphs showing the percentages of spindle-shaped cells that were 3$\beta$HSD-positive on days 7 and 14 (*C*) and BrdUrd-labeled on those days (*D*). (Scale bars, 10 $\mu$m.)

stained and unstained for 3$\beta$HSD were also tabulated (Fig. 1*D*, lower-right-hand graph). These results showed that, whereas on day 7, no BrdUrd-labeled PLCs were seen, 2 ± 1% were BrdU-labeled on day 14. Based on these *in situ* observations of the peritubular and perivascular interstitial boundary layer, we postulated that the 3$\beta$HSD-negative spindle-shaped cells seen on day 7 were putative SLCs, and that the PLCs identified on day 14 derived from the SLCs.

**Isolation and Characterization of LHR-Negative–PDGFR$\alpha$-Positive Putative SLCs.** Putative SLCs were isolated from the testes of pups on postnatal days 0, 7, 14, and 21 (13). Most (95–98%) cells obtained from day 0 and 7 testes did not stain for 3$\beta$HSD, as would be expected from the *in situ* analyses described above. At both ages, dispersion-resistant clusters, representing 5% and 2% of the cells isolated from day 0 and 7 testes, respectively, stained intensely and were identified as FLCs (Fig. 2*A*). The presence of these cells was consistent with *in situ* analyses of 3$\beta$HSD-stained testis sections (Fig. 1*A*). The yields of the 3$\beta$HSD-negative cells differed at the two ages: on day 7, $2.0 \pm 0.2 \times 10^6$ cells were obtained from the testes of 60 pups (average 17,000 cells per testis). This was more than double the yield from comparable numbers of day 0 testes. In contrast to cells from days 0 and 7, 90% of the cells harvested from day 14 and 21 testes stained faintly for 3$\beta$HSD, and 5% were intensely stained (data not shown), as would be expected of PLCs (13).

With the knowledge that FLCs express LHR, we used an LHR antibody to selectively remove the cells that express LHR. We found that this removed the cells that had stained intensely for 3$\beta$HSD. The remaining cells were incubated with an anti-PDGFR$\alpha$ antibody. The PDGFR$\alpha$-positive cells obtained were purified further by plating them onto anti-PDGFR$\alpha$-coated dishes. Approximately 99% of the cells obtained in this way were PDGFR$\alpha$-positive and 3$\beta$HSD-negative (Fig. 2*B*). Of the two million 3$\beta$HSD-negative cells (putative SLCs) that had been isolated from the testes of 60

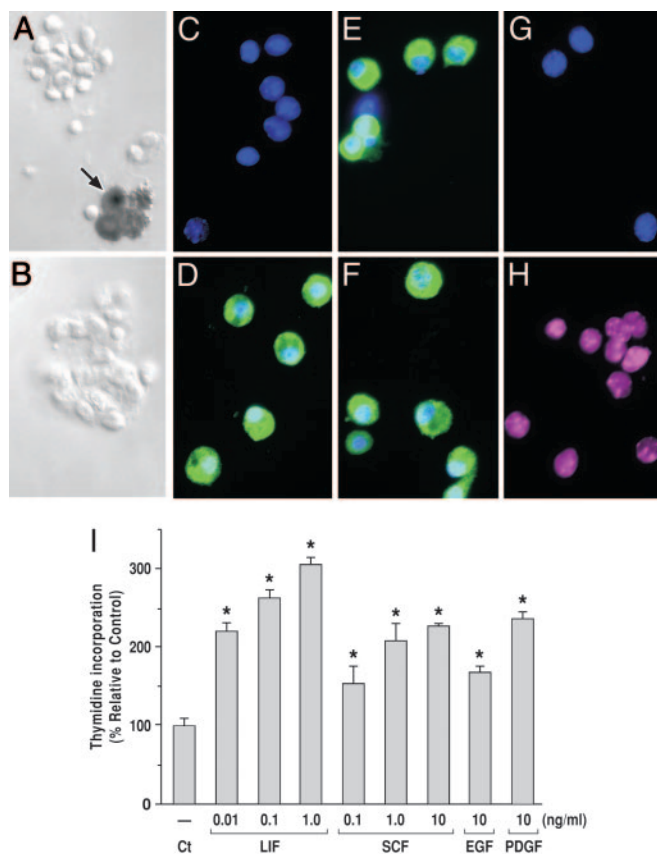


**Fig. 2.** Proliferation of putative SLCs. Freshly isolated spindle-shaped cells (putative SLCs and PLCs) contaminated with FLCs stained strongly for 3$\beta$HSD (arrow) are shown in *A*. The cells remaining after elimination of LHR-positive cells, and then selection of the PDGFR$\alpha$-expressing cells, the putative SLCs, did not stain for 3$\beta$HSD (*B* and *C*) or LHR (*G*). They were, however, positively stained for PDGFR$\alpha$ (*D*), c-kit (*E*), LIFR (*F*), and GATA4 (*H*). DAPI staining (blue) was used to provide nuclear contrast. Cells were incubated in varying concentrations of LIF, stem cell factor (SCF), EGF, or PDGF-BB, and [$^3$H]thymidine incorporation was measured in a scintillation counter. Compared with control (basal LCM medium alone), LIF, SCF, EGF, and PDGF significantly stimulated SLC proliferation activity (*I*). Mean ± SE (*n* = six samples of two experiments). *, Significant difference compared with control.

pups, ≈50% remained after the LHR/PDGFR$\alpha$ antibody purification, or ≈8,500 cells per testis.

The LHR-negative–PDGFR$\alpha$-positive cells were plated on coverslips and characterized for the stem markers leukemia inhibitory factor (LIF) receptor (LIFR) and c-kit, and the LC lineage markers GATA4, 3$\beta$HSD, LHR, P450scc, and P450c17 (Table 1). The LHR-negative–PDGFR$\alpha$-positive cells were unstained for 3$\beta$HSD (Fig. 2*C*) or LHR (Fig. 2*G*), indicating that the isolated cells were not contaminated with FLCs. In contrast, 100% of these cells expressed LIF receptor (LIFR) (Fig. 2*E*), and most (84 ± 5%, mean ± SEM, *n* = six separate preparations) also expressed c-kit

**Table 1. Percentage of positive cells stained after immunoselection of interstitial cells**

| Protein staining | % positive cells (mean ± SE) |
|---|---|
| 3$\beta$HSD | 0 |
| LHR | 0 |
| PDGFR$\alpha$ | 99.36 ± 0.36 |
| c-kit | 83.84 ± 4.75 |
| LIFR | 97.32 ± 1.72 |



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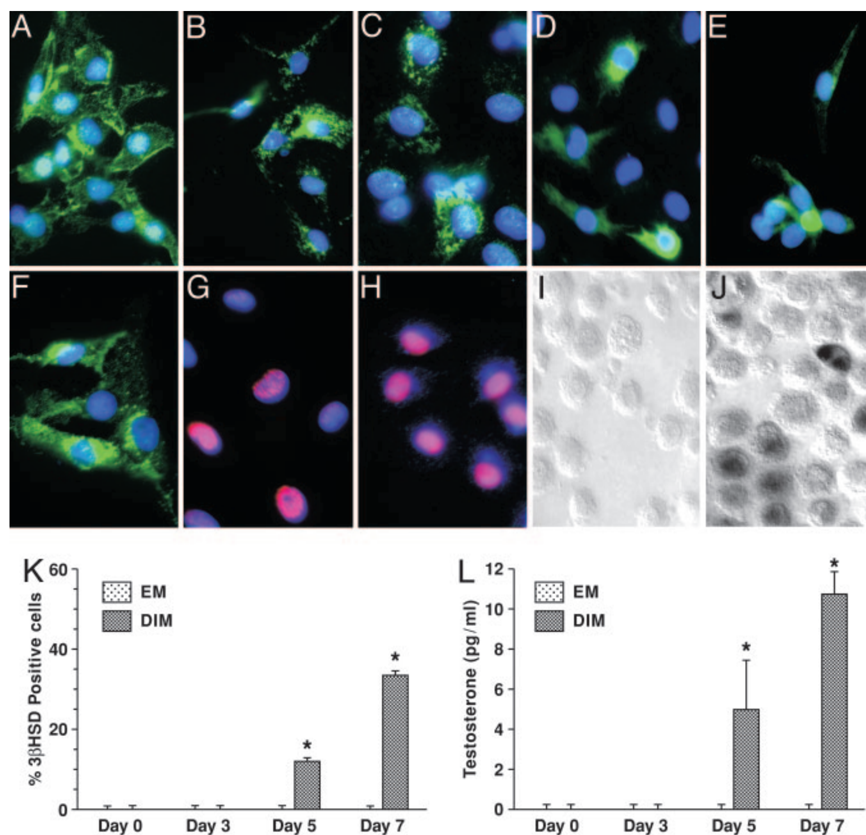


**Fig. 3.** Differentiation of putative SLCs into LCs *in vitro*. Expression of lineage-specific markers was seen after culture of the putative SLCs in DIM (LH + IGF-I and T3) for 7 days. Representative preparations were stained as follows: LHR (*A*), StAR (*B*), P450scc (*C*), 3βHSD (*D*), P450c17 (*E*), PDGFRα (*F*), steroidogenic factor 1 (SF-1) (*G*), and GATA4 (*H*). LHR-negative–PDGFRα-positive SLCs are unstained for 3βHSD enzyme (*I* and *K*) and become 3βHSD-positive (*J* and *K*) after culture. Testosterone production progressively increased with the culture in DIM (*L*). Mean ± SE ($n = 6$). *, Significant change compared with control.

(Fig. 2*F*), two common markers for stem cells (14, 15). The majority were also positive for the GATA4 transcription factor (Fig. 2*H*), which has been implicated in LC development (16). The LHR-negative–PDGFRα-positive cells were plated in an enriched cell culture medium designed for expansion of cell number. The attached cells were continuously grown for >6 months at subconfluent densities. Under these conditions, the cells maintained a stable LHR-negative–PDGFRα-positive phenotype and had a doubling time of 3 days.

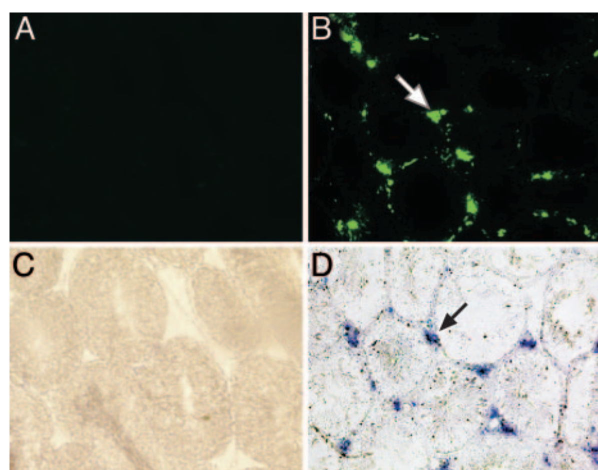


**Fig. 4.** Differentiation of putative SLCs *in vivo* in adult rat testes that had been depleted of LCs by EDS injection of the rats. (*A* and *C*) Testis section from a control rat 4 days after the rat received an injection of vehicle under epifluorescent illumination (*A*) and after histochemical staining for 3βHSD enzyme activity (*C*). (*B*) Fluorescent interstitial staining in rat testis 10 days after implantation of labeled donor SLCs into the testes of EDS-injected rats. (*D*) Many of the labeled donor cells were also positively stained for 3βHSD (arrow).

**Proliferation of LHR-Negative–PDGFRα-Positive Putative SLCs.** Given that the LHR-negative–PDGFRα-positive cells expressed LIFR, PDGFRα, and c-kit, and that in other systems, these receptors are known to mediate stem cell renewal, we determined whether the growth factors LIF, PDGF, and stem cell factor, would stimulate proliferative activity. As seen in Fig. 2*I*, these factors significantly increased the proliferation in a dose-dependent manner, reaching a maximum of a threefold increase with 1 ng/ml LIF.

To assess whether LHR-negative–PDGFRα-positive putative SLCs could be induced to differentiate into 3βHSD-positive LCs *in vitro*, subconfluent cultures at different passages were placed in a DIM. By 7 days in DIM, many cells exhibited signs of differentiation, as judged by expression of proteins and enzymes involved in androgen biosynthesis, including LHR (Fig. 3*A*), StAR (Fig. 3*B*), P450scc (Fig. 3*C*), 3βHSD (Fig. 3*D*), and P450c17 (Fig. 3*E*). In addition, they stained positively for PDGFRα (Fig. 3*F*) and the nuclear transcription factors steroidogenic factor 1 (Fig. 3*G*) and GATA4 (Fig. 3*H*), which are known to be involved in LC development (Fig. 3 *A*–*D*). When cells from separate passages were used to induce differentiation, the proportion 3βHSD-positive cells ranged from 29% to 40% after 7 days of culture (Fig. 3 *J* and *K*), indicating the stability of the process. The media were collected and the concentration of testosterone was measured. The data showed that, as a function of time in DIM [LH, insulin-like growth factor

I (IGF-I), thyroid hormone (T3), and PDGF], testosterone synthesis is induced in parallel with the increased percentage of 3βHSD-staining cells (Fig. 3*K*).

**Putative SLCs Colonize the Interstitium of LC-Depleted Host Rat Testes and Express 3βHSD.** The LC cytotoxin ethane-dimethanesulfonate (EDS) was administered to adult rats, and fluorescently labeled LHR-negative–PDGFRα-positive SLCs were injected into the parenchyma at the cranial pole of the testis 4 days later, when ALCs in the host testes had been eliminated. On day 10 after implantation, the testes were removed for analysis. Fluorescently labeled cells were found exclusively in the interstitium. Significant numbers of the fluorescent cells became positively stained for 3βHSD activity by 10 days after their introduction into the testes (Fig. 4*D*). In contrast, EDS-treated rats that received saline control injections lacked fluorescence (Fig. 4*C*). Importantly, in EDS-treated control rats, 3βHSD histochemical staining was not observed, indicating the depletion of mature LCs from the host testes was successful (Fig. 4 *A* and *C*).

## Discussion

We hypothesized herein that the precursor LCs ultimately derive from undifferentiated SLCs, and that SLCs must be present in the rat testis before the onset of precursor cell differentiation. Well characterized stem cells have several features in common that define them as stem cells, important among which are the ability for self renewal and for differentiation (17, 18). Thus, SLCs would be expected to be present in the testis only in small numbers and to be maintained by renewal cell divisions. One or both of the progeny of an SLC division would be expected to be capable of undergoing commitment to the LC lineage, a still poorly understood process that restricts developmental fate. The numbers of committed SLCs would be expected to increase through amplification, creating a pool of cells that undergoes differentiation into cells that are able to synthesize and secrete testosterone. Thus, to test the hypothesis that the precursor cells that give rise to adult LCs ultimately derive from undifferentiated SLCs, we searched for cells in the neonatal testis with three distinctive characteristics: (*i*) the ability to proliferate and expand indefinitely *in vitro* (self renew) without showing signs of differentiation; (*ii*) the ability to differentiate (i.e., to express 3βHSD and ultimately synthesize testosterone); and (*iii*) when transplanted into host rat testes, the ability to colonize the interstitium and subsequently differentiate *in vivo*.

Herein we describe the harvesting of an enriched fraction of cells with the above properties, putative SLCs, from the testis by selecting LHR-negative–PDGFRα-positive cells from an interstitial cell fraction on day 7 postpartum. Day 7 postpartum was selected, because this is before the onset of the steroidogenic enzyme and LHR gene expression seen in the spindle-shaped interstitial cells 4 days later, on day 11 postpartum (4). The putative SLCs displayed essential characteristics that define cells as stem cells in that they (*i*) expanded their numbers during prolonged culture *in vitro*; (*ii*) could be induced to express proteins associated with LC differentiation, including LHR, StAR, P450scc, 3βHSD, and P450c17; and (*iii*) colonized a host tissue that had been depleted of the mature LCs.

Morphologically, the putative SLCs are similar in morphology to PLCs, but they do not express LC lineage-specific markers, including P450scc, 3βHSD, P450c17, StAR, and LHR. Given that the cells in the putative stem cell fraction did not produce testosterone in response to LH, we conclude there was little or no contamination by fetal and later-stage intermediates of the postnatal LCs (PLCs, ILCs, and ALCs). Thus, this study identifies, isolates, and documents the characteristics of a cell population (putative SLCs) and elicits their transition from an undifferentiated to a differentiated state, as defined by the ability to express steroidogenic activity. Based on the labeling of cells *in situ* for 3βHSD and BrdU immunoreactivity, we are confident that the putative SLCs isolated from postpartum day 7 testes are the undifferentiated spindle-shaped cells seen in testis sections.

If the spindle-shaped cells were directed toward the LC lineage in a uni- or pluripotent fashion, they would be expected to express lineage-specific transcription factors. GATA4, a nuclear transcription factor that has been shown to be present in steroidogenic cell lineages (19, 20), is expressed by the SLCs, but whether these cells are uni- or pluripotential will require further analysis.

LIFR was found to be present in putative SLCs. This is notable because LIF, a member of the IL-6 family of cytokines, mediates self renewal of stem cells. LIF is required for long-term self renewal of neural stem cell cultures (21) and for maintenance of primordial germ cells *in vitro* (22). In the rat testis, LIF is detectable from 13.5 days of gestation onward and is predominantly expressed by peritubular myoid cells surrounding the seminiferous tubules (23). In the first week postnatally, the peritubular cells have a fibroblastic ultrastructure, appearing spindle-shaped by light microscopy (2), and form a two- to three-cell-layer-thick boundary tissue (lamina propria). The putative SLCs are situated in the outermost layer of the boundary tissue, in the interstitial space (2), and therefore are likely targets of LIF. The LHR-negative–PDGFRα-positive cell population was found to express LIFR, and LIF stimulated their proliferation *in vitro*. LIFR and its intracellular signaling partner subunit gp130 are expressed at their highest levels in PLCs relative to expression levels in ILCs and ALCs (6), thus linking the identified putative SLCs to later stages of LC differentiation. LIF actions on the later LC stages are not well understood but may cause decreased steroidogenesis (24) through lowering of cholesterol substrate availability in the mitochondria (25). In most stem cells (e.g., mouse embryonic stem cells), LIF withdrawal is a stimulus for differentiation (26). These data suggest that the LIF signaling pathway is present in the LC lineage, and that it may have a role in stimulating renewal of the identified putative SLCs.

It is established that the development of LC steroidogenic capacity requires stimulation by LH (9). In genetically hypogonadal LH-deficient GnRHr$^{hpg}$ mice, for example, ALC numbers reach only 10% of control (27). Similarly, although 3βHSD-expressing PLCs are formed in LHR knockout mice (28), LCs fail to develop further and remain severely hypoplastic (29, 30). These results indicate that factors other than LH may act on the putative SLCs to induce LHR expression. Evidence from mouse knockout studies suggests a role of IGF-I (31) and PDGF (12) in SLC proliferation and differentiation. Within the SLC, the transcription factors GATA4 and steroidogenic factor 1 (32) are involved in the induction of LC-specific gene expression.

PDGFRα expression is found in neural stem cells and is a characteristic of this stem cell type (33, 34). Expression of PDGFRα was observed in the isolated LHR-negative–PDGFRα-positive cells, corroborating an increasing body of evidence that PDGF signaling is necessary for stem cell commitment and differentiation in the LC lineage of mice and neonatal rats (35, 36). It will now be possible to ask whether the FLCs that differentiate *in utero* and that have been shown to respond to PDGF share a common stem cell origin with the ALCs that begin differentiation on day 10 postpartum. In mice, PDGFRα is expressed at low levels in the mesenchyme of the mesonephros at day 11.5 postconception in both sexes. It is also highly expressed in the coelomic epithelium and at the gonad-mesonephros border at 11.5 days in both sexes. By day 12.5, expression is confined to the male and is very strong in the interstitial cells of the fetal testis (35). In neonatal testis, PDGFRα is located in the interstitial areas near peritubular cells (37) and, in adult testes, PDGFRα is localized exclusively in LCs. This supports the hypothesis that PDGFRα can be used as a LC lineage marker. PDGFs can be added to a lengthening list of factors of Sertoli cell origin that modulate LC function in the neonatal testis, along with proteins such as IGF-I (31) and Kit ligand (KL) (38).

In the present study, we demonstrate that PDGFRα-positive cells in day 7 of postnatal are putative SLCs: they are proliferative and

self renewing and can propagate up to at least 6 months. They have no properties of LCs, such as steroidogenic enzyme expression, but are able to differentiate into LCs *in vitro* and *in vivo*. PDGFR$\alpha$ signaling is now seen to be crucial for the development of both fetal and ALC populations (12, 35). Whether the SLCs that give rise to regenerating LC in the EDS model [reportedly from nestin-positive vascular stem cells (3)] also express PDGFR$\alpha$ is worthy of further investigation.

*In vitro* induction of differentiation of the isolated putative SLCs into the LC lineage showed that only $\approx$40% of the cells become 3$\beta$HSD-positive in 7 days of culture. Although the identity of the remaining cells is not known, we favor the hypothesis that the undifferentiated cells are SLCs that have yet to differentiate. Our rationale for favoring this hypothesis is that stem cells normally maintain a certain proportion of their numbers in the stem cell niche, whereas the remaining proportion commit to lineage specific differentiation.

## Materials and Methods

**Chemicals.** DMEM (DMEM-Ham's F-12, D-2906) was purchased from Sigma. Avian myeloblastosis virus reverse transcriptase was purchased from Promega. The following antibodies were purchased from Santa Cruz Biotechnology: rabbit polyclonal antiserum against PDGFR$\alpha$ (catalog no. sc-338); c-kit (catalog no. sc-13508); LIFR (catalog no. sc-659); goat polyclonal antiserum against SF1 (catalog no. sc-6035); and GATA4 (catalog no. sc-1237). Other antibodies were: 3$\beta$HSD (provided by Van Luu-The, Laval University, Quebec); P450 side-chain cleavage enzyme (P450scc, RDI-P450sccabr; RDI Research Diagnostics, Flanders, NJ); 17$\alpha$-hydroxylase (P450c17) and StAR (provided by D. B. Hales, University of Illinois, Chicago); and LHR (provided by Jay Wimalasena, University of Tennessee, Knoxville).

**Animals.** Sprague–Dawley rats (1 and 2 wk and 3 mo old) were purchased from Charles River Laboratories. To label dividing cells, all animals received an i.p. injection of BrdUrd (40 $\mu$g/g body weight; catalog no. 280879; Roche Molecular Biochemicals) at 1 or 2 h before death. The animals were killed by asphyxiation with $CO_2$. The animal protocol was approved by the Institutional Animal Care and Use Committee of Rockefeller University, New York (protocol no. 01-041).

**Immunohistochemistry.** The immunohistochemical detection of 3$\beta$HSD and BrdUrd was performed as described (39, 40). Briefly, after vascular perfusion with Bouin's solution, the testes were postfixed overnight in the same fixative, then embedded in paraffin. Double staining for 3$\beta$HSD and BrdUrd was performed by using 6-$\mu$m-thick transverse sections of testis that were mounted on glass slides (catalog no. 12-550-15; Fisher Scientific). Avidin–biotin immunostaining was performed by using a kit (catalog nos. PK-2200 for BrdUrd and PK-6101 for 3$\beta$HSD; Vector Laboratories) according to the manufacturer's instructions. Antigen retrieval was carried out by microwave irradiation for 10 min in 10 mM (pH 6.0) citrate buffer, and endogenous peroxidase was blocked with 0.5% $H_2O_2$ in methanol for 30 min. The sections were then incubated with a monoclonal anti-BrdUrd antibody (RPN 202; Amersham Pharmacia Biosciences) for 30 min at room temperature. The antibody bound to the nuclei was visualized with diaminobenzidine (catalog no. sk-4100; Vector Laboratories), and the labeled nuclei were stained black by adding a nickel solution to the chromogen. After washing, the sections were double-labeled by incubation with a 3$\beta$HSD polyclonal antibody diluted 1:3,000 for 1 h at room temperature. The antibody–antigen complexes were visualized with diaminobenzidine alone, resulting in brown cytoplasmic staining in positively labeled LCs. The sections were counterstained with Mayer hematoxylin, dehydrated in graded concentrations of alcohol, and coverslipped with resin (Permount, SP15–100; Fisher Scientific). In control experiments, sections were incubated with nonimmune rabbit IgG (3$\beta$HSD) or mouse IgG (BrdUrd) by using the same working dilution as the primary antibody.

Using testis sections, labeling indices for 3$\beta$HSD staining and BrdUrd incorporation were determined to provide relative measures of the numbers of differentiated and proliferative LCs, respectively. Spindle-shaped cells in the interstitial space were easily distinguishable from round testicular macrophages and more mature LCs and from peritubular cells immediately adjacent to the seminiferous tubule by previously published criteria (10). Counts of at least 500 cells per animal were made from the testes of each of five different animals. The 3$\beta$HSD and BrdUrd labeling indices were then calculated as the number of labeled cells divided by the total count (labeled + unlabeled) multiplied by 100.

Immunofluorescent staining was performed on isolated LCs (see below) by using LCs grown on microscope cover glasses. Cells were fixed with 4% formaldehyde, washed with PBS, and permeabilized with 0.1% wt/vol Saponin detergent in PBS plus 10% normal serum. Nonspecific binding was blocked by incubation with 10% normal serum before addition of the primary antibody. Cells were incubated with different antibodies for 1 h at room temperature. Cells were then incubated with Alexa488-conjugated second antibody for 1 h. Afterward, the cells were counterstained with DAPI, mounted onto glass microscope slides, and coverslipped. The slides were examined under a Nikon fluorescence microscope with a filter suitable for detecting the fluorescence of FITC (green). A total of 500 cells were counted for each of six separate cell preparations.

**Percoll Purification of SLCs.** Testes from 60 1-wk-old rats were removed and decapsulated. Interstitial cells were dissociated from the seminiferous tubules with 0.25 mg/ml collagenase (collagenase-D; Roche Molecular Biochemicals) in medium 199 for 10 min at 34°C with shaking. The separated cells were filtered through two layers of 100-$\mu$m pore-size nylon mesh, centrifuged at 250 $\times$ *g*, and resuspended in 55% isotonic Percoll. After density gradient centrifugation at 22,000 $\times$ *g* for 45 min at 4°C, a fraction of spindle-shaped cells was collected between densities of 1.064 and 1.070 g/ml. Approximately 2 million cells were obtained at this step. The cells were washed with HBSS, centrifuged at 250 $\times$ *g*, and resuspended in phenol red-free 1:1 DMEM:F12 supplemented with 1 mg/ml bovine albumin. FLC contamination was evaluated by histochemical staining for 3$\beta$HSD activity, with 0.4 mm of etiocholanolone as the steroid substrate (41). FLCs were present in the preparations as clusters that were intensely stained by 3$\beta$HSD histochemistry.

**Immunoselection.** Immunoselection of the putative SLCs from among the spindle-shaped cell fraction was carried out by a modification of published procedures (42). Our objective was to select for cells that were LHR-negative and PDGFR$\alpha$-positive. Three 60-mm-diameter Petri dishes were incubated overnight at 4°C with 2 ml of anti-IgG antibody solution-two with 10 $\mu$g/ml goat anti-mouse IgG (Jackson ImmunoResearch) and one with 10 $\mu$g/ml goat anti-rabbit IgG (Jackson ImmunoResearch) in 50 mM Tris, pH 9. The dishes were washed three times with calcium- and magnesium-free PBS and incubated for several hours at room temperature with the appropriate antibodies for immunoselection. The interstitial cells were washed by centrifugation and resuspended in minimal essential medium with Hank's balanced salt solution (MEM-H) containing 0.5% FCS, passed over an uncoated Petri dish to remove macrophages, and then sequentially passed over two dishes coated with anti-LHR antibody (1:200) to remove LHR-positive FLCs. The resulting cell preparation was incubated in a dish coated with a 1:200 dilution of anti-PDGFR$\alpha$ rabbit serum. Each selection step was for 30 min at room temperature with occasional gentle swirling. The final preparation was washed with LCM, and the immunoselected PDGFR$\alpha$-positive cells were removed with trypsin (0.125%), washed, and resuspended in expan-

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sion medium (LCM, 2% FCS). These cells, designated putative SLCs, were cultured and passaged.

**SLC Culture.** After immunoselection, the putative SLCs were cultured in an expansion medium (EM) adapted for embryonic stem cell culture (43) with modification. In brief, EM consisted of DMEM:F12 supplemented with 2% FBS (FCS), 1 nM dexamethasone, 1 ng/ml LIF, 10 ng/ml human PDGF $\beta$ homodimer (PDGF-BB), 10 ng/ml mouse EGF, and the insulin–transferrin–sodium selenite (ITS) cell culture supplement (5 mg/liter insulin, 5 mg/liter transferrin, 5 $\mu$g/liter sodium selenite; Sigma, I-1884). SLCs were maintained in this medium for at least 6 months. To determine whether these cells could be induced to differentiate into the LC lineage, the cells were replated in a new medium containing differentiation-inducing factors. Several hormones were evaluated individually for their ability to induce differentiation, including 10 ng/ml PDGF-BB, 1 nM thyroid hormone, 1 ng/ml LH, and 70 ng/ml IGF-I. Individually, no inductive activity was detectable and, therefore, a mixture was devised to induce differentiation (DIM) that contained phenol red-free DMEM:F12, 2% FCS, 10 ng of PDGF-BB, 1 ng/ml LH, 1 nM thyroid hormone, 70 ng/ml IGF-I, and the ITS supplement.

**SLC Proliferative Activity.** Thymidine incorporation was used to assess cell proliferation, as described (44). The putative SLCs were treated with DMEM:F12 (LCM) alone or in combination with different growth factors including 2% FCS, 0.1–1.0 ng/ml LIF, 0.1–10 ng/ml KL, 10 ng/ml PDGF-BB, or 10 ng/ml EGF. Cells were labeled with [$^{3}$H]thymidine (DuPont–New England Nuclear) at 1 $\mu$Ci/ml (1 Ci = 37 GBq) (specific activity, 104.7 Ci/mmol) during the last 16 h of incubation. After labeling, the cells were washed twice with Dulbecco's PBS and harvested. Cells were lysed in 0.5 ml of hyamine hydroxide (ICN), and radioactivity was measured in a liquid scintillation counter.

**Assay of Testosterone Concentration.** Testosterone concentration in the medium was measured with a tritium-based radioimmunoassay (RIA), as described (45). Interassay variation of the testosterone–RIA was between 7% and 8%.

**SLC Transplantation.** Cultured SLCs were tagged with a fluorescent lineage tracking dye, carboxyfluorescein diacetate, and succinimidyl ester (Cell Trace CFSE cell Proliferation Kit; Molecular Probes/Invitrogen, no. C34554), as described in the manufacturer's instructions. In brief, SLCs were removed from fibronectin-coated culture plates with trypsin, resuspended in warm PBS, and incubated for 15 min at 34°C with the CFSE dye (final dye concentration was 5 $\mu$M). After incubation, the stained cells were washed with warm PBS and centrifuged. The cells were resuspended in warm PBS and incubated for 30 min at 34°C to complete modification of the dye. The stained SLCs were then washed two additional times with warm PBS and loaded into a 0.3-ml syringe with a 29½-gauge needle for injection into the testis.

SLCs were transplanted into the testes of EDS-treated adult Sprague–Dawley male rats. EDS was administered in a single dose of 75 mg/kg body weight. This treatment resulted in a rapid elimination of the LCs present in the adult testis by 4 days after treatment (46). Approximately 1,000,000 SLCs in a 25-$\mu$l volume of PBS were injected into the parenchyma of recipient testes 4 days after the rats had received EDS. The control animals for the experimental group were EDS-treated rats that received a testicular injection of the PBS vehicle. Tissues from all animals were examined 10 days after transplantation (day 14 after EDS). Our rationale for choosing 14 days after EDS is that, at this time, fluorescently tagged SLCs that are transplanted into rat testes show no 3$\beta$HSD activity at 14 days but do so thereafter (unpublished data).

**Statistics.** Between-group differences were determined by one-way ANOVA. In cases of $P < 0.05$, Duncan's multiple comparison test was used to identify significant differences between groups (47). The testosterone production data were analyzed by Student's $t$ test, with a Sidak adjustment to the $P$ value for planned comparisons to identify significant differences between treatment and control (47). All data are expressed as mean ± SEM. Differences were regarded as significant at $P < 0.05$.

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