Regulation of seminiferous tubule-associated stem Leydig cells in adult rat testes

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Testicular Leydig cells are the primary source of testosterone in males. Adult Leydig cells have been shown to arise from stem cells present in the neonatal testis. Once established, adult Leydig cells turn over only slowly during adult life, but when these cells are eliminated experimentally from the adult testis, new Leydig cells rapidly reappear. As in the neonatal testis, stem cells in the adult testis are presumed to be the source of the new Leydig cells. As yet, the mechanisms involved in regulating the proliferation and differentiation of these stem cells remain unknown. We developed a unique in vitro system of cultured seminiferous tubules to assess the ability of factors from the seminiferous tubules to regulate the proliferation of the tubule-associated stem cells, and their subsequent entry into the Leydig cell lineage. The proliferation of the stem Leydig cells was stimulated by paracrine factors including Desert hedgehog (DHH), basic fibroblast growth factor (FGF2), platelet-derived growth factor (PDGF), and activin. Suppression of proliferation occurred with transforming growth factor β (TGF-β). The differentiation of the stem cells was regulated positively by DHH, lithium- induced signaling, and activin, and negatively by TGF-β, PDGFBB, and FGF2. DHH functioned as a commitment factor, inducing the transition of stem cells to the progenitor stage and thus into the Leydig cell lineage. Additionally, CD90 (Thy1) was found to be a unique stem cell surface marker that was used to obtain purified stem cells by flow cytometry.

Leydig cell | stem cell | DHH | CD90 | testosterone

Testicular Leydig cells are the primary source of testosterone (T) in males. T is essential for the development of the male reproductive system and the maintenance of male reproductive functions (1, 2). In addition to defects in reproductive system, its deficiency in the adult contributes to other symptoms that include increased body fat, decreased muscle mass, increased fatigue, depressed mood, decreased cognitive function (3, 4), and reduced immune response (5, 6). In aged men, low T has been reported to contribute to mortality (7). Thus, the formation and maintenance of a functional Leydig cell population throughout adult life is of fundamental importance.

In rodents and humans, T production gradually increases from the peripubertal period through the adult, coincident with the development of adult Leydig cells. There now is compelling evidence that most, if not all, of the adult Leydig cells arise from stem cells, not from the transdifferentiation of fetal Leydig cells (8, 9). In previous studies, we and others isolated cells from neonatal testes that expressed platelet-derived growth factor receptor-α (PDGFα) or nestin (9–11). Depending on culture conditions, these cells were shown to be capable of proliferating indefinitely or of differentiating into T-producing Leydig cells and, thus, were identified as stem Leydig cells (9–11). During their differentiation, these cells proceeded through two intermediate stages, progenitor Leydig cells and immature Leydig cells, before becoming adult Leydig cells (12). The gene expression pattern by the stem Leydig cells was similar to that of bone marrow stem cells and quite different from the patterns of the cells in the Leydig cell lineage (13).

The adult Leydig cells, once established, turn over slowly during adult life. However, when these cells are eliminated from adult testes by treating the rats with ethane dimethanesolfonate (EDS), new, fully functional adult Leydig cells reappear (14, 15). In initial efforts to localize the precursor cells, seminiferous tubules were isolated from EDS-treated testes and cultured with luteinizing hormone (LH) (16). Cells on the tubule surfaces first underwent division, and then differentiated and produced T (17). These results suggested that in addition to reported perivascular locations in the interstitial compartment (18), stem cells also were located on the surfaces of the seminiferous tubules (16, 19, 20).

Studies of a number of tissues have shown that stem cell self-renewal and differentiation are regulated by the interactions between cues that are intrinsic to the cells and extracellular signaling from the local environment, the latter referred to as the niche. In many tissues, including the testis, anatomic complexity combined with the inability to specifically mark stem cells make it difficult to identify these cells, characterize the niche, or determine the extrinsic factors involved in stem cell functions. Thus, as yet the extent to which the testicular environment influences the ability of the stem cells to proliferate and/or differentiate remains unknown. In the present study, we used a unique in vitro system of cultured seminiferous tubule to identify stem Leydig cells on the surface of seminiferous tubules and to assess the ability of factors associated with the tubules to regulate their proliferation and entry into the Leydig cell lineage (i.e., their differentiation). We provide evidence that the proliferation and subsequent differentiation of the stem Leydig cells are regulated by multiple niche factors from the seminiferous tubules, including PDGF, basic fibroblast growth factor (FGF2), transforming growth factor β (TGF-β), activin, Notch, Wnt, and most importantly, Desert hedgehog (DHH). Additionally, we report

Significance

Leydig cells, derived from stem cells, are the primary source of testosterone in males. Testosterone deficiency has been linked to sexual dysfunction and decreased bone density, muscle mass, and cognition. Thus, the formation and maintenance of the Leydig cell population is of fundamental importance. Through the use of a unique tubule culture system, we have identified critical niche factors that control the proliferation and differentiation of the Leydig stem cells. Additionally, we report on the isolation and purification of these cells through a specific cell surface marker protein.

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on the isolation of the stem cells by flow cytometric sorting through a specific cell surface marker protein, CD90.

Results

Differentiation of Stem Leydig Cells Associated with Seminiferous Tubules. We showed previously that functional Leydig cells can be generated by culturing Leydig cell-free seminiferous tubules with LH (16, 17). With the intent to identify the tubule-associated cells that give rise to T-producing Leydig cells, seminiferous tubule fragments of comparable lengths were cultured in medium containing LH for up to 4 wk. In the presence of LH, little or no T was produced by the tubules for the first week (Fig. 1*A*). By the end of 2 wk, T began to appear in the medium, and then increased with further culture through 4 wk (Fig. 1*A*). Examination of the morphology of freshly isolated and cultured tubules revealed a continuous layer of cells surrounding the tubules, on the outer surface of which were scattered, spindle-shaped cells (Fig. 1*B*). By 2 wk in culture, the continuous layer of cells had not changed in appearance, but lipid droplets had become apparent in the cytoplasm of the cells located on their surface (Fig. 1*C*). By 4 wk, the outer cells had become round and had far fewer lipid droplets (Fig. 1*D*).

The cells in the continuous layer were identified as myoid cells by their location and staining for α-smooth muscle actin (α-SMA), a well-established myoid cell marker (Fig. 1*E*). The cells on the surface of the myoid cell layer did not stain for α-SMA (Fig. 1*F*, white arrows) and also did not stain for CYP11A1, a marker for steroidogenic cells. By 4 wk of tubule culture with LH, CYP11A1-positive cells (red) were seen on the surface of the α-SMA–positive (green) myoid cells (Fig. 1*G*). The relationship of the α-SMA–positive myoid cells and the CYP11A1-positive cells is evident in whole mount tubules before (Fig. 1*H*) and after (Fig. 1*I* and Fig. S1) culture for 4 wk with LH. The cells outside the myoid cell layer also became 3βHSD-positive after 4 wk of culture with LH, consistent with their positive staining for CYP11A1 (Fig. S2).

Putative Niche Factors Involved in the Regulation of Stem Leydig Cell Proliferation and Differentiation. We hypothesized that there are circulatory and local factors that regulate the proliferation and differentiation of the tubule-associated stem Leydig cells. To test this hypothesis, we examined the effects of hormones, growth factors, and cytokines (Table S1) that had been shown by microarray analysis to be up-regulated or down-regulated significantly in the differentiation of stem to progenitor Leydig cells (Fig. S3), or that had been suggested to play roles in Leydig cell development and/or adult stem cell function in other organs. Identified factors were tested by using the cultured seminiferous tubule system that we developed (16, 17). The advantage of this system is that proliferation and differentiation of the stem cells occur sequentially, with most proliferation occurring during the first week of culture and most differentiation occurring subsequently (17). This same sequence occurs in vivo (21, 22).

To assess the effects of particular factors on stem cell proliferation, equivalent lengths of tubules were incubated with LH plus a given factor during week 1, the period during which active proliferation of the stem cells occurs, and then switched to LH-only

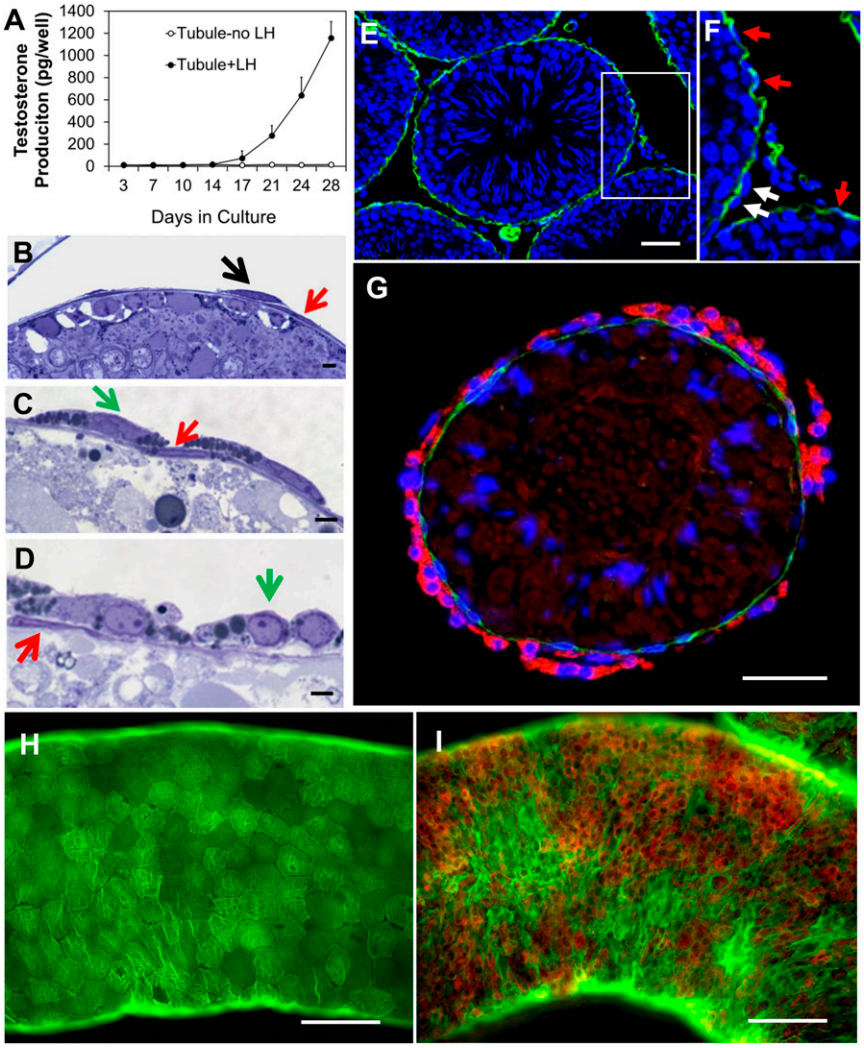

Fig. 1. Differentiation of Leydig cells associated with seminiferous tubules in vitro. (*A*) T production by tubules cultured with LH. (*B*) Section showing continuous layer of cells surrounding freshly isolated tubules (red arrow), with scattered spindle-shaped cells outside these cells (black arrow). (*C* and *D*) Morphological changes in cells on the outside of the tubules (green arrows) after tubule culture with LH for 2 or 4 wk, respectively. (*E*) α-SMA–positive myoid cells (green) on tubule surfaces 4 d after EDS. (*F*) α-SMA–positive myoid cells (red arrows) outside of which are α-SMA–negative putative stem Leydig cells (white arrows). (*G–I*) Tubules costained for α-SMA (green, myoid cells) and CYP11A1 (red, Leydig cells). (*G*) Cross-section of tubule cultured for 4 wk with LH. (*H*) Whole-mount tubule before culture. (*I*) Whole-mount tubule cultured for 4 wk with LH. (Scale bars: white, 100 μm; black, 10 μm.)

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medium for weeks 2 and 3. To assess the effects of given factors on stem cell differentiation, equivalent lengths of tubules were incubated in medium containing LH alone during week 1, and then with LH plus the factor to be tested during weeks 2 and 3, the period during which differentiation of the cells occurs. After week 3, T was assayed in the media, and the results were compared with tubules that had been cultured with LH alone.

When added to cultures during week 1, SAG (an agonist of DHH), FGF2, activin, PDGFBB, and PDGFAA had stimulatory effects on T production, whereas TGF-β, DHT, dbcAMP, and inhibitors of PDGFR and DHH were among the factors that had inhibitory effects (Fig. 2*A*). To determine whether the stimulatory or inhibitory effects of these factors on T production were the result of their effects on stem cell proliferation during week 1 and, thus, on the number of cells capable of differentiating to form T-producing cells during weeks 2 and 3, dividing cells were labeled with EdU after tubules had been cultured for 5 d. Treatment with TGF-β inhibited cell proliferation in comparison with incubation with LH alone (compare Fig. 2*B* vs. Fig. 2*C*), whereas treatment with FGF2 stimulated cell proliferation (Fig. 2*D*). The effects of various factors on cell proliferation were quantified (Fig. 2*E*). PDGFAA, PDGFBB, activin, FGF2, and SAG, the five most active factors found to stimulate T production, resulted in two- to fivefold increases in EdU-positive cells in comparison with LH alone, whereas treatment with TGF-β and dbcAMP, which reduced T production significantly, decreased EdU-positive cells (Fig. 2*E*). Treatment with inhibin and lithium, factors that did not affect T production when applied during week 1, also did not affect cell proliferation (Fig. 2*E*). Increased numbers of 3βHSD-stained Leydig cells (Fig. S4*A*) were seen at 3 wk after treatment of tubules during week 1 with PDGFBB, FGF2, or SAG, consistent with the cell proliferation data seen at 1 wk.

When added to cultured tubules during weeks 2 and 3, the period during which cell differentiation occurs, SAG and lithium resulted in at least 10-fold stimulation of T production (Fig. 3*A*). Activin, PDGFAA, DHT, and antagonists for TGF-β (SB431542; SB525334), Notch (DAPT), and Wnt (XAV939) also had mild stimulatory effects. In contrast, TGF-β, Wnt activator I, and DHH inhibitors had negative effects on T production. Interestingly, FGF2 and PDGFBB, which had stimulatory effects on cell proliferation during week 1, were found to have inhibitory effects on T production when administered during weeks 2 and 3.

To examine interactions among factors that regulate stem cell proliferation and differentiation, tubules were treated during week 1 with LH plus factors that affect proliferation, and then were switched during weeks 2 and 3 to medium containing LH plus SAG, the most potent differentiation stimulator (Fig. 3*B*). Incubation of the tubules during week 1 with PDGFAA, FGF2, or activin and then with LH alone for weeks 2 and 3 resulted in relatively modest increases in T production by the end of week 3. However, when tubules that had been incubated with any of the three factors during week 1 were incubated with SAG during weeks 2 and 3, the tubules increased their T production dramatically compared with tubules incubated with SAG alone (Fig. 3*B*). Adding lithium to SAG-treated tubules during weeks 2 and 3 increased T production compared with SAG alone.

Although LH is essential for the development of adult Leydig cells, stem Leydig cells do not express the LH receptor (9, 23, 24). Therefore, we reasoned that there must be a Leydig cell lineage commitment factor, as yet unidentified, that triggers the transition from stem Leydig cells to LH-responsive progenitor cells. Having found that SAG and lithium have profound stimulatory effects on differentiation during weeks 2 and 3 of culture (Fig. 3*A*), we examined the possibility that one or both of these factors might induce the transition from stem to progenitor cells in the absence of LH. To that end, the tubules were incubated in culture without LH for 1 wk, and then with SAG or lithium from weeks 2 to 3. At the completion of week 3, the ability of the tubules to produce T in response to a brief (2-h) treatment with LH, dbcAMP, 22-hydroxycholesterol (22HC), or progesterone (P5) was determined. As seen in Fig. 3*C*, tubules cultured in medium alone for 3 wk did not produce any T in response to brief exposure to LH, dbcAMP, 22HC, or P5. Similarly, treatment with lithium during weeks 2 and 3 failed to increase T production.

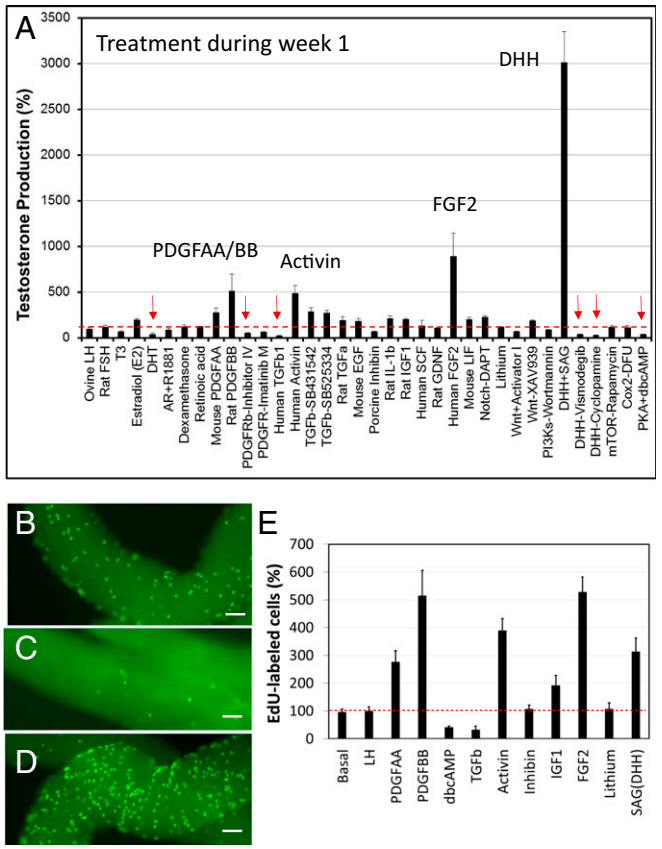

Fig. 2. (*A*) Effects on T production of potential regulatory factors added to tubules during week 1 of culture with LH. The tubules were then switched to LH-only medium for weeks 2 and 3. T was assayed in the media after 3 wk and expressed as the percentage of T production by tubules cultured with LH alone in week 1. (*B–E*) Dividing cells were labeled with EdU after 5 d of cultures with LH-only medium (*B*), LH plus TGF-β (*C*) or LH plus FGF2 (*D*), and cell numbers were quantified (*E*). Data are expressed as mean ± SEM of 4–7 separate experiments. Red arrows indicate significant inhibition. (Scale bars: 100 μm.)

When tubules were cultured with SAG from weeks 2 to 3, however, there was significant elevation of T production in response to each of LH, dbcAMP, 22HC, and P5, indicating that DHH signaling, even in the absence of LH, can stimulate stem cells to enter the Leydig cell lineage and ultimately become able to produce T.

Sertoli cells have been reported to produce paracrine factors, including DHH, that are involved in the regulation of Leydig cell development (25, 26). We asked whether DHH signaling alone can commit stem Leydig cells to differentiate, or whether commitment also requires other factors produced by the seminiferous tubules. To address this question, the peritubular stem cells were separated from the tubules by collagenase treatment of the tubules. Then, the isolated cells and stripped tubules were cultured separately (Fig. S4*B*). Neither the tubules nor the isolated peritubular cells produced T in response to 3-wk culture with LH alone, or with LH for 1 wk and then with LH plus PDGFAA, DHT, activin, antagonists of Notch or Wnt, or lithium during weeks 2 and 3. In contrast, SAG (DHH agonist), when added during weeks 2 and 3, induced the isolated cells, but not the stripped tubules, to produce T. These results support the conclusion that DHH may be the long-sought, tubule-derived niche factor required for the commitment of stem cells to Leydig cells. The factors that affect stem Leydig cell proliferation and differentiation are summarized in Fig. S4*C*.

CD90 as a Specific Surface Marker for the Isolation of Stem Leydig Cells from Adult Testis. In the studies described above, stem cells obtained by collagenase treatment of seminiferous tubules were

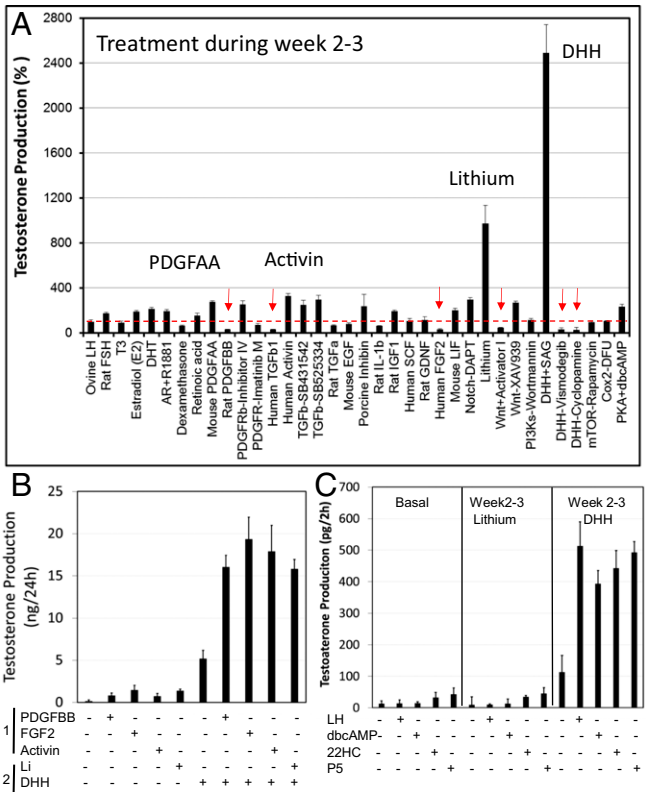

Fig. 3. Effects on T production of potential regulatory factors. (*A*) Factors were added during weeks 2 and 3 after culture of the tubules with LH alone during week 1. T was assayed in the media after 3 wk and expressed as the percentage of T production by tubules cultured with LH alone in weeks 2 and 3. Red arrows indicate significant inhibition. (*B*) T production by tubules stimulated with proliferating factors during week 1 and then with or without SAG and/or lithium during weeks 2 and 3. (*C*) Effects on T production of treating tubules with SAG or lithium for weeks 2 and 3, in the absence of LH, after which the tubules were incubated (2 h) with LH, dbcAMP, 22HC, or P5. Data are expressed as mean ± SEM of 4–7 separate experiments.

shown to differentiate in response to SAG (DHH). We asked whether there might be other cells in these preparations (e.g., germ, Sertoli, myoid) that might contribute factors involved in the differentiation of the SAG-treated stem cells, or whether DHH signaling alone was sufficient. To address this question required the identification of a Leydig stem cell-specific surface marker with which to purify the cells. Analysis of our previously published microarray data (13) identified 15 cell surface proteins whose message levels were highly expressed in stem cells and then turned off at the progenitor stage (Fig. S5). Among these proteins, we tested four (CD90, p75NTR, CD51, and PDGFRα) for their specificity. We found CD90 to be of greatest use for purifying stem Leydig cells. In particular, CD90 localized to cells on the surface of freshly isolated seminiferous tubules (Fig. 4*A*), which is where stem cells reside. In tubules costained for CD90 (Fig. 4*B*) and for the myoid cell protein desmin (Fig. 4*C*), cells on the surfaces of the tubules were stained for one or the other protein, with little overlap (Fig. 4 *D* and *E*). This result indicated that the myoid cells do not express CD90.

Peritubular cells obtained from collagenase-treated freshly isolated tubules (Fig. S6*A*) were stained for CD90 (Fig. S6*B*) and sorted by flow cytometry (Fig. S6*C*). As shown in Fig. 4*G*, the $CD90^+$ cells separated into a population not seen in unstained cell preparations (Fig. 4*F*). The $CD90^+$ cells represented about 0.4% of the total cells. The $CD90^+$ and $CD90^-$ cells were cultured with LH for 3 wk plus or minus SAG during weeks 2 and 3, and their ability to differentiate into steroidogenic cells was assessed (Fig. 4*H*). $CD90^+$ but not $CD90^-$ cells were able to form T-producing cells in the presence of LH plus SAG. Staining by 3βHSD of cultured cells was consistent with T production; the $CD90^+$ (Fig. 4*J*) but not $CD90^-$ cells (Fig. 4*I*) stained positively after the 3-wk period of LH/SAG. These results indicate that in the presence of LH, DHH signaling alone is sufficient to induce the Leydig stem cell to differentiate.

Discussion

In the normal rat testis, the growth and differentiation of the adult Leydig cell population is largely completed by approximately day 70 postpartum. The number of Leydig cells present in the adult testis (approximately 25 million) does not change with aging. However, there is evidence that although the Leydig cell number is relatively stable, there is some turnover. For example, ^{3}H-thymidine has been shown to be incorporated into cells in the interstitial compartment (27), and injections of rats with high doses of hCG to result in the formation of new Leydig cells (28). These early studies, based on ^{3}H-thymidine labeling, in fact may have underestimated the turnover rate of adult Leydig cells. For example, cells around the tubules were shown to divide actively, but were classified as myoid cells (27).

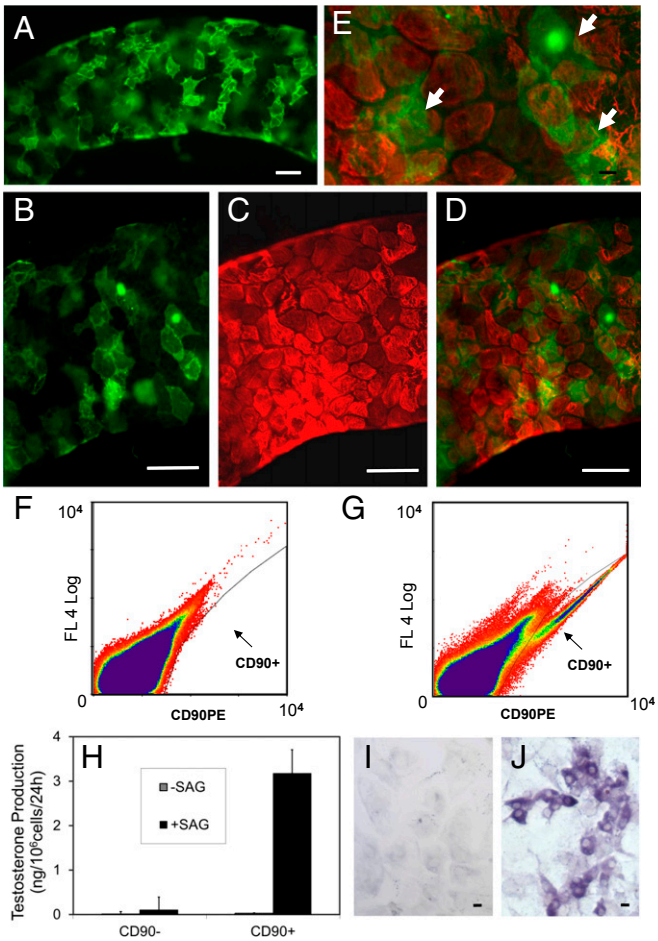

Fig. 4. Identification and isolation of CD90-positive cells. (*A*) Peritubular cells in freshly isolated seminiferous tubules stained for CD90. (*B* and *C*) Tubules costained for CD90 (*B*, green) and the myoid cell marker desmin (*C*, red). (*D* and *E*) Few cells were positive for both CD90 (green) and desmin (red). White arrows indicate the green $CD90^+$ cells on top of red myoid cells. (*F*) Unstained cell population sorted by flow cytometry. (*G*) $CD90^+$ cell fraction sorted by flow cytometry after staining with CD90 antibody. (*H*) T production by $CD90^+$ and $CD90^-$ cells cultured with LH plus or minus SAG (DHH agonist) for weeks 2 and 3. (*I* and *J*) Staining by 3βHSD of $CD90^-$ (*I*) and $CD90^+$ (*J*) cells treated with LH for week 1 and then SAG for weeks 2 and 3. Data are expressed as mean ± SEM of three separate experiments. (Scale bars: white, 100 μm; black, 10 μm.)

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We now know that stem Leydig cells are located both on the tubule surfaces (9, 16, 19, 20) and associated with blood vessels (18, 24). In the event of loss of the adult Leydig cells (14, 15), stem cells in both locations are capable of regenerating new Leydig cells (16, 20–22; Fig. S7*A*). The peritubular stem cells, however, play a particularly significant role in reestablishing the Leydig cell population when testes are regressed (20; Fig. S7*B*).

In the present study, we identified a group of cells on the surface of the seminiferous tubules of the adult testis that are distinguishable from myoid cells and are capable of giving rise to functional Leydig cells. In contrast to the continuous layer of α-SMA–staining myoid cells surrounding the tubules, the cells do not express α-SMA and are located outside the layer of cells that do so. These cells became CYP11A1-positive and 3βHSD-positive in association with T production that is induced by culturing tubules with LH for 3–4 wk, indicating that the peritubular cells had become steroidogenic. To begin to assess whether the seminiferous tubules provided niche factors needed for the peritubular cells to differentiate, mild collagenase treatment of the tubules was used to isolate cells from the surface of the tubules. In contrast to LH-cultured tubules, the isolated cells failed to differentiate into steroidogenic cells when cultured with LH, suggesting that the tubules might indeed provide factors needed for the differentiation, and perhaps the proliferation, of the peritubular putative stem cells.

In an earlier study, we had conducted microarray analysis of gene expression during the differentiation of neonatal stem Leydig cells to cells that entered the Leydig cell lineage as progenitor Leydig cells (13). The significantly up-regulated or down-regulated genes for signaling molecules and receptors were among those in which we took particular interest. Others were proteins identified in previous studies that were suggested to play roles in Leydig cell development or in stem cell function in organs outside the testis. We used the cultured seminiferous tubule system that we developed to screen for the effects of these factors. We found that, as is the case in vivo (21, 22), the proliferation of the stem cells on the surfaces of the tubules occurred during the first week of culture, which was followed by their differentiation during weeks 2 and 3 (17). With this system, therefore, factors associated with the tubules that might be involved in cell proliferation, cell differentiation, or both can be tested.

Five factors (PDGFAA, PDGFBB, FGF2, activin, DHH) were identified which, when added to tubules during week 1, resulted in stimulated T production by the end of week 3, and three (TGF-β, androgen, dbcAMP) in suppressed T production. These results suggest that these factors had differential effects on cell proliferation during week 1, resulting in different numbers of cells able to differentiate during weeks 2 and 3 into steroidogenic cells. The effects of these factors on proliferation were assessed in three ways. First, proliferation was tested directly by labeling the dividing cells with EdU during the first week of treatment. Second, the tubules treated with the stimulatory factors during week 1 were further treated during weeks 2 and 3 with the DHH agonist SAG, a potent stem Leydig cell differentiation inducer, and T was measured at the end of week 3. This latter approach was based on the assumption that if there were more stem cells resulting from treatment with proliferation factors during week 1, there would be more T-producing cells formed, and more T produced, in the presence of differentiation inducer. Third, 3βHSD-positive cells were directly quantified after 3 wk of treatment. The results obtained by the three approaches were largely in agreement, except for SAG. In that case, the substantial increase in T production and in 3βHSD-positive cells seen after 3 wk cannot be explained entirely by increase in EdU-labeled cells during week 1, suggesting that in contrast to other factors tested, SAG treatment during week 1 has effects in addition to increasing cell proliferation. With respect to differentiation, DHH, lithium, activin, PDGFAA and androgen were found to stimulate the differentiation of stem cells during weeks 2 and 3, and TGF-β, Wnt, and Notch to be inhibitory. Interestingly, PDGFBB and FGF2, the two most potent stem Leydig cell proliferation stimulators, inhibited stem Leydig cell differentiation, suggesting that these factors may play roles in controlling the balance between stem and differentiated cells. The factors that affect stem Leydig cell proliferation and differentiation are summarized in Fig. S5*C*.

The critical role of LH in adult Leydig cell steroidogenic function is well established. However, it is unlikely that LH alone could commit stem Leydig cells to the differentiation pathway because the stem cells do not express the LH receptor (9, 23, 24). We found SAG, which stimulates DHH, to have strong effect on differentiation. Consequently, we hypothesized that DHH signaling might play an important role in the early commitment of stem cells into the Leydig cell lineage. When tubules were cultured without LH for 3 wk, no Leydig cells were formed. However, when tubules were incubated with SAG in the absence of LH from weeks 2 to 3, differentiation of the stem cells into LH-responsive, T-producing cells occurred. These results suggest that DHH may be the critical commitment factor that triggers the transition of stem Leydig cells into the Leydig cell lineage. The expression of LH receptors by the progenitor cells makes these cells responsive to LH signaling, which is essential for the further maturation of the cells.

Previous studies indicated that DHH and PDGF are involved in the normal development of the fetal and adult Leydig cell populations (25, 26, 29–32). The specific roles of these factors in the functions of stem Leydig cells in adult testes were not elucidated. Using the tubule culture system that we developed, DHH and PDGF were found to increase both stem cell proliferation and differentiation. Although both PDGFAA and PDGFBB stimulated stem cell proliferation, they played opposite roles in stem cell differentiation, with PDGFAA stimulatory and PDGFBB inhibitory. We also found that there is a robust stimulatory effect of lithium on stem cell differentiation. This metal is of particular interest because of its reported effects on the activation of Wnt signaling by inhibiting GSK3β (33). As yet, the molecular mechanism by which this factor acts remains uncertain.

As discussed above, we had obtained results suggesting a critical role for DHH in differentiation of stem Leydig cells. The isolation of pure stem cells was required to address the question of whether DHH, by itself, is sufficient to induce stem Leydig cell differentiation in the absence of other testicular (niche) cells. The isolation of pure stem cells requires specific cell surface markers. Several proteins had been reported in previous studies to be expressed by stem Leydig cells in neonatal or adult testes, including nestin (11, 18), COUP-TFII (24, 34), Arx (35), CD51 (11), p75NTR (11), and PDGFRα (9, 10, 16). Nestin, COUP-TFII, and Arx are intracellular proteins and, therefore, could not readily be used as markers to purify the cells. PDGFRα, a cell surface protein, had been used to concentrate stem Leydig cells from neonatal (9, 10) and adult (16) rats. CD51 and p75NTR also are cell surface proteins whose expressions were found to overlap with that of nestin in the neonatal mouse testis (11). By reanalysis of an earlier array study (13), we also identified a number of cell surface proteins whose mRNAs were highly expressed by stem cells but turned off with the transit of these cells to progenitor Leydig cells.

Among the proteins that we examined, CD90 was found to be most promising for the specific isolation of stem Leydig cells. Staining freshly isolated seminiferous tubules with CD90 antibody resulted in its specific localization to cells on the outer surface of tubules and not by other seminiferous tubule-associated cells, including myoid cells. Additionally, when the $CD90^+$ and $CD90^-$ cells were separated by flow cytometric sorting and then cultured with LH/SAG, the $CD90^+$ cells differentiated into 3βHSD-staining cells and produced T. These observations establish CD90 as a specific cell surface marker for the isolation of the seminiferous tubular-associated stem Leydig cells in the adult rat testis. Using these cells, we found that in the absence of SAG (DHH), the $CD90^+$ cells failed to differentiate, but did so when exposed to SAG. These results suggest that DHH is a critical steroidogenic lineage trigger factor in the commitment of stem to Leydig progenitors and able to induce the stem cells to differentiate even in the absence of other niche cells.

In summary, we have shown that the stem cells associated with the surface of seminiferous tubules are capable of giving rise to new adult Leydig cells. Both the proliferation and differentiation of the

stem Leydig cells occur under the apparent influences of the seminiferous tubular cells. Multiple factors have been found that affect the proliferation and differentiation of these stem cells. Among the factors that affect differentiation, DHH signaling apparently is necessary and sufficient. The stem Leydig cells of the rat testis were found to express the surface protein CD90 specifically, and that allows the cells to be isolated. The current study, focused as it is on the stem cells associated with tubules, does not rule out the possibility that there also are stem cells present in the interstitial compartment of the testis, the regulation (and functions) of which may or may not be the same as the stem cells associated with the tubules.

Materials and Methods

Culture of Seminiferous Tubules. All animal procedures were performed in accordance with NIH Guide for the Care and Use of Laboratory Animals, according to protocols approved by the Johns Hopkins Animal Care and Use Committee. Equal lengths (5 cm) of isolated tubules were cultured in 24-well plates with or without LH (10 ng/mL) in M199 medium supplemented with 0.1% BSA, 15 mM Hepes, 2.2 mg/mL sodium bicarbonate, penicillin/streptomycin (100 U/mL and 100 μg/mL) and insulin/transferrin/selenium for up to 4 wk at 34 °C and 5% (vol/vol) CO_2. For screening experiments (Figs. 2*A* and 3*A*), each of the 36 different factors (Table S1) was added to the media during either week 1 or weeks 2–3. LH was included in the media all of the times. At the end of 3 wk, the abilities of the cultures to produce T were assayed by incubation of the tubules with LH (10 ng/mL) for 24 h. Each factor was tested at three different concentrations (ranges used are reported in Table S1). The concentrations reported herein (Figs. 2*A* and 3*A*) were either the lowest that elicited maximal response, or the highest that had no effect. These were the concentrations also used in subsequent experiments (Figs. 2 *B–E*, 3 *B* and *C*, and 4*H* and Fig. S4 *A* and *B*). For experiments to examine interactions among proliferation and differentiation factors (Fig. 3*B*), tubules were treated during week 1 with PDGFBB, FGF2, or activin and then were switched to lithium or SAG (DHH agonist) during weeks 2 and 3. T production was assayed by incubation of the tubules with LH for 24 h. For experiments to examine the effects of lithium and SAG on the differentiation of stem Leydig cells in the absence of LH (Fig. 3*C*), the tubules were cultured with or without lithium or SAG alone for weeks 2 and 3. At the end of the 3 wk, the ability of the cells to produce T was assessed after 2-h incubation with LH (10 ng/mL), dbcAMP (1 mM), 22-hydroxycholesterol (22HC, 25 μM) or pregnenolone (P5, 25 μM). For all experiments, media were frozen for T assay, and tubules were used for either morphology or stained for 3βHSD, CYP11A1, desmin, or α-SMA. The antibodies used in these studies are listed in Table S2.

Isolation and Culture of Peritubular Cells. To isolate peritubular cells, the tubules were digested with 1 mg/mL collagenase-D in DMEM/F12 medium at 34 °C for 30 min with slow shaking (90 cycles per min) and allowed to settle. The dispersed cells were filtered through a 100-μm pore nylon mesh and were plated in 24-well plates in DEME/F12 medium containing 5% (vol/vol) FBS for 2 h, washed, and expanded by DEME/F12 medium containing 2.5% (vol/vol) FBS, 10 ng/mL FGF2, and 10 ng/mL PDGFBB. When the cells reached 80% confluence, they were switched into M199 medium containing LH (10 ng/mL) for a week. Then the cells were treated with LH plus PDGFAA (10 ng/mL), activin (10 ng/mL), DHT (10 ng/mL), DAPT (2 μM), XAV939 (0.1 μM), lithium (5 mM), or SAG (0.5 μM) from weeks 2 to 3 to assess the ability of these factors to induce differentiation. At the end of 3 wk, differentiation was determined by assessing the ability of the cells to produce testosterone in response to LH (24 h).

Statistical Analyses. Data are expressed as the mean ± SEM. Group means were evaluated by one-way ANOVA. If group differences were revealed by ANOVA ($P < 0.05$), differences between individual groups were determined with the Student–Neuman–Kuels test, using SigmaStat software (Systat Software). Values were considered significant at $P < 0.05$.

See *SI Materials and Methods* for additional procedures.

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