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The Helix-Loop-Helix Inhibitor of Differentiation (ID) Proteins Induce Post-Mitotic Terminally Differentiated Sertoli Cells to Re-Enter the Cell Cycle and Proliferate

Jaideep Chaudhary, Ingrid Sadler-Riggleman, Jacquelyn M. Ague, and Michael K. Skinner[1]

Center for Reproductive Biology, School of Molecular Biosciences, Washington State University, Pullman, Washington 99164-4231

ABSTRACT

Prior to puberty the Sertoli cells undergo active cell proliferation, and at the onset of puberty they become a terminally differentiated postmitotic cell population that support spermatogenesis. The molecular mechanisms involved in the postmitotic block of pubertal and adult Sertoli cells are unknown. The four known helix-loop-helix ID proteins (i.e., Id1, Id2, Id3, and Id4) are considered dominant negative regulators of cellular differentiation pathways and act as positive regulators of cellular proliferation. ID proteins are expressed at low levels by postpubertal Sertoli cells and are transiently induced by serum. The hypothesis tested was that ID proteins can induce a terminally differentiated postmitotic Sertoli cell to reenter the cell cycle if they are constitutively expressed. To test this hypothesis, *ID1* and *ID2* were stably integrated and individually overexpressed in postmitotic rat Sertoli cells. Overexpression of *ID1* or *ID2* allowed postmitotic Sertoli cells to reenter the cell cycle and undergo mitosis. The cells continued to proliferate even after 300 cell doublings. The functional markers of Sertoli cell differentiation such as transferrin, inhibin alpha, Sert1, and androgen binding protein (ABP) continued to be expressed by the proliferating Sertoli cells, but at lower levels. FSH receptor expression was lost in the proliferating Sertoli cell-Id lines. Some Sertoli cell genes, such as cyclic protein 2 (cathepsin L) and Sry-related HMG box protein-11 (*Sox11*) increase in expression. At no stage of proliferation did the cells exhibit senescence. The expression profile as determined with a microarray protocol of the Sertoli cell-Id lines suggested an overall increase in cell cycle genes and a decrease in growth inhibitory genes. These results demonstrate that overexpression of *ID1* and *ID2* genes in a postmitotic, terminally differentiated cell type have the capacity to induce reentry into the cell cycle. The observations are discussed in regards to potential future applications in model systems of terminally differentiated cell types such as neurons or myocytes.

bHLH, cell cycle, inhibitor of differentiation, postmitotic, SC-Id lines, Sertoli cells, terminal differentiation, testis

INTRODUCTION

Terminal differentiation is the state achieved when a cell exits the cell cycle to become postmitotic, and the differentiated gene expression profile allows a specialized function for the cell. Often, these terminally differentiated cells cannot be replenished, and once lost, cause abnormal tissue function. Examples of terminally differentiated cells include neurons [1], myocytes [2, 3], and Sertoli cells [4]. Abnormalities or loss of these terminally differentiated cells causes corresponding neurodegeneration [5, 6], muscle degeneration [7], or infertility disease states [8]. The cellular mechanisms that promote and maintain terminal differentiation are poorly understood. The speculation is that altered signal transduction and cell cycle pathways influenced by unique transcriptional events allow a cell to irreversibly exit the cell cycle and promote a unique spectrum of gene expression required by the cell [1, 2, 9, 10]. The current study investigates a factor that can influence terminal differentiation using the Sertoli cell as the model cell type.

Sertoli cell fate is established in the embryonic gonad at the time of sex determination [11, 12] and is followed by a phase of rapid cell proliferation and differentiation. During puberty, the final phase of Sertoli cell differentiation occurs, which is marked by cessation of proliferation, and irreversible changes in Sertoli cell morphology and physiology [13]. The changes associated with terminal differentiation of Sertoli cells at puberty include exit from the cell cycle and the formation of the blood-testis barrier. This differentiated phenotype is needed for the proper microenvironment and cytoarchitectural support of the developing spermatogenic cells. Sertoli cell differentiation is accompanied by the expression of many gene products that are not present in immature cells [14–19].

In general, hormones and growth factors such as FSH [20], interleukin-1 alpha [21], and transforming growth factor (TGF) alpha [21] increase proliferation of Sertoli cells obtained from neonatal and prepubertal testes. Early postpubertal Sertoli cells also remain responsive to these hormones and growth factors, but they fail to proliferate and enter the cell cycle [8, 22]. The molecular mechanisms involved in this switch to a postmitotic and irreversible exit from the cell cycle at puberty are largely unknown. The proposal is made that regulatory signaling networks altered by hormones and growth factors will influence the cell cycle [16, 22]. The activation of the extracellular-regulated kinase (ERK)-mitogen-activated protein (MAP) kinase pathway and subsequent up-regulation of cyclin D1 in response to FSH [23], the induction of the growth inhibitor p27 (i.e., cyclin-dependent kinase inhibitor 1B, Cdkn1b) by steroid factors [16], and the activation of the protein kinase A (PKA)-cAMP pathway [23, 24] are examples of hormone-responsive signal transduction pathways involved. Quantitative spermatogenesis is dependent on the total number of Sertoli cells established prepubertally [25, 26]. Optimum Sertoli cell number and function are maintained through the activation of various signal transduction pathways [27–30] and induction of a number of transcription factors [31–36]. The activation of specific combinations of these transcription factors is in part responsible for stage-dependent proliferation and differentiation of Sertoli cells.

[1]Correspondence: Michael K. Skinner, Center for Reproductive Biology, School of Molecular Biosciences, Washington State University, Pullman, WA 99164-4231. FAX: 509 335 2176; e-mail: Skinner@mail.wsu.edu

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Sertoli cells have been shown to express members of the basic helix-loop-helix (bHLH) transcription factor family [37–39]. The bHLH family of transcription factors is a critical cell-type determinant and plays important roles in cellular differentiation. The bHLH domain consists of two amphipathic helixes separated by a loop that mediates homodimerization and heterodimerization adjacent to a DNA binding region rich in basic amino acids [40, 41]. The bHLH dimers bind to an E Box (CANNTG) DNA consensus sequence present in a wide variety of tissue-specific promoters [42, 43]. The E box domain has been shown to influence the promoters of a number of Sertoli cell genes including transferrin (*Trf*) [37], *Fos* (i.e., c-fos) [44], SF-1 (i.e., *Nr5a1*) [45], and FSH receptor (*Fshr*) [46]. The ubiquitously expressed class A bHLH proteins consist of E2-2 [47]; HEB (i.e., transcription factor 12) [48]; and the differentially spliced products of the E2A (i.e., *Tcfe2a2*) gene, E12 and E47 [43]. The class A bHLH dimerize with tissue-restricted and developmentally regulated class B proteins such as MyoD and NeuroD (i.e., *Neurod1*) [49, 50]. Previous observations suggest that the Sertoli cells express the class A proteins E47 [38] and REBα (i.e., the rat isoform of human HEB) [39]. These bHLH proteins regulate FSH-stimulated Sertoli cell gene expression [37, 44, 51].

The members of the ID (inhibitor of differentiation/DNA binding) (i.e., *Idb* or *Id*) family modulate the transcriptional activity of class A and class B bHLH heterodimers. The four known ID proteins (ID1, ID2, ID3, and ID4) share a homologous HLH domain, but lack the basic DNA binding region [45, 52]. Thus, the ID proteins act to sequester bHLH proteins by forming inactive dimers to prevent binding of bHLH proteins to the E-box responsive elements [53–55]. Therefore, ID proteins are largely considered as dominant negative regulators of differentiation pathways [56–58], but positive regulators of cellular proliferation. The biphasic expression patterns of ID1 and ID2 corresponding to the G1 cell cycle phase and the G1-to-S phase transition supports their role in proliferation [57, 59]. The mechanisms by which ID proteins promote the cell cycle are diverse, but appear to involve suppression of cell cycle inhibitors p21 and p27 (i.e., cyclin-dependent kinase inhibitor 1A and 1B), cyclin A, cyclin E, cyclin-dependent kinase-2 (CDK2), and interactions with the retinoblastoma protein (pRb) [60]. Previous observations suggest that differentiated Sertoli cells express ID proteins [22, 61, 62]. The functional significance of ID protein expression in terminally differentiated and postmitotic Sertoli cells is unclear. Recent observations suggest that long-term (72 h) stimulation of Sertoli cells in culture with FSH down-regulates ID1 and ID3. In contrast, serum up-regulates ID1 and ID3 expression [62]. Short-term stimulation of Sertoli cells with FSH (30 min to 12 h), up-regulates ID2 in a biphasic manner [63] and mimics the effect of mitogens on other cell systems [60]. The transient up-regulation of *ID* genes in response to FSH suggests that differentiated Sertoli cells may be competent to reenter the cell cycle if *ID* gene expression is sustained. The current study was designed to investigate whether an altered expression of the members of the ID family, specifically ID1 and ID2, can allow reentry of postmitotic Sertoli cells into the cell cycle.

MATERIALS AND METHODS

Isolation of Sertoli Cells

Sertoli cells were isolated from the testes of 20-day-old rats by a modified procedure described earlier [64, 65]. Sertoli cells from 60-day-old adult rats were isolated by a modified procedure previously described [66]. The Washington State University Animal Care Committee approved all animal use and procedures. The isolated Sertoli cells were more than 98% pure and were plated under serum-free conditions. Cells were maintained in a 5% CO_2 atmosphere in Ham-F12 medium (Life Technologies, Inc., Rockville, MD) at 32°C. Sertoli cells were treated with either FSH (25 ng/ml; o-FSH-16; National Pituitary Program, Torrance, CA), dibutryl cAMP (200 μM), or 10% bovine calf serum (BCS). These concentrations of FSH and cAMP have previously been shown to optimally stimulate cultured Sertoli cell differentiated functions [67, 68]. Cell number, purity, and viability do not change during the culture in the absence or presence of treatment [67, 68]. Peritubular cell contamination was assessed with a microarray analysis, and by comparison of primary Sertoli cells, primary peritubular cells, and Sertoli cell-Id lines. Observations demonstrated negligible peritubular cell-specific gene expression in the Sertoli cell lines.

Plasmids

The eukaryotic expression plasmid pCI-neo-*Id1* and *Id2* were constructed. The human *ID1* (GenBank accession number NM-002165) and *ID2* (GenBank accession number NM-002166) polymerase chain reaction (PCR) primers were designed to amplify the full-length coding region. The coding sequences of *ID1* and *ID2* were obtained through reverse transcription (RT)-PCR of human cell line RNA. The fragments were first subcloned into a pGEM-T-EZ (Promega, Madison, WI) plasmid. The pGEM-T-EZ plasmid containing the *ID1* and *ID2* fragments were digested with *Eco*RI and the resulting fragment was ligated into *Eco*RI-digested pCIneo expression plasmid (Promega).

Establishment of a Stably Integrated Sertoli Cell Line

Rat Sertoli cells were cultured in 24-well plates at a density of 10^6 cells for 48 h and then transfected with the *ID1* or *ID2* expression plasmid by the calcium phosphate method coupled with hyperosmotic shock (10% glycerol) as previously described [62]. Briefly, 1.5 μg of the plasmid in 150 μl of transfection buffer (250 mM $CaCl_2$, mixed 1:1 vol/vol with 2× Hepes [28 mM NaCl, 50 mM Hepes, and 1.47 mM Na_2HPO_4 pH 7.0]) was added to each well of a 24-well plate containing 1×10^6 Sertoli cells in 1 ml of Ham-F12 with 0.01% BSA, and incubated at 32°C for 4 h. After incubation, the cells were subjected to a hyperosmotic shock. The medium was aspirated, and 1 ml of 10% glycerol in Hanks balanced salt solution (HBSS; Life Technologies) was added. The cells were incubated for 3 min and the wells were washed twice with HBSS before fresh Ham-F12 was added. After several cell doublings the same transformed cells were subjected to G418 selection (0–500 μg/ml). At the G418 concentration (43 μM), selecting for the presence of neomycin, all the primary nontransformed Sertoli cells failed to survive, whereas the cell lines survived. The transformed cell sensitivity to G418 was established, but Sertoli cell-Id lines were not selected with G418 so as not to characterize a clonal, isolated cell population. Unselected SC-Id lines were used for further analysis and subsequently maintained in the Ham-F12 medium.

The cells were routinely reseeded at a concentration of approximately 4×10^5 cells in 100-mm tissue culture dishes and incubated in the F12-Ham medium with 10% BCS. Sertoli cell-Id lines were maintained in the medium and passaged on 100-mm tissue culture dishes. In vitro growth curves were obtained by plating the transformed cells in 100-mm plates at 10^5 cells/plate. Sertoli cell-Id line cells were grown to confluency, trypsinized, counted, and plated at a 1:4 dilution in new 100-mm plates.

Cell Proliferation Assay

The proliferation rate as reflected by the rate of DNA synthesis was performed using ^{3}H-thymidine incorporation assays. Approximately 10^4 cells/well were seeded in 24-well plates. Twenty-four hours after seeding, or when the cells reached ~50% confluency, cells were incubated in F12 medium without calf serum for 48 h before treatment in Dulbecco modified Eagle medium containing 0.1% BCS for 24 h. After the 24-h treatment cells were then incubated with ^{3}H-thymidine for 6 h before harvest. Cells were then sonicated, applied to diethylaminoethyl filters, washed, and then dissolved in 1 M NaOH. Radioactivity in the samples was determined by liquid scintillation counting. The amount of DNA in the well was assessed and data expressed as thymidine incorporation per microgram of DNA.

Western Blotting

Confluent Sertoli cells, peritubular cells, and Sertoli cell-Id lines were washed twice with HBSS and lysed with lysis buffer (Promega). Lysates

were centrifuged at 10 000 × *g* for 10 min at 4°C, and supernatants were collected. Samples were electrophoretically separated on a 15% mini-SDS gel (Bio-Rad Laboratories, Hercules, CA). The protein was subsequently transferred onto nitrocellulose membrane and probed with specific antibodies to ID1 and ID2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The specific antigen-antibody complex was visualized using a horseradish peroxidase chemiluminescent detection kit (Pierce, Rockford, IL).

RNA Preparation and PCR

Freshly isolated, cultured, or transformed Sertoli cells were lysed directly using Trizol Reagent (Invitrogen, Carlsbad, CA). Total RNA was isolated from the cell lysate following the manufacturer's protocol for RNA isolation. The final RNA pellet was dissolved in distilled water. Total RNA (2 μg) was reverse transcribed in a final volume of 25 μl containing 20 U RNasin (Promega); 1.25 mM of each dNTP, 250 ng oligo(dT) (Pharmacia, Peapack, NJ), 10 mM dithiothreitol, and 200 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen) in the MMLV first-strand synthesis buffer supplied by the manufacturer (Invitrogen). The RNA was denatured for 10 min at 65°C, then cooled on ice before adding the RT mix and enzyme. The reverse transcriptase reaction was carried out at 42° C for 1 h. Each PCR contained 250 pg of reverse-transcribed DNA, 1 μM of each 5′ and 3′ oligonucleotide primers, 0.5 U *Taq* polymerase, and 0.1 mM of each dNTP. The primer pair sequences used were obtained from published sequences of rat cyclophilin, α-actin, transferrin, androgen-binding protein (ABP), FSH receptor (FSHR), and Sert1. The primer sequences were as follows: cyclophilin, 5′ primer (ACA CGC CAT AAT GGC ACT GGT GGC AAG TCC ATC) and 3′ primer (ATT TGC CAT GGA CAA GAT GCC AGG ACC TGT ATG); α-actin 5′ primer (GAG CAT CCG ACC TTG CTA AC) and 3′ primer (AGA TGG CTG GAA GAG GGT CT); rat transferrin, 5′ primer (ATC TGG GAG ATC CTC AAA GTG GCT C) and 3′ primer (GGC ACT AGT CCA CAC TGG CCT GCT A); FSH receptor, 5′ primer (CTG CCA AGA CAG CAA GGT GA) and 3′ primer (AGC CAT GGT TTG GTA AGG AA); ABP, 5′ primer (GAC GGA CCC TGA GAC ACA TT) and 3′ primer (GAA CAG TCC AGG TTG CAG GT); SERT-1, 5′ primer (TCC TGC TCT GAC ACT TCC AGT T) and 3′ primer (AGC TGA CCC ATA ATT GAT GCA C); and inhibin alpha, 5′ primer (TCT GAA CCA GAG GAG GAG GA) and 3′ primer (GGC CTG AGC AAG AAC AGA GT).

The possible contamination of RNA with DNA was distinguished by performing the RT reaction without MMLV reverse transcriptase. The absence of any product in the amplification reaction using such a reverse-transcribed preparation indicated the absence of any contaminating DNA in our RNA samples. Each RT reaction was performed using three different samples. The PCR-based amplification reactions were carried out in duplicate on each reverse-transcribed RNA sample. Simultaneous PCR reactions were also carried out using primers designed to rat cyclophilin or α-actin to monitor the efficiency of the RT-PCR reactions. Cyclophilin or α-actin was faithfully amplified in all the PCR reactions, indicating consistency in the quality of RT and PCR reactions. The number of cycles used was approximately 30, which is within the linear range, and cyclophilin or α-actin expression was assessed as a constitutive gene for data normalization. The identity of the corresponding PCR products was size- and the sequence-confirmed by the Center for Reproductive Biology Molecular Biology Laboratory. The data presented are representative of at least three different RT-PCR reactions and DNA preparations.

Microarray Analysis

RNA was hybridized to the Affymetrix (Santa Clara, CA) U34A 8799 gene chip. The Genomics Core at the Center for Reproductive Biology at Washington State University performed the analysis as previously described [69, 70]. Briefly, RNA from the cells was reverse transcribed into cDNA, and cDNA was transcribed into biotin-labeled RNA. Biotin-labeled RNA was then hybridized to the Affymetrix U34A 8799 gene chips. Each gene set is composed of 16 pairs of 24-mer oligonucleotides, with one sense strand specific for the gene and one antisense strand with single point mutations for use as a comparative negative control. Biotinylated RNA was then visualized by labeling with phycoerythrin-coupled avidin. The microarray was scanned on a Hewlett-Packard Gene Array Scanner (Hewlett-Packard Co., Palo Alto, CA). Two microarray chips from two different RNA samples were analyzed for each of the primary Sertoli cell, SC-Id1, and SC-Id2 preparations. The microarray data set can be observed at www.skinner.wsu.edu.

Bioinformatics

The majority of the data analysis was performed using the Affymetrix Microarray Suite Software. Most of this study used the comparison analysis software offered and analysis parameters previously described [70]. Two repeats for each developmental stage were performed, which allowed four comparisons in the experiment. Only genes that displayed a consistent change in expression over all four comparisons, an average fold change of two or greater, and had a relative hybridization intensity of at least 100 were included in the analysis. A one-way analysis of variance parametric test of variances was performed, and only genes found to be statistically different ($P < 0.05$) at one or more developmental stages are presented. Basic gene clustering was determined by accessing the Affymetrix database through the Microarray Suite Software. A simple clustering analysis was performed by the Gene Spring Software (Silicon Genetics). The algorithm identified genes whose transcriptional profiles were similar and placed them into separate gene clusters.

RESULTS

Generation of Sertoli Cell-ID Lines

As discussed in *Materials and Methods,* Sertoli cells were isolated and used to stably integrate human *ID1* or *ID2*. Initially, a Sertoli cell preparation from 20-day-old rats was used. These cell lines are characterized below. Subsequently, separate Sertoli cell preparations from 20-day-old and 60-day-old rats were used to stably integrate *ID1*. All the Sertoli cell preparations that were stably integrated resulted in cell populations that proliferated and could be passaged. Therefore, Sertoli cell preparations from postmitotic developmental stages, both midpubertal 20-day-old and adult 60-day-old, could be induced to proliferate after stable integration of *ID1*. The second 20-day-old Sertoli cell-Id1 line generated is now at 100 doublings. Two 60-day-old Sertoli cell-Id1 lines have doubled more than 20 times. This second 20-day and two 60-day Id1 lines have been shown to contain Sertoli cell genes, but they have not been analyzed with a microarray procedure. The initial Sertoli cell-Id1 and -Id2 lines were found to be resistant to G418, but clonal isolates were not used. The unselected Sertoli cell-Id lines that proliferated were used for subsequent analysis due to the inability of nontransformed primary cultures of Sertoli cells to proliferate. This avoids the use of a selected clonal isolate and allows the entire transfected cell population to be characterized. The 20-day Sertoli cell-Id lines were not found to contain contaminating peritubular cells using a microarray analysis and comparison with peritubular cells. In contrast to negligible differences between Sertoli cells and the Id lines, observations demonstrated that more than 5700 highly expressed peritubular cell genes were not found to be expressed in the Sertoli cell-Id lines (data not shown).

Effect of ID1 *and* ID2 *Overexpression on Sertoli Cell Proliferation*

As shown in Figure 1A, overexpression of *ID1* and *ID2* initiated proliferation of the Sertoli cells, which was not possible in their primary, nontransformed counterparts. Primary Sertoli cell cultures had negligible ^{3}H-thymidine incorporation, confirming their inability to proliferate. Treating the primary Sertoli cell cultures with 10% serum or EGF (Fig. 1, A and B) did not increase thymidine incorporation or cell proliferation. In contrast, the proliferation of the control, nonstimulated *ID1* or *ID2* overexpressing Sertoli cells measured after 50 doublings was 7- and 20-fold higher, respectively, compared with that of the primary Sertoli cells (Fig. 1, A and B). Cellular proliferation was assessed in Sertoli-Id cell lines before and after 50 dou-

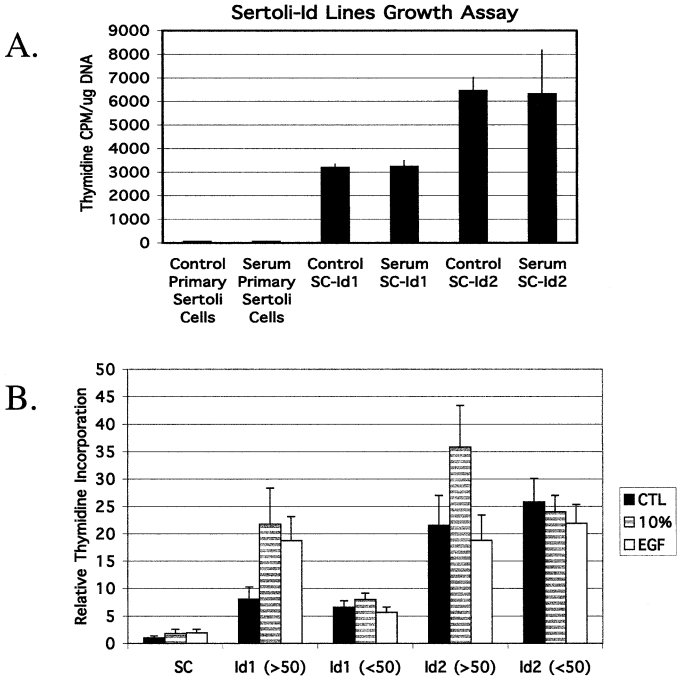

FIG. 1. Effect of ID1 and ID2 overexpression on Sertoli cell ^{3}H-thymidine incorporation. **A**) Sertoli cell proliferation rate expressed in terms of ^{3}H-thymidine incorporation per microgram of DNA. A representative of three experiments performed in triplicate is shown. **B**) Sertoli cell proliferation measured before (<50) and after (>50) 50 cell doublings in response to the growth factors EGF and 10% serum. Data similar to that shown in (**A**) were normalized to the relative rate of proliferation compared with primary control Sertoli cells (**B**). The proliferation rate (relative thymidine incorporation) of primary control Sertoli cells was negligible and essentially the same as the background, which was approximately 400 cpm/μg DNA. SC-Id1, Sertoli cells overexpressing Id1; SC-Id2, Sertoli cells overexpressing Id2; above, >50 doublings; below, <50 doublings; EGF, epidermal growth factor; 10%, 10% serum. Data in (**B**) are the mean ± SEM from three different experiments performed in replicate.

blings to investigate whether the rate of proliferation changed over time. Previous studies have shown many cell populations senesce at approximately 40–50 doublings, so 50 doublings was selected as the point of transition to immortalization. The comparative data from these experiments show a difference between the *ID1* and *ID2* transformants. The data shown in Figure 1B are normalized to thymidine incorporation (cpm/μg DNA) in primary control, nontransformed Sertoli cells set to 1. Thymidine incorporation in primary control Sertoli cell cultures was at or within 10% of the assay background counts, suggesting that these cells failed to proliferate or enter the S phase of the cell cycle. This normalization allowed comparison of proliferation experiments performed over a period of time. At less than 50 cell doublings, the *ID1* transformants had a 3-fold lower rate of thymidine incorporation compared with *ID2* transformants. Both the *ID1* and *ID2* transformants failed to respond to mitogenic stimulation. A significant increase in the proliferation rate of *ID1* transformants was observed when measured after 50 cell doublings. The rate of proliferation between control untreated cultures was similar before and after 50 cell doublings, but, interestingly, a significant (3-fold) increase in proliferation was observed after stimulation of *ID1* transformants with mitogens. A similar increase in proliferation in response to serum as a mitogen, but not EGF, was observed with the *ID2* transformants after 50 doublings (Fig. 1B). At around 50 cell doublings the cell lines appear to involve an alteration in cellular transformation (i.e., immortalization). The nontransformed primary Sertoli cell cultures did not proliferate or incorporate thymidine in the absence or presence of mitogens.

The cell doubling times during an extended culture period were also examined to determine whether the Sertoli-Id transformants continued to grow indefinitely or undergo senescence. The rate (i.e., time in days) of cell doubling was recorded soon after transformation of the Sertoli cells with *ID1* or *ID2* plasmids. As shown in Figure 2A, the transformed Sertoli cells continued to proliferate indefinitely (at least 700 days post-transfection). The doubling time (i.e., time required for cell doubling) as shown in Figure 2A was between 3 and 5 days for Sertoli-Id1 and between 2 and 4 days for Sertoli-Id2. A plateau was not observed when the number of cell doublings was plotted against days post-transfection, suggesting the Sertoli cells did not undergo senescence at any time after transformation (Fig. 2B). Generally, the cells that can continue to proliferate beyond 70 cell doublings are considered immortalized [71]. As

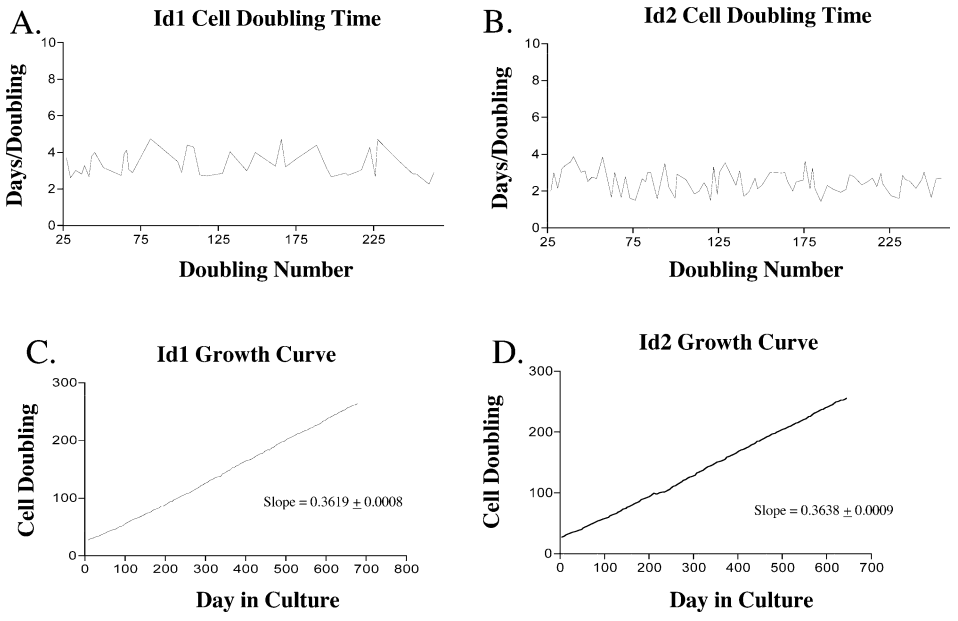

FIG. 2. Growth characteristics of Sertoli cells overexpressing ID1 or ID2. **A** and **B**) The cell doubling time involving the number of cell doublings over a period of time plotted against time (days) required for each cell doubling. The number of days required for each cell doubling were recorded to determine the average doubling time for Sertoli cells overexpressing either ID1 (**A**) or ID2 (**B**). **C** and **D**) A growth curve involving each progressive cell doubling plotted against the number of days in culture to determine whether cells were undergoing senescence. A linear and continuous increase in doubling over the time period (days) in Sertoli cells overexpressing ID1 (**C**) or ID2 (**D**). The data are an integration of cells counted before and after each doubling over a period of more than 700 days.

shown in Figure 2, the Sertoli cells stably transfected with *ID1* or *ID2* plasmid have continued to proliferate beyond 250 cell doublings. These results suggest that overexpression of *ID1* and *ID2* appears to transform the Sertoli cells and prompts them to reenter the cell cycle.

Confirmation of Overexpression of ID1 *and* ID2

Western blot analysis using specific antibodies to ID1 and ID2 were used to confirm the overexpression of *ID1* and *ID2* in the Sertoli cell lines. Because *ID1* and *ID2* are expressed by primary Sertoli cells [62], the Western blot experiments were designed using optimum protein concentrations at which endogenous ID levels become undetectable in the primary, nontransformed Sertoli cell cultures [62]. Sertoli cells after several days in culture have no ID expression, and lysates from these cells were compared against those of the Sertoli-Id lines. The Western blot analysis demonstrated that the ID1 and ID2 proteins were not detected in primary Sertoli cells, as shown in Figure 3. ID1 and ID2 proteins were both detected in Sertoli cells transformed with either *ID1* or *ID2*, respectively. The ID antibodies were isoform specific and had no cross-reactivity between ID1 or ID2 [62] (Fig. 3). Of interest, increased levels of ID2 and ID1 were also observed in cells immortalized with the opposing ID1 or ID2 proteins. These experiments confirm that Sertoli cells transformed with ID1 and ID2 expression plasmids have the cognate proteins at a level significantly higher than the nontransformed cells (Fig. 3). Therefore, the stable transfection of either *ID1* or *ID2* induced the overexpression of both *ID1* and *ID2* in both Sertoli cell-Id lines. This novel observation suggests that ID1 and ID2 can influence the expression of the other.

Characterization of Sertoli Cell Line Morphology

The Sertoli cell lines will be designated SC-Id1 and SC-Id2, respectively. The primary Sertoli cells (i.e., nontransformed) stained with hematoxylin and eosin showed a cuboidal-like morphology (Fig. 4). The large Sertoli cell nucleus showed the presence of dense nucleoli (Fig. 4). The irregular shape of the primary Sertoli cell cultures was changed to a smooth, elongated, and flattened morphology following overexpression with *ID1* or *ID2* (Fig. 4, C and E). The presence of dense nucleoli in cells overexpressing *ID1* or *ID2* was consistent with the primary Sertoli cell (Fig. 4). A similarity in the morphology was observed after treatment of primary Sertoli cells and SC-Id cell lines with cAMP. This included an increase in the nuclear density and appearance of long cytoplasmic extensions. In the *ID1* or *ID2* overexpressing cells, cAMP treatments also increased nuclear density with the appearance of long cytoplasmic extensions (Fig. 4, D and F). These observations suggest that the cAMP response in terms of morphological changes is retained by the Sertoli cells overexpressing *ID1* or *ID2*. Observations support a Sertoli-like morphology in the Id cell lines.

Characterization of Sertoli Cell Differentiated Genes

RT-PCR of the genes normally expressed by Sertoli cells was used to confirm that the corresponding ID overexpressing cells were derived from the primary postmitotic Sertoli cells and maintained Sertoli cell differentiated function. The specific primers used are presented in *Materials and Methods*. The *Sert1* gene is expressed primarily by Sertoli cells, (Fig. 5, pSC), and was expressed by SC-Id lines over-

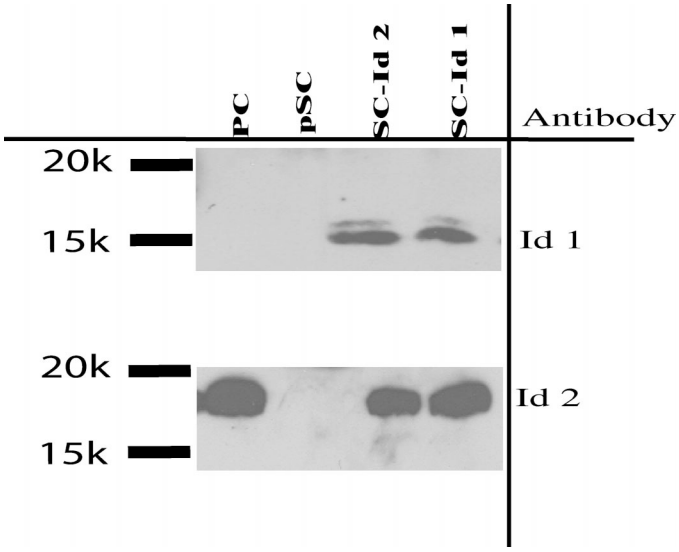

FIG. 3. Western blot analysis of ID1 and ID2 using the corresponding antibodies on cell lysates obtained from the primary Sertoli cells (pSC) or Sertoli cells overexpressing either ID1 (SC-Id1) or ID2 (SC-Id2) at 40 and 120 doublings, respectively. Also shown is the Western blot of peritubular cells (PC). The approximate size of the specific immunoprotein complex is shown on the right. A representative of three different experiments is shown.

expressing either ID1 or ID2 (Fig. 5; SC-ID1 and SC-ID2). Consistent with the observation that Sert1 is Sertoli cell-specific, the testis peritubular cells did not express Sert1 (Fig. 5, PC). This result suggests that the SC-Id lines overexpressing ID retain Sert1 expression and confirms the identity of *ID* overexpressing cells as derived from Sertoli cells. The Sertoli cells overexpressing either *ID1* or *ID2* also expressed androgen binding protein (ABP), transferrin, and inhibin alpha (Fig. 5). All these are specific gene products of Sertoli cells and demonstrate that SC-Id lines retain some Sertoli cell-differentiated functions. The expression of the FSH receptor was observed in the primary Sertoli cells, but was lost following overexpression of *ID1* or *ID2* (Fig. 5). All the cell lines generated have been shown to lose FSH receptor expression. To normalize gene expression, a constitutively expressed gene cyclophilin (i.e., peptidylprolyl isomerase A; *Ppia*) was used and was present in all cellular RNA preparations (Fig. 5). Collectively, these results suggest that the cells overexpressing either *ID1* or *ID2* are derived from Sertoli cells and retain the ability to express many of the genes normally expressed by primary Sertoli cells, with the exception of the FSH receptor *(Fshr)*.

Quantitative Microarray Analysis of Changes in Gene Expression

Microarray analysis was performed to quantitate the changes in the Sertoli cell transcriptome after *ID* overexpression (Table 1 and Chart 1). Comparisons were made with primary (nontransformed Sertoli cells) and SC-Id1 or SC-Id2 lines taken at approximately 80 doublings. In the SC-Id1 line, 526 genes increased and 788 genes decreased by greater than 2-fold compared with the primary Sertoli cells. In the SC-Id2 line, 568 genes increased and 764 genes decreased compared with primary Sertoli cells. As shown in Chart 1, a decrease in transferrin, inhibin alpha subunit, and Wilms Tumor Antigen (WT1) transcription factor were observed in the SC-Id lines in comparison to primary Sertoli cells. Müllerian inhibiting substance (MIS) gene ex-

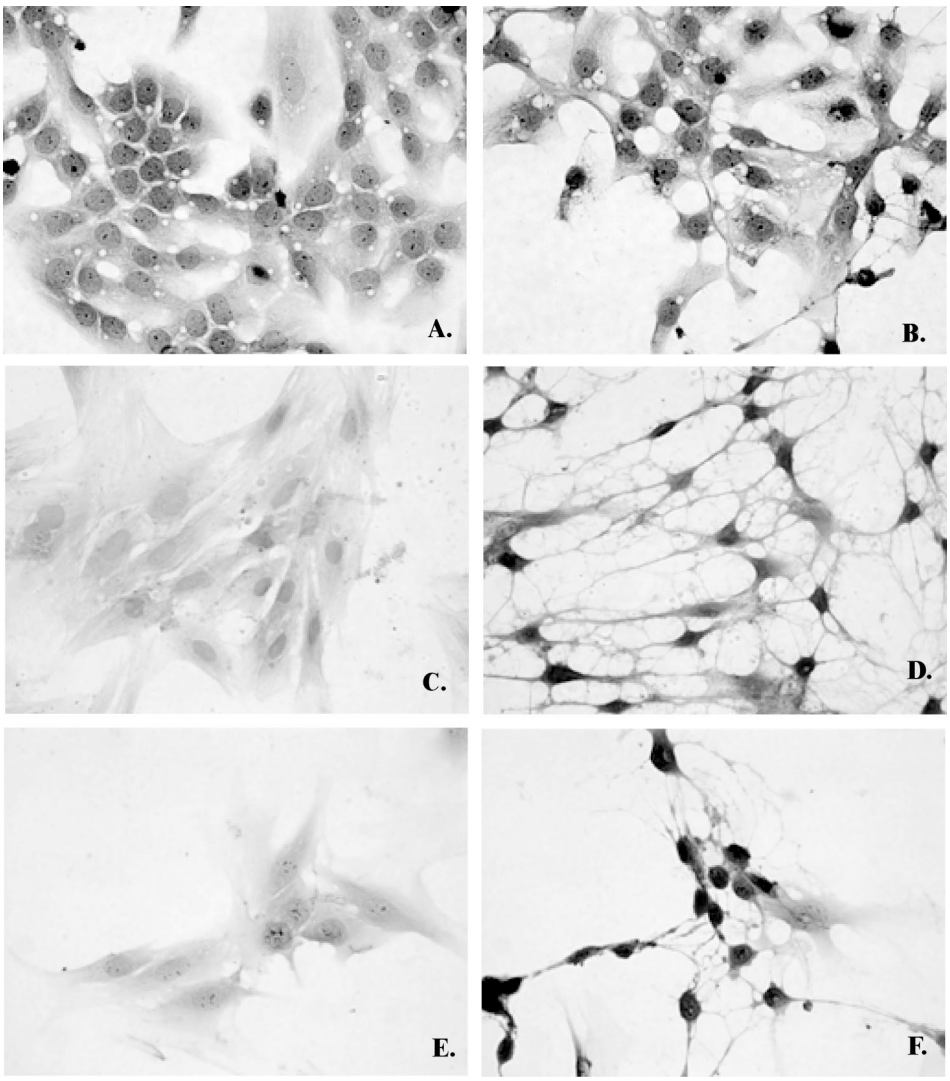

FIG. 4. Morphology of Sertoli cells as observed under the light microscope) (×100 magnification). **A** and **B**) Primary Sertoli cells; (**C** and **D**) Sertoli cells overexpressing ID1 at 50 doublings; (**E** and **F**) Sertoli cells overexpressing ID2 at 50 doublings. Representative images of untreated control cell cultures are shown in (**A**, **C**, and **E**), whereas (**B**, **D**, and **F**) are those following cAMP stimulation for 72 h. Data are representative of a minimum of three experiments.

pression did not change significantly (Table 1 and Chart 1). Overexpression of ID1 or ID2 was accompanied by increased expression of Sry-related HMG box protein Sox11 and Cathepsin L. Cluster analysis demonstrated that a number of cell cycle genes increased in the SC-Id lines, including the cyclins D and G, and cdk4 and cdc37 (Chart 1). The cell cycle inhibitor p21 decreased, and p27 slightly decreased, while p16 showed a small increase (Table 1 and Chart 1). The retinoblastoma (*Rb*) gene and Rb binding protein (*Rbbp9*) gene were decreased, suggesting the cell cycle may have abnormal feedback mechanisms. Unfortunately, telomerase was not present on the microarray chip, so a preliminary experiment involved RT-PCR. Observations demonstrated the presence of telomerase reverse transcriptase (*Tert*) gene expression in the SC-Id lines, but at reduced levels compared with that of the primary Sertoli cells (data not shown). Other gene clusters demonstrated general decreases in transcription factors, and both increases and decreases in signal transduction and metabolic enzyme gene expression (Chart 1). The semiquantitative data presented for PCR in Figure 5 confirm the trends in data obtained from the microarray (Table 1) for selected genes. A statistical analysis of the microarray data was performed, and all data presented in Table 1 and Chart 1 for specific genes are statistically different between the primary Sertoli cell and Sertoli cell-Id lines ($P < 0.05$).

A detailed cluster analysis indicates the relationships (i.e., gene tree dendrograms) of altered gene expression after ID transformation of the Sertoli cell is shown in Figure 6. The changes in color represent no change (yellow), increases (red), and decreases (blue) in related gene clusters with the gene tree dendrogram. This is a comparison between primary Sertoli, SC-Id1, and SC-Id2, and does not relate to the primary Sertoli data in Table 1 and Chart 1. Although most changes in gene expression were similar in the SC-Id1 and SC-Id2 lines (yellow), some differences are observed. Most of the gene expression in primary Sertoli cells was either increased (red) or decreased (blue) in relation to the SC-Id lines. Several of the specific gene clusters (i.e., cell cycle, signal transduction, and transcription factor genes) involved are also indicated in Figure 6.

DISCUSSION

Previous studies to stimulate terminally differentiated postmitotic Sertoli cells to undergo cell proliferation have been unsuccessful. Previously developed rat and mouse Sertoli cell lines such as MSC-1, RTS3-3, TM-4, ASC-17D, and TTE3 [72–81] have been derived from tumorigenic models or by overexpressing viral oncogenes such as SV40 large T antigen in proliferating prepubertal Sertoli cells. Adult postmitotic Sertoli cells have also been shown to be

transformed by SV40 T antigen to promote cell proliferation [73]. Therefore, the molecular events associated with Sertoli cell terminal differentiation have a dominant phenotype and are unable to proliferate without cellular transformation. Overexpression of genes involved in proliferation, differentiation, or both that are normally expressed by Sertoli cells in response to hormones and growth factors may provide more direct information on the associated molecular events involved in Sertoli cell terminal differentiation.

Previous observations have shown that bHLH proteins are involved in regulating differentiated functions such as transferrin [37, 44] and ABP promoter activation [82] in postmitotic Sertoli cells. The postpubertal Sertoli cells were also shown to express the dominant negative HLH proteins ID1 and ID2 [61, 62]. In primary Sertoli cell cultures the levels of ID1 and ID2 expression decreased in response to FSH [62]. FSH is generally considered a differentiation promoting agent for Sertoli cells. This corresponds to ID proteins generally being considered as proliferation-promoting and differentiation-inhibiting factors. The ability of FSH to suppress ID expression correlates with an increased differentiation and decreased proliferation of the cells [62]. These observations led us to hypothesize that stable overexpression of either ID1 or ID2 (or both) may allow postmitotic Sertoli cells to reenter the cell cycle and promote cell proliferation. Our hypothesis was supported by recent observations that ID overexpression can transform different cell types such as keratinocytes [71]. Unlike Sertoli cells, most of these cell types are quiescent, and after appropriate mitogenic stimuli, can enter the cell cycle. The cellular transformation by ID proteins is mediated by stimulation of the cell cycle and activation of telomerase [83–85]. Consistent with these observations, an increased expression of ID proteins is also observed in many forms of cancer [86–91]. The current study considered the effects of ID on a terminally differentiated cell that is postmitotic. The dogma would suggest that these cells would not be able to reenter the cell cycle due to molecular events promoting terminal differentiation.

Overexpression of either ID1 or ID2 promoted Sertoli cell proliferation, which is not possible in pubertal or adult Sertoli cells. ID1 overexpression promoted the proliferation of both pubertal (i.e., 20-day-old) and adult (i.e., 60-day-old) Sertoli cells. The observed increase in DNA synthesis is higher in cells expressing ID2 than in Id1. In addition, the proliferation rate may be dependent on mitogenic stimulation in cells overexpressing Id1, but it may be relatively independent in cells expressing Id2. The specific mechanism by which ID proteins initiate Sertoli cell proliferation requires further investigation. However, ID overexpression is expected to involve alterations in cell cycle control genes such as p27, p21, c-Myc, p16, and Rb activity. This hypothesis is based on the mechanism by which ID promotes proliferation in other cell systems such as endothelial cells, keratinocytes, and mammary epithelial cells (Fig. 7). In general, ID may regulate the expression or function of Myc [92], Rb proteins [93], related proteins p107 and p130 [94], and cyclin-dependent kinase (CDKN2) [60]. Reversing the cell cycle arrest through ID binding to the Rb family of proteins releases E2F to then promote cell cycle gene (e.g., S-phase factors) expression [93]. ID also antagonizes the growth-suppressive activities of cyclin-dependent kinase inhibitors p16 [95, 96] and p21 [97] (Fig. 7). C-myc activity in response to FSH has been reported in prepubertal and early pubertal Sertoli cells to be involved in cell proliferation [98]. The cMyc-Id-pRb pathway has been shown to be active in many proliferative cells [99]. Based on these observations, it is speculated that ID overexpression inhibits Rb [93] and decreases the expression of growth inhibitors [94, 96, 97] to allow Sertoli cells to reenter the cell cycle (Fig. 7).

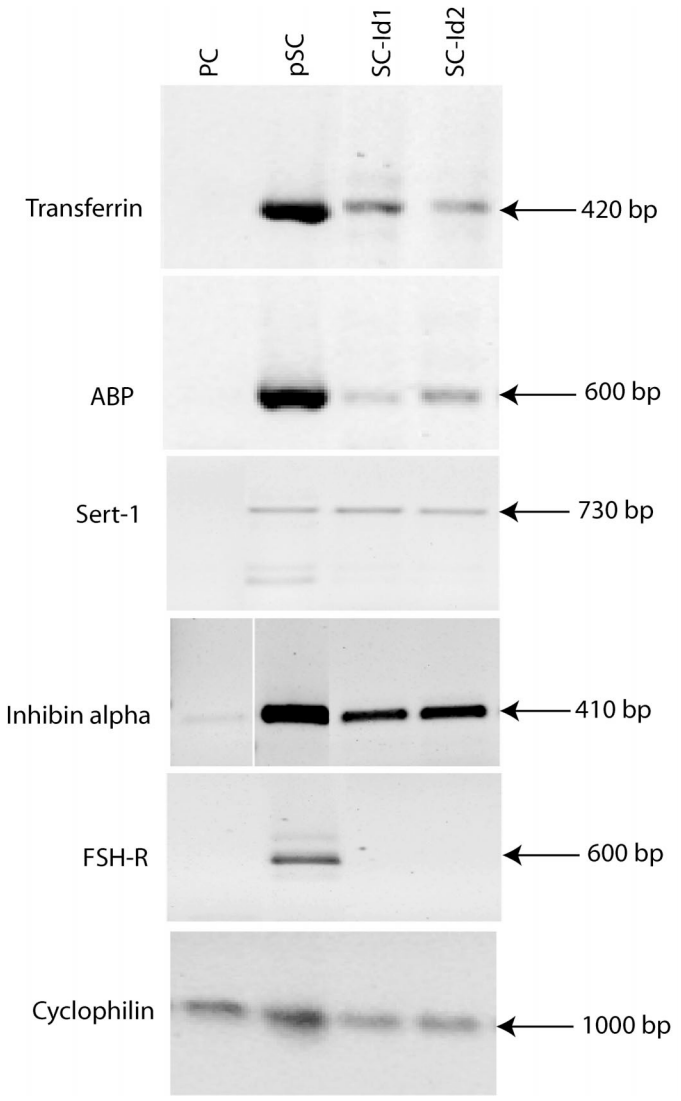

FIG. 5. RT-PCR of genes normally expressed by Sertoli cells. RT-PCR was performed on total RNA using gene-specific primers listed in Chart 1. The primary peritubular cells (PC) as a negative control, primary Sertoli cells (pSC) as a positive control, and Sertoli cell ID1 (SC-Id1) and ID2 (SC-Id2) lines at 50–120 doublings were assayed. The genes analyzed were transferrin, androgen binding protein (ABP), SERT-1, inhibin-alpha, and FSH receptor (FSH-R). Cyclophilin (RT-PCR) was used to monitor the reverse transcription loading. The size (in base pairs) of each RT-PCR product is shown on the right. The RT-PCR is a representative of at least six experiments.

The increase in both ID1 and ID2 levels following transformation with either ID1 or ID2 is an intriguing observation and suggests that both isoforms may be required to promote Sertoli cell proliferation. This explains why the cell phenotype is similar between the SC-Id1 and SC-Id2 lines. Observations suggest an interaction between the ID family members. The speculation is that the actions of either ID1 or ID2 altered transcriptional activity such that the promoters for both ID genes are activated. Further investigation is needed to elucidate the transcriptional interaction

TABLE 1. Sertoli-Id lines selected microarray gene expression analysis.

Genbank accession No.	Common name	Signal		
		SC primary*	Id1*	Id2*
	Sertoli cell specific genes			
D38380	Transferrin	3370.8 (375.2)	20.2 (1.7)	37.2 (11.5)
M58040	Transferrin receptor	75.1 (18.3)	20.9 (6.7)	37.0 (7.2)
S98336	Mullerian inhibiting substance	167.0 (20.5)	166.5 (8.8)	150.8 (9.1)
M36453	Inhibin alpha-subunit	446.0 (34.5)	81.4 (3.2)	69.7 (4.0)
AF044058	Androgen receptor interacting protein	271.9 (8.9)	143.1 (6.8)	164.4 (5.8)
S63358	WT1 zinc-finger homolog [rats, testis]	374.9 (36.4)	2.3 (0.9)	9.8 (0.7)
M34043	Thymosin beta-10 (testis-specific) gene	3156.1 (198.4)	3739.8 (80.0)	2418.2 (55.6)
X15705	Testis-specific heat shock protein-related gene hst70	90.4 (42.9)	4.1 (0.6)	4.0 (0.1)
AB009662	Testis specific protein	61.9 (43.7)	2.6 (0.6)	1.3 (0.8)
L33869	Ceruloplasmin mRNA	347.8 (151.1)	25.8 (1.4)	12.1 (1.7)
AJ004858	Sry-related HMG-box protein Sox11	21.3 (3.1)	75.6 (16.2)	71.6 (3.9)
J01435	Cytochrome oxidase subunit I [Sertoli cells]	7889.4 (326.1)	8014.5 (702.4)	7252.9 (111.5)
M38759	Androgen binding protein	467.0 (121.3)	9.1 (5.2)	7.6 (1.1)
S85184	Cyclic Protein-2=cathepsin L proenzyme [Sertoli cells]	260.1 (10.4)	665.1 (30.9)	340.2 (46.7)
M37482	Inhibin beta-subunit	68.2 (9.5)	84.4 (14.5)	76.2 (4.3)
AF077195	Sertoli cell protein (SC4)	35.4 (4.6)	11.2 (5.0)	7.9 (1.5)
M23264	Androgen receptor	28.7 (4.0)	24.3 (4.5)	13.7 (0.4)
L02842	FSHR	24.9 (16.9)	2.6 (1.1)	1.8 (0.9)
	Cell Cycle Genes			
D14014	Cyclin D1	70.3 (9.9)	232.9 (25.1)	117.0 (6.1)
D16308	Cyclin D2	154.5 (78.1)	493.2 (42.9)	524.5 (7.2)
D16309	Cyclin D3	55.6 (36.5)	359.1 (18.2)	232.1 (18.3)
D38560	Cyclin G	219.3 (34.8)	833.3 (33.8)	796.7 (25.0)
L11007	cdk4	315.2 (4.7)	861.0 (101.7)	763.2 (78.2)
D26564_at	cdc34	200.6 (7.8)	380.7 (9.3)	403.0 (59.0)
L41275	p21	49.8 (3.8)	19.6 (11.5)	26.6 (4.8)
S79760_at	p16	49.2 (2.3)	59.1 (15.6)	68.8 (0.3)
D83792_at	p27	13.4 (1.0)	11.3 (4.1)	17.1 (1.5)
D25233cds_at	Rb	63.8 (3.8)	23.5 (1.0)	25.3 (0.3)
AF025819	Rb binding protein	4.0 (1.3)	0.8 (0.1)	1.1 (0.2)
U31668_at	E2F	41.8 (0.1)	20.4 (2.8)	20.8 (3.5)

* Values in parentheses indicate ± SEM.

between ID1 and ID2. An interesting observation made in this study was that the Sertoli cells transformed with ID1 or ID2 failed to undergo senescence even after 200 cell doublings. Previous studies have proposed that ID overexpression may delay replicative senescence in human endothelial cells [100], keratinocytes [96], and fibroblasts [101]. A similar study using retroviral based overexpression of ID1 in human keratinocytes delayed the onset of replicative senescence [102]. Unlike the study by Alani et al. [96] who selected the *ID-1* transformants that had elevated telomerase activity and inactivated Rb/p16 pathway, Nickoloff et al. used unselected *ID-1* transformants [102]. This experimental discrepancy may have led to different conclusions in the ability of ID-1 to immortalize [96] or delay [102] replicative senescence of human keratinocytes. These results led to the hypothesis that immortalization requires the activation of telomerase, TERT. Our experimental approach was similar to that of Nickoloff et al. [102] in that the Sertoli cells were unselected for activation of telomerase and inactivation of p16/Rb pathway. The ability of Sertoli cells that overexpress ID to survive and proliferate was the only selection performed. Preliminary studies support that telomerase (i.e., Tert) is present in the SC-Id lines, but at a reduced level than in primary Sertoli cells. Previously high levels of telomerase reverse transcriptase (Tert) have been shown in other terminally differentiated cells such as neurons [103]. The role of *Id* genes in regulating senescence is also evident from the *Id1* null mouse embryo fibroblasts that senesce prematurely because of increased expression of p16 [96]. The molecular mechanisms involved in bypassing the senescence by Sertoli cells overexpressing *Id1* and *Id2* may be unique and needs further evaluation. It is likely ID proteins will influence telomerase activity and have a role in the block in senescence. Future studies will need to investigate the role of telomerase in these immortalized SC-Id lines. The speculation is that the terminal differentiated postmitotic nature of the cell may promote or allow this immortalization. The molecular control of terminal differentiation may involve a permanent alteration in the cell cycle pathways. ID overexpression could be used to study this process in more detail. ID overexpression could also be used to promote and investigate cell proliferation in postmitotic cells such as neurons or muscle. In the event an inducible promoter could be used, then regeneration of these terminally differentiated cells could provide therapeutic strategies for neurodegenerative or muscle degenerative diseases. The focus of the current study on Sertoli cells provides insight into a link between ID proteins and the postmitotic state of other terminally differentiated cells.

The presence of Sertoli genes such as inhibin alpha, transferrin, Sert, and ABP suggests that ID1/ID2 overexpression allowed the expression of these differentiated genes. The levels of their expression were diminished compared with that of primary Sertoli cells, as demonstrated in the microarray and RT-PCR experiments. The magnitude of the change in gene expression is sometimes different between the two procedures. This is likely due in part to PCR using two oligonucleotide primers, while the microarray procedure involves 16 different oligonucleotide primers for the specific gene. The observed decrease in gene expression can be explained in part based on the observations that

many gene expressions, such as that of transferrin, are regulated by an E-Box present in the proximal promoter region [37]. Previous experiments have shown that transferrin promoter activity can be blocked by either mutating the E-box or by transiently overexpressing ID [37]. The decrease in transferrin expression in these SC-Id cell lines further confirms the previous results that bHLH proteins are involved in regulating the expression of the transferrin gene. Ectopically expressed ID1 or ID2 may titer out the positively acting bHLH protein or proteins, making them unavailable for binding and activating differentiated gene expression (Fig. 7). Similar mechanisms are responsible for the downregulation of ABP [82]. The complete loss of FSH receptor in Sertoli cells overexpressing Id1/Id2 may also be due to the lack of available bHLH transcription factors required for inducing FSH receptor expression [104].

The analysis of the Sertoli cell transcriptome in primary Sertoli cells and the Sertoli-Id lines demonstrates the presence of a number of Sertoli cell differentiated genes, but at reduced levels of expression. Therefore, the cells had a lower level of differentiated function, but they did not dramatically alter cellular differentiation. A number of differentiated markers increased in expression (i.e., Sox11, cyclic protein 2), suggesting these genes may have a distinct role and regulation in contrast to those that are decreased. As expected, a number of cell cycle genes were increased, as discussed. Other cellular pathways influenced by the overexpression of ID1 or ID2 were transcriptional regulation pathways, signal transduction pathways, and metabolic pathways (Chart 1 and Fig. 6). The relationships between the gene clusters are shown in Figure 6 and demonstrate that the majority of changes were similar between SC-Id1 and SC-Id2. The genes that are distinct between ID1 and ID2 likely reflect differences in the actions of ID1 versus ID2. In general, cell cycle gene clusters were increased, while differentiated genes and transcription factor gene clusters were decreased in response to ID overexpression. This correlates with the decrease in cellular differentiation and increase in cell cycle induction summarized in Figure 7. This analysis provides insights into how these cellular pathways respond between the terminally differentiated state and active cell cycle proliferative state.

A distinction needs to be made between Sertoli cell lines generated by overexpression of Id1/Id2 versus those obtained by SV40 T antigen or other oncogenes. The SC-Id cell lines were developed using overexpression of a gene normally expressed by Sertoli cells in response to mitogens, and these genes are required to maintain Sertoli cell differentiated functions. *ID* genes are also a normal gene involved in regulating the cell cycle [60]. The functional morphology, physiology, and gene expression profile of these cells closely resemble the primary Sertoli cells, compared with those of the cell lines developed by SV40 T antigen. This is evident from the similar morphology between primary Sertoli cells and Sertoli cells overexpressing *ID1*/*ID2*. However, certain similarities between all the cell lines exist, such as loss of FSH receptor [105]. The ID proteins do interact with Rb in a manner similar to that of SV40 T antigen, but the T antigen can also interact with a variety of other cellular pathways that are distinct from ID proteins [106]. The current study used a constitutively expressed ID construct. Future studies will use an inducible promoter to effectively turn the ID expression on and off. This would allow a more careful analysis of the transition of Sertoli cells from a postmitotic to mitotic state. Terminal differentiation of the cells in regard to gene expression could be more precisely controlled and monitored.

CHART 1. Sertoli-Id lines microarray gene expression analysis.

Rat 13,000 Gene Chip (Affimetrix)

	SC-Id1 line	SC-Id2 line
(Total) Genes Increased (>2x)	526	568
(Total) Genes Decreased (>2x)	788	764
Sertoli cell differentiation genes	Fold increase (+) or decrease (-)	
Transferrin	-168.5	-90.7
ABP	-1.9	-1.7
FSHR	-9.6	-13.8
Ceruloplasmin	-13.5	-28.7
Inhibin-Alpha	-5.5	-6.4
MIS	-1.0	-1.1
WT1	-163.0	-38.3
SOX11	+3.6	+3.4
CP2/Cathepsin L	+2.6	+1.3
Cell cycle genes	Fold increase (+) or decrease (-)	
Cyclin D1	+3.3	+1.7
Cyclin D2	+3.2	+3.4
Cyclin D3	+6.5	+4.2
Cyclin G	+3.8	+3.6
cdk4	+2.4	+2.6
Cdc37	+1.9	+2.0
p21	-2.5	-1.9
p16 (CDKN2A, INK4a)	+1.3	+1.4
p27 (CDKN1B)	-1.2	-1.3
Rb binding protein	-5.0	-3.6
Rb	-2.7	-2.5
E2F	-2.0	-2.0
	Transcription factor genes	
(Total) Genes Increased (>2X)	15	14
(Total) Genes Decreased (>2X)	44	43
	Signal transduction genes	
(Total) Genes Increased (>2X)	42	37
(Total) Genes Decreased (>2X)	57	52
	Metabolic genes	
(Total) Genes Increased (>2X)	10	8
(Total) Genes Decreased (>2X)	29	36

The efficiency of spermatogenesis is largely dependent on the number of Sertoli cells in the testis [107]. These observations are supported by the FSH receptor and FSH beta subunit knockout models [108, 109]. In these knockouts, a decrease in Sertoli cell numbers correlates with decreased spermatogenesis [25]. These observations support the notion that an increased Sertoli cell number may lead to quantitatively increased spermatogenesis. In the future, this model system can be exploited to study the effect of increased Sertoli cell proliferation on spermatogenesis in an inducible transgenic model in which *ID1* or *ID2* gene expression is directed to Sertoli cells using Sertoli cell specific promoters such as Sert1 [110]. In the event that ID overexpression increased Sertoli cell number, then a larger testis and greater sperm output would be expected. This model would be dependent on being able to turn off ID expression and allow normal cellular differentiated gene expression to be present. The current observations suggest the potential to alter the postmitotic nature of terminally differentiated cells. Applications in a number of disease states

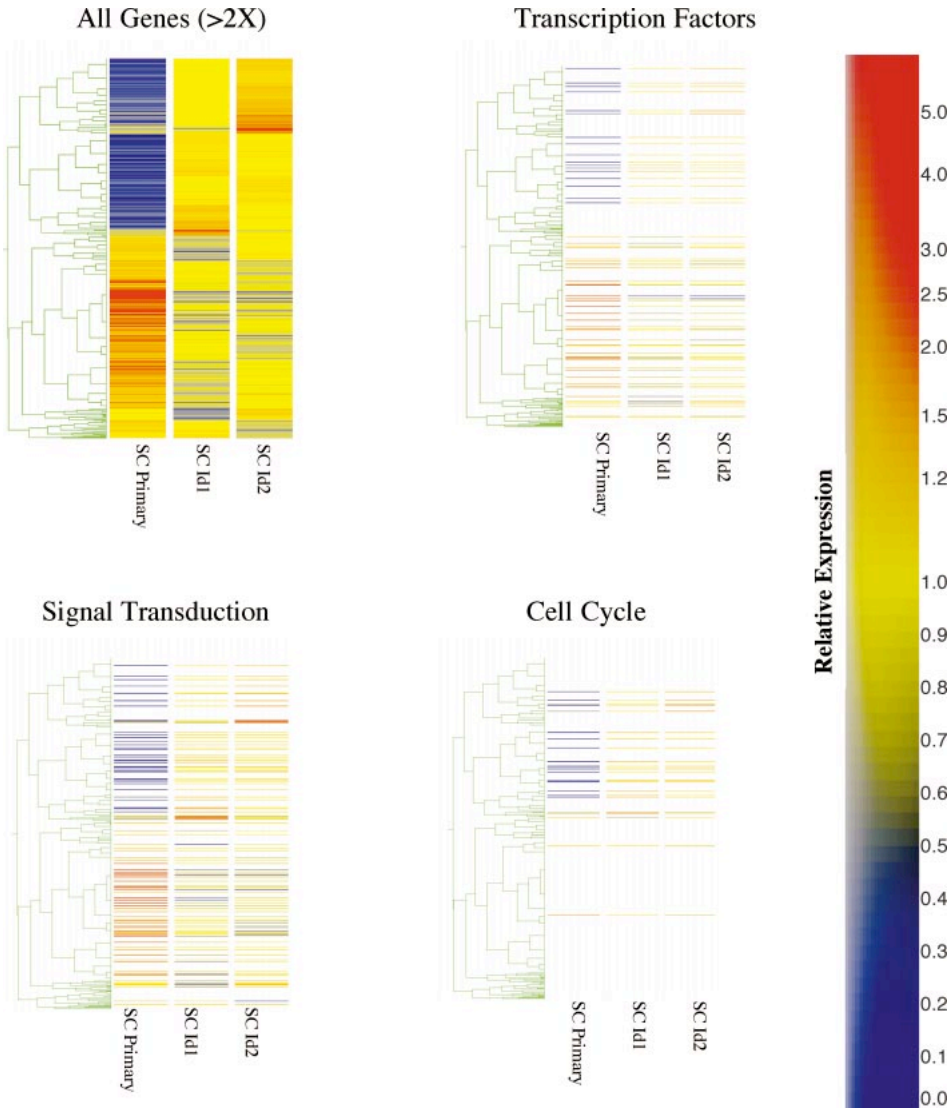

FIG. 6. Gene tree dendrogram smooth correlation cluster analysis of changes in the transcriptomes of primary Sertoli cells (SC Primary), Sertoli cell-Id1 (SC-Id1), and Sertoli cell-Id2 (SC-Id2) at 80 doublings. A comparison is made between each separately. The dendrogram with relative expression relationships on the right correlate with increased (red) and decreased (blue), as indicated by the correlation color panel. Data for all genes (>2×), minus the cell cycle genes of EST, transcription factor genes, and signal transduction genes are presented. Analysis used a GeneSpring software procedure using the data summarized in Table 1 and Chart 1.

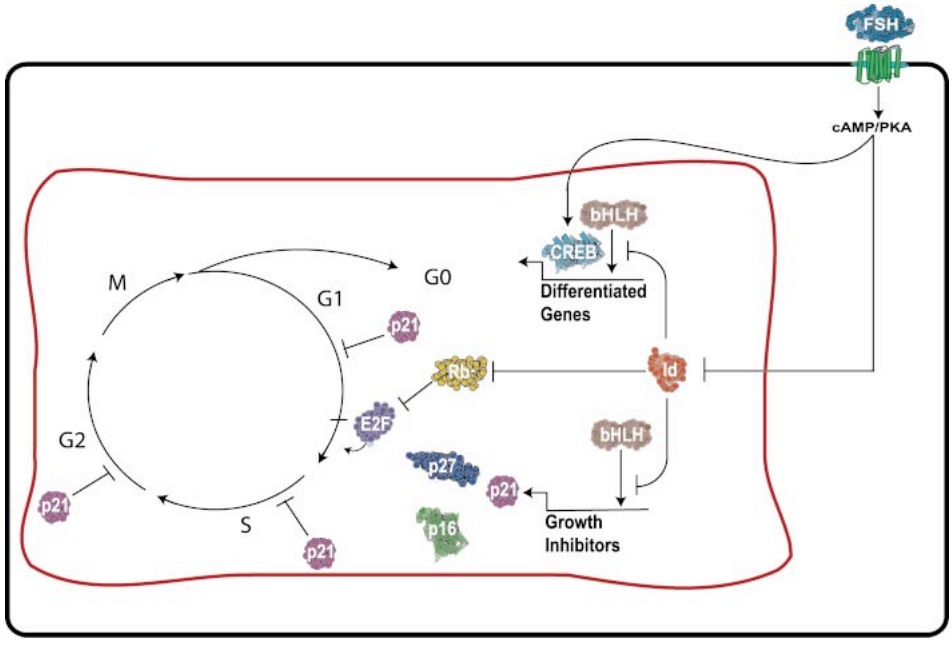

FIG. 7. Schematic of proposed ID interactions with the cell cycle proteins and cell differentiation proteins in the Sertoli cell. FSH acting at the FSH receptor activates cAMP and protein kinase A (PKA), which activates Sertoli cell differentiated genes and inhibits ID expression. ID binds retinoblastoma protein (Rb), which allows E2F to promote the cell cycle. ID blocks bHLH proteins needed for expression of growth inhibitors p16, p21, and p27, and decreases growth inhibition.

such as neuroregeneration and muscle regeneration, as well as fertility enhancement, now need to be considered. The concept would be to promote a postmitotic cell such as a neuron to proliferate in response to *ID* expression and allow neural regeneration to occur. The key would be to regulate *ID* expression and promote neural differentiation following cell proliferation.

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