Optimal culture conditions are critical for efficient expansion of human testicular somatic and germ cells in vitro

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Objective: To optimize culture conditions for human testicular somatic cells (TSCs) and spermatogonial stem cells.
Design: Basic science study.
Setting: Urology clinic and stem cell research laboratory.
Patient(s): Eight human testicular samples.
Interventions(s): Testicular tissues were processed by mechanical and enzymatic digestion. Cell suspensions were subjected to differential plating (DP) after which floating cells (representing germ cells) were removed and attached cells (representing TSCs) were cultured for 2 passages (P0–P1) in StemPro-34– or DMEM-F12–based medium. Germ cell cultures were established in both media for 12 days.
Main Outcome Measure(s): TSC cultures: proliferation doubling time (PDT), fluorescence-activated cell sorting for CD90, next-generation sequencing for 89 RNA transcripts, immunocytochemistry for TSC and germ cell markers, and conditioned media analysis; germ cell cultures: number of aggregates.
Result(s): TSCs had significantly prolonged PDT in DMEM-F12 versus StemPro-34 (319.6 ± 275.8 h and 110.5 ± 68.3 h, respectively). The proportion of CD90-positive cells increased after P1 in StemPro-34 and DMEM-F12 (90.1 ± 10.8% and 76.5 ± 17.4%, respectively) versus after DP (66.3 ± 7%). Samples from both media after P1 clustered closely in the principle components analysis map whereas those after DP did not. After P1 in either medium, CD90-positive cells expressed TSC markers only, and fibroblast growth factor 2 and bone morphogenetic protein 4 were detected in conditioned medium. A higher number of germ cell aggregates formed in DMEM-F12 (59 ± 39 vs. 28 ± 17, respectively).
Conclusion(s): Use of DMEM-F12 reduces TSC proliferation while preserving their unique characteristics, leading to improved germ cell aggregates formation compared with StemPro-34, the standard basal medium used in the majority of previous reports. (Fertil Steril® 2017;107:595–605. ©2017 by American Society for Reproductive Medicine.)
Key Words: Human testicular somatic cells, germ cells, spermatogonial stem cells, male fertility preservation

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Cancer treatment outcomes among children have improved tremendously in recent years, allowing the majority of them to survive (1). Consequently, the prevalence of adults who were previously treated with the use of chemotherapy and/or radiotherapy has increased significantly (2). One of the unfortunate complications of cancer treatment is infertility (3). Unlike adolescents (4) and adults, semen cryopreservation before gonadotoxic exposure is not possible for boys, because spermatogenesis does not commence until puberty (5). This has led to worldwide initiatives to develop spermatogonial stem cell (SSC)–based strategies for fertility preservation.

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SSC homeostasis requires a balance between self-replication to maintain the stem cell pool and meiotic differentiation to fuel spermatogenesis (6). This equilibrium is tightly regulated by the testicular microenvironment (7). Testicular somatic cells (TSCs), including Sertoli and peritubular cells, play essential roles during spermatogenesis by providing structural and nutritional support for differentiating germ cells (8). The frequency of SSCs is estimated to be low in mammalian testes; it is thought to represent 0.03% of testicular cells in mice (9). Therefore, propagation of SSCs in vitro would likely be required before attempting fertility restoration by either autotransplantation to the testis (5) or in vitro spermatogenesis (10). Unfortunately, early reports of long-term human SSC expansion have been controversial (1, 11, 12).

Human testicular cell suspension cultures are characterized by two main populations: fibroblastic-like somatic cells which tend to attach to culture dishes, and small round cells which are presumed to be germ cells and tend to adhere to the attached cells. The latter may divide and form colonies which disappear in early cell passages because the adherent cells quickly grow and become confluent (13). In keeping with this, there is an observed loss of SSC-associated marker expression (UTF1, FGFR3, and DAZL) during early culture stages, together with a concomitant increase in the expression of TSC markers (VIM, ACTA2 and GATA4) (12). TSCs are crucial for SSC expansion in vitro, but their overgrowth remains a major challenge (13). Therefore, TSC overgrowth in vitro is one of the major hurdles to overcome when attempting to achieve efficient SSC expansion. Previous studies tried to characterize the different testicular cell subpopulations according to specific markers (14, 15). CD90 has been suggested to be exclusively expressed in human somatic cells compared with germ cells, especially in the postnatal period (16).

Media composition is a critical aspect of cell culture. Medium formulations used for ex vivo human SSC propagation by the majority of researchers (1, 12, 13, 17, 18) have not changed significantly from the StemPro-34–based medium used in early mouse studies, where efficient long-term expansion of SSCs was reported (19). A recent study found that human SSCs display limited proliferation in vitro under murine SSC StemPro-34–based medium culture conditions (20). In contrast, the use of DMEM-F12–based medium has rarely been reported (21). To our knowledge, despite the crucial role that media composition plays in such systems, there are no publications directly comparing these two types of media for human testicular cell culture. Because TSC overgrowth remains a crucial obstacle for human SSC expansion in vitro, we decided to compare the effects of these two types of media on testicular somatic and germ cell growth. Specifically, our aim was to compare the proliferation rates and phenotypes of human TSCs expanded in culture with the use of growth factor–supplemented StemPro-34 or DMEM-F12 medium. In addition, we contrasted the effect of these two types of media on germ cell survival.

METHODS

Tissue Collection and Cell Dissociation

Human testicular samples were obtained from seven patients who underwent orchiectomy (four owing to testicular malignancy and three because of testicular pain) and one patient that underwent microscopic testicular sperm extraction (microTESE) for nonobstructive azoospermia (NOA; Supplemental Table 1, available online at www.fertstert.org). All patients provided written informed consents to participate in this study (University of Toronto Research Ethics Board [REB] no. 30252 and Mount Sinai Hospital REB no. 14-0032-E). After pathologic analysis and/or reproductive use (microTESE), the remaining testicular tissue was transferred to the research lab in isolation medium composed of Dulbecco Modified Eagle Medium, Nutrient Mix F-12 (DMEM-F12; Life Technologies) with 7.5% sodium bicarbonate, nonessential amino acids (Invitrogen), 1% penicillin/streptomycin, 1 μl/mL gentamicin (G1397-10MI; Sigma-Aldrich), and 10 mg/mL DNAse (cat. no. d-5025 150KU; Sigma-Aldrich). Two of the orchiectomy tissue samples were immediately cryopreserved and used subsequently for these studies. Samples were washed three times with the use of phosphate-buffered saline solution (PBS) followed by chopping with scissors in Modified Eagle Medium (MEM) + 20% fetal bovine serum (FBS) to get 1–2-mm pieces; 10–20 pieces were transferred to 1 mL freezing medium consisted of MEM + 20% FBS + 5% dimethylsulfoxide. Vials were thawed in water at 37°C for 3 minutes. The thawed tissue was washed in isolation medium and then subjected to mechanical and enzymatic digestion as performed in the four fresh samples. Briefly, mechanical digestion was conducted with the use of fine scissors and needles to disrupt the tissue and to separate the seminiferous tubules as much as possible. Tissue pieces were transferred to a 50-mL tube containing isolation medium and washed repetitively until the supernate became clear. Five mL Enzyme Mix I containing 2 mg/mL collagenase type I (cat. no. LS004196; Warthington), 2 mg/mL hyaluronidase (cat. no. H2126; Sigma-Aldrich), 2 mg/mL trypsin (cat. no. T1005; Sigma-Aldrich), and 4 μL DNAse (cat. no. d-5025 150KU; Sigma-Aldrich) was added to the tube containing 5 mL isolation medium and testicular tissue (total volume of 10 mL) and incubated in a shaker-incubator at 32°C and 120 rpm for 15 minutes. The cell suspension was centrifuged for 5 minutes at 400 rpm without brake, and the supernate was removed. Tubules were washed in isolation medium, followed by centrifugation for 5 minutes at 400 rpm without brake. The supernate was removed and 5 mL Enzyme Mix II containing 2 mg/mL collagenase type I, 2 mg/mL hyaluronidase, and 4 μL DNAse in 5 mL isolation medium (total 10 mL) was added to the tissue pellet, following by incubation at 32°C on a shaker at 120 rpm. Microscopic observation was performed to assess the thinning and perforation of the peritubular cell layer. Accordingly, up to 10 additional minutes of enzymatic incubation time was included. Undigested tissue was removed by subjecting the cell suspension to sequential filtration with 70-μm and 40-μm nylon filters, followed by centrifugation for 5 minutes at 1,800 rpm without brake. To lyse red blood cells, testicular cells suspensions were incubated with a 1:4 isolation medium to ammonium chloride ratio on ice for 10 minutes and washed twice with isolation medium with 10% FBS (lot no. AWK24007; Hyclone) and without DNAse.

Differential Plating

Dishes were coated with 0.2% gelatin (cat. no. G1393; Sigma-Aldrich) by incubation for 30 minutes at 37°C followed by solution aspiration. Testicular cells were plated at a density of 20,000 cells per cm^2 and incubated overnight in isolation medium with 10% FBS and without DNAase at 37°C with 20% O_2 and 5% CO_2. Floating cells were removed the following morning without any additional washing. Attached cells were resuspended with the use of TrypleE (Gibco) and transferred to 15-mL tubes, followed by centrifugation for 5 minutes at 12,000 rpm. The supernate was aspirated and the remaining cells counted with the use of an automated Cell Countess (Life Technologies).

Cell Cultures

Somatic cells, obtained after DP, were cultured at a density of 5,000 cells per cm^2 in 12-well plates (BD Biosciences) coated with 0.2% laminin (VWR International Co.). StemPro-34 (Life Technologies/Invitrogen) and DMEM-F12 (Life Technologies) media were supplemented with growth factors as previously described (1, 12, 13), including 2% FBS (Hyclone), 1% penicillin-streptomycin, 1 μL/mL gentamicin, and 15 mmol/L/10 mL HEPES. The growth factors included were glial cell–derived neurotropic factor (GDNF; 10 ng/mL, cat. no. 212-GD-010; R&D Systems), epidermal growth factor (EGF; 20 ng/mL, cat. no. AF-100-15; Peprotch), fibroblast growth factor (FGF) 2 (10 ng/mL, cat. no. Af-100-18B; Peprotech), and leukemia inhibitory factor (LIF; 10 U/mL, cat. no. GF342; EMD Millipore) (22).

The medium was replaced with freshly prepared medium every 2 days. When cells became 80%–90% confluent, they were counted and passaged in laminin-coated 6-well plates (passage 1; P1). At 80%–90% culture confluency, cells were counted and subjected to characterization procedures. Population doubling time (PDT) was calculated to assess cell proliferation and survival with the use of the following formula: $PDT = t \cdot \log 2/(\log Nt - \log N0)$, where t = the culture time (h), N0 is the number of cells seeded, and Nt is the number of cells harvested (23).

Fluorescence-Activated Cell Sorting

Samples of 50,000–100,000 cells were incubated with phycoerythrin-conjugated anti–human CD90 (1:40; BD Bioscience) for 30 minutes at 4°C and then washed with 3% FBS-PBS. One mg/mL propidium iodide (Sigma-Aldrich) was added to 200 μL cell suspension which was then sorted directly into RNA lysis buffer (Norgen Bioteck Corp.) with the use of the fluorescence-activated cell sorting (FACS) on the Aria II-SC BRV (Becton Dickinson) at the Hospital for Sick Children Flow Cytometry Facility (Toronto, Ontario).

Next-Generation Sequencing of Targeted RNAs

RNA was extracted from samples with the use of the RNA/DNA Purification Micro Kit (Norgen), according to the manufacturer's instructions. Concentration of extracted RNA was quantified with the use of the Qubit 2.0, and quality was assessed by the RNA Pico Kit on the 2100 Bioanalyzer (Agilent Technologies) at the Princess Margaret Genomic Centre in Toronto, Canada. In total, four samples after DP, three after P1 with StemPro-34–based medium, and four after P1 with DMEM-F12–based medium had adequate RNA quality to perform next-generation sequencing (NGS). cDNA libraries were prepared from 10 ng RNA with the use of the Ion Ampliseq RNA library kit (Life Technologies), according to the manufacturer's instructions. In brief, RNA was reverse transcribed into cDNA with the use of the Veriti thermocycler (Applied Biosystems). Target sequences were amplified with the use of a custom panel for targeted RNA sequencing that included 89 sets of primers. Markers used for NGS were chosen based on their known markers of specific cell lineages within the testicular niche, including as SSCs and meiotic, spermatid, and somatic cells according to previous reports (Supplemental Fig. 1, available online at www.fertstert.org). Libraries were quantified with the use of the 2100 Bioanalyzer (Agilent Technologies) and the Agilent High-Sensitivity DNA kit. Samples containing quality DNA within the region of 125–300 bp were diluted to 30–75 pmol/L and loaded onto an Ion 316 v2 chip. Library amplification and sequencing preparation were conducted on the Ion Chef System with the use of the Ion PGM Hi-Q Chef Kit and then sequenced on the Ion PGM Sequencer (Thermo Fisher) according to the manufacturer's instructions. Sequences were aligned by means of Ion Reporter and analyzed with the use of Partek Flow.

Immunocytochemistry

Cells were passaged to 8-well-chamber laminin-coated slides, and at 80% confluency they were fixed with 4% paraformaldehyde (PFA) for 20 minutes and then washed with cold PBS (cat. no. p3813-10PAK; Sigma-Aldrich) and stored at 4°C for up to 1 month. Slides were rinsed three times with PBS and then permeabilized in 0.1% Triton X (cat. no. 9410; Millipore) and 1% bovine serum albumin (BSA; cat. no. 23208; Medstore) in PBS for 15 minutes. Blocking was performed with the use of 5% normal goat serum (GS, Jackson Immunoresearch Laboratories) and 1% BSA in PBS for 1 hour at room temperature. Primary antibodies diluted in PBS containing 5% GS + 1% BSA were added and incubated overnight at 4°C. Primary antibodies included rabbit anti–human CD90 (1:50, Ab133350; Abcam), rabbit anti–human FSH receptor (1:500, Sc-13935; Santa Cruz), rabbit anti–human smooth muscle actin (1:200, Ab32575; Abcam), rabbit anti–human DAZL (1:800, Ab34139; Abcam), and mouse anti–human vimentin (1:500, M0725; Dako). After three washes with PBS containing 1% BSA, cells were incubated with the following secondary antibodies diluted 1:500 in PBS containing 5% GS + 1% BSA: Alexa555-conjugated goat anti–rabbit IgG (cat. no. 4413; Cell Signaling Technologies) and Alexa488-conjugated goat anti–mouse IgG (cat. no. 4408; Cell Signaling Technologies). Slides were washed three times with PBS containing 1% BSA and counterstained with Hoechst (1:2,000; Invitrogen) in PBS containing 1% BSA for 3–5 minutes. After three washes, Permafluor (TA030FM; Thermo Fisher Scientific) was added with the slide coverslip.

Fluorescence images were observed and captured with the use of the EVOS fluorescence microscope (Thermo Fisher Scientific). Negative control samples were processed under identical conditions with the omission of primary antibodies. Human umbilical cord perivascular cells served as positive control samples (23).

Enzyme-Linked Immunosorbent Assay of Conditioned Media

To assess growth factor secretion after culture, cells were passaged to P2 at a density of 5,000 cells per cm^2 on 6-cm^2 laminin-coated dishes (BD Biosciences) and cultured in parallel with fully supplemented media until they reached 80% confluency. At that point, they were rinsed twice with PBS and then incubated in basal (unsupplemented) StemPro-34 or DMEM-F12 medium for 1 hour, after which fresh basal medium was replaced for 72 hours. Conditioned medium was collected, centrifuged at 3,000 rpm for 10 minutes, and snap frozen in 1–5-mL aliquots. Cells were then harvested and counted.

Medium samples were thawed and concentrated with the use of protein concentrators (cat. no. PI87748; Pierce). ELISA for human FGF2, BMP4, BMP6, LIF, and GDNF was performed according to the manufacturer's instructions (Ray Biotech) and analyzed in duplicate medium samples, including basal medium control samples, on a Multi-Mode Microplate Reader F5 (Molecular Devices). The concentration of each factor was calculated with the use of the standard curve equation, dilution factor, and final volume collected and normalized for cell counts.

Assessment of Human Testicular Germ Cell Survival

To investigate the possible effect of medium on germ cell survival, we evaluated germ cell aggregation after 12 days in culture. After DP, germ and somatic cells from two human orchiectomy samples (patients 4 and 6) were cultured under six different culture conditions for each medium group. As described in Supplemental Table 2 (available online at www.fertstert.org), one-half of the cultures included floating cells only, and the others combined attached and floating cells in predefined ratios: R2, R13, and R25. This study design enabled us not only to compare media, but also to assess the effect of somatic cells on germ cell survival in the different floating/attached cell ratios. Human SSC-like aggregates ($\geq$ 10 cells) were imaged with the use of bright-field microscopy (Olympus) and quantified on day 12 as an indicator of germ cell survival. Two additional samples (patients 7 and 8) were cultured identically to perform germ cell phenotyping by means of NGS as described above.

Data Analysis

The results were presented as mean ± SD, unless otherwise indicated. Graphpad (Prism) was used for statistical analysis. Statistical significance was determined by means of Student *t* test or, when more than two conditions were compared, analysis of variance followed by Tukey multiple comparisons test. Differences were considered to be significant when $P<.05$.

RESULTS

Testicular Somatic Cell Population Doubling Time Is Reduced in DMEM-F12–Based Compared with StemPro-34–Based Culture Conditions

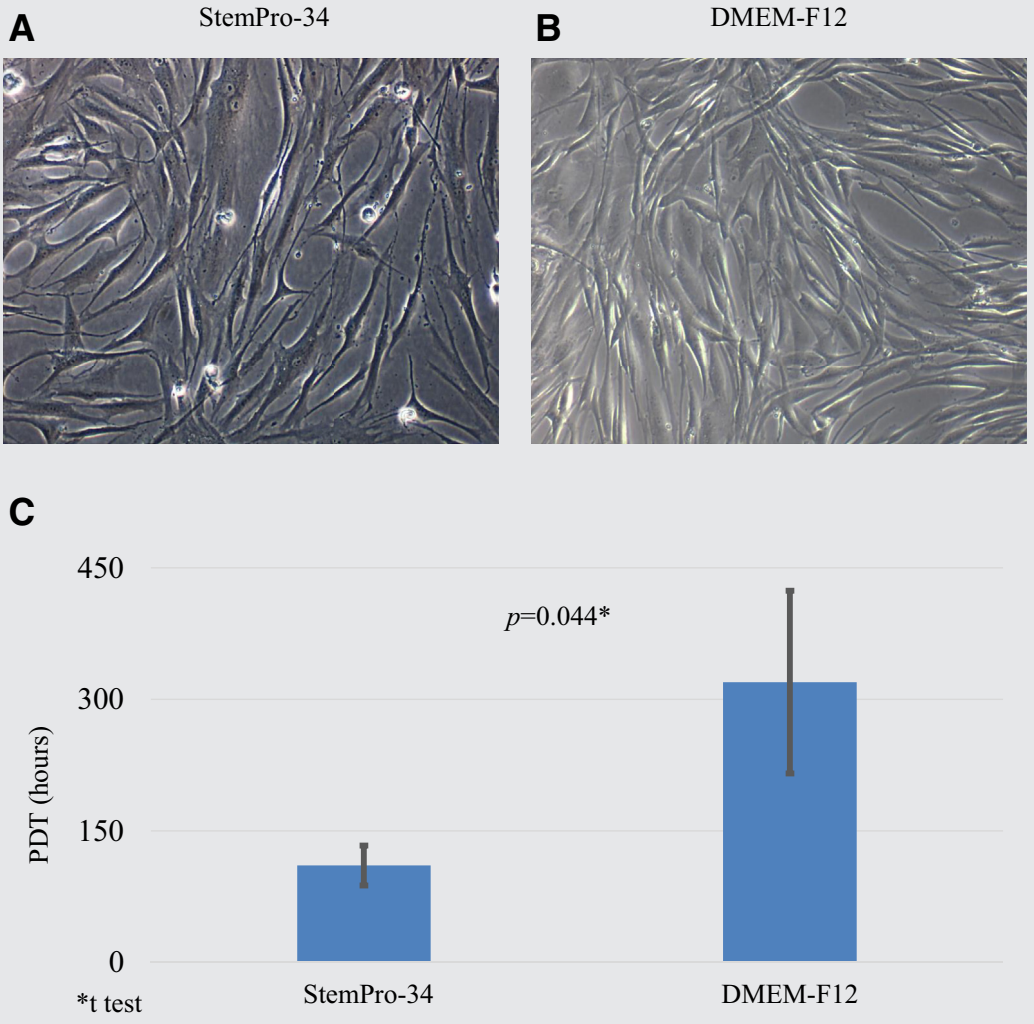
The mean number of attached cells collected after tissue harvesting and DP was 193,000 ± 116,000 cells. Cells adhered to the plates and exhibited fibroblast-like morphology throughout P0 and P1 in both StemPro-34– and DMEM-F12–based cultures (Fig. 1A). Interestingly, cells cultured with StemPro-34 became confluent and had to be passaged earlier than those cultured in DMEM-F12. The average days in culture for P0, P1, and total culture duration were 8.4 ± 2.1 vs. 9.75 ± 2.9, 5.6 ± 1.1 vs. 7.8 ± 3.6, and 14 ± 1.2 vs. 17.5 ± 4.1, respectively ($P>.05$ for all). In contrast, PDT at P0 and P1 was significantly reduced in StemPro-34 compared with DMEM-F12 culture conditions (110.5 ± 68.3 h vs. 319.6 ± 275.8 h, respectively; $P=.044$; Fig. 1B).

Testicular Somatic Cells Showed Similar Gene and Protein Expression Profiles after Expansion in StemPro-34 and DMEM-F12 Culture Conditions

CD90-positive adherent cells were sorted by means of FACS at the start of P0 (after DP) and at the end of P1. Although the proportion of CD90-positive cells increased after culture in both StemPro-34 and DMEM-F12 (90.1 ± 10.8% and 76.5 ± 17.4%, respectively), the difference did not reach statistical significance compared with after DP (66.3 ± 7%; $P>.05$; Supplemental Fig. 2, available online at www.fertstert.org). Targeted RNA sequencing was performed to assess the expression of 89 testicular cell lineage–associated markers in CD90-positive cells. In a principle component analysis (PCA), samples of CD90-positive testicular adherent cells grown in StemPro-34 and DMEM-F12 culture conditions clustered very closely (Fig. 2A). A more detailed PCA mapping of samples after P1 demonstrated separate sample clusters for each medium, revealing minor differences (Fig. 2B). This illustrated that DMEM-F12 and StemPro-34 CD90-positive cells are more similar to each other than to CD90-positive cells after DP. The complete comparison of targeted gene expression is shown in Supplemental Figure 3 (available at www.fertstert.org). In summary, CD90-positive cells isolated after P1 showed multiple significant differences in gene expression compared with after DP, including an almost complete elimination of germ cell markers and significant changes in several somatic cell markers. Fewer changes were observed between media after P1.

Although somatic and mesenchymal cell–associated genes were expressed in all groups of CD90-positive cells (after DP and after P1 in both media), several differences could be noted between samples after DP (before culture) compared with after P1 in both media. Normalized reads (reflecting transcript expression levels) for *THY1*, *STAR*, and *GATA4* were significantly higher after P1 in StemPro-34 (33,339 ± 3,931, 24,064 ± 21,943, and 4,506 ± 1,325, respectively)

FIGURE 1



Somatic cells in culture with (**A**) StemPro-34 and (**B**) DMEM-F12. Cell morphology in both media was characterized by elongated appearance and confluency. (**C**) However, population doubling time (PDT) was significantly higher in DMEM-F12, representing a lower proliferation rate compared with StemPro-34.

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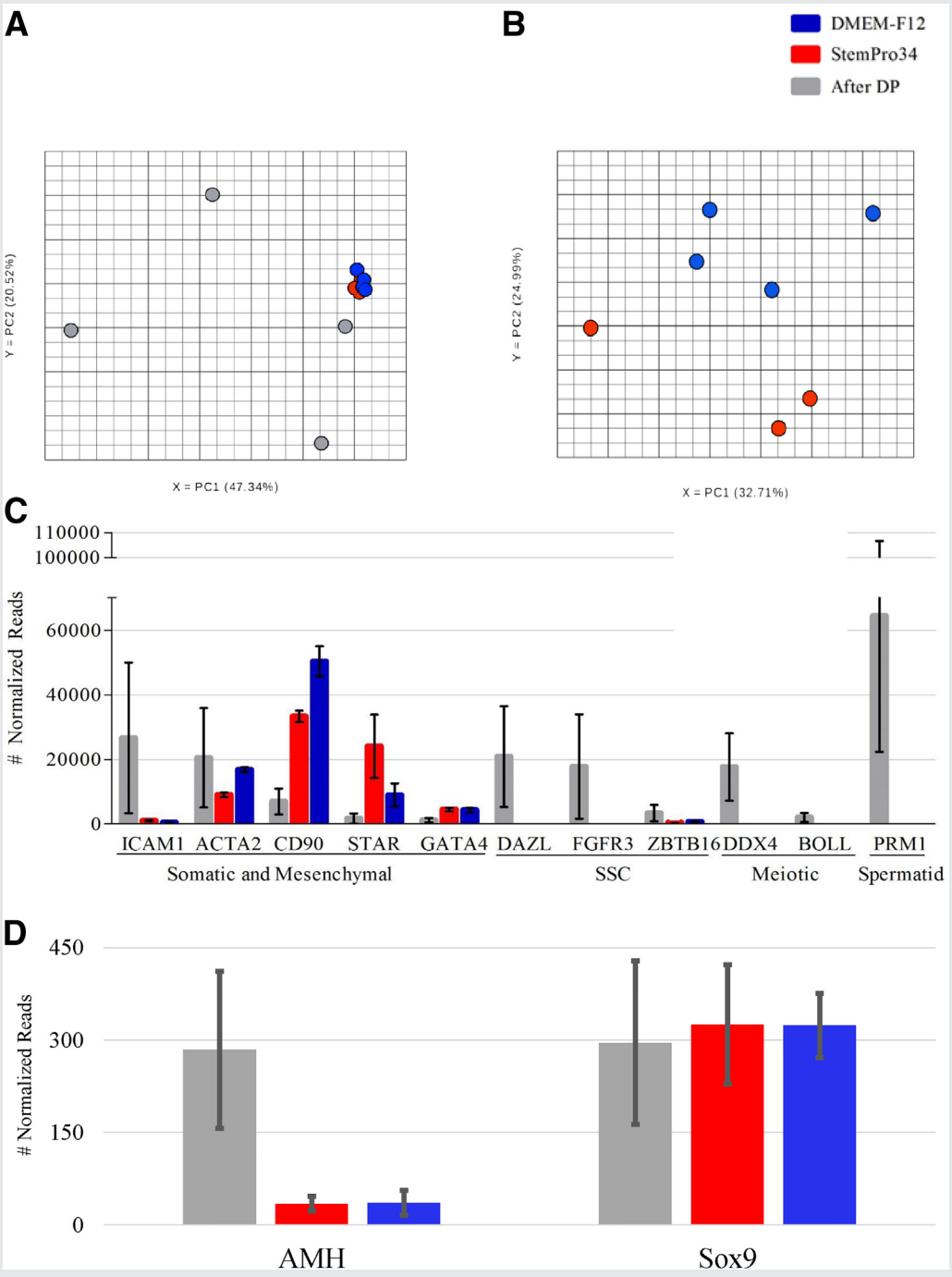
and DMEM-F12 (50,372 ± 12,338; 8,953 ± 1,856, and 4,321 ± 1,856, respectively) compared with after DP (6,964 ± 3,981, 1,688 ± 1,549, and 1,174 ± 677, respectively; all $P<.05$), whereas *ICAM1* expression declined significantly in expanded CD90-positive cells (1,129 ± 189 and 416 ± 57 in StemPro-34 and DMEM-F12, respectively, vs. 26,658 ± 23,330 after DP; $P<.05$). Interestingly, germ cell markers, including hSSC-associated *FGFR3*, meiotic cell–associated *DDX4*, and spermatid-associated *PRM1/PRM2*, were detected in the primary CD90-positive adherent cell population after DP but were not detected after P1 in both media (Fig. 2C). Sertoli cell–associated transcripts *SOX9* and antimüllerian hormone (*AMH*) showed different RNA expression changes throughout culture. *AMH* expression decreased significantly after P1 in both media, whereas *SOX9* expression remained similar (Fig. 2D).

A few significant differences in gene expression were observed between media samples after P1. Notably, mesenchymal stem cell–associated *THY1* levels were significantly increased in DMEM-F12 (50,372 ± 12,338) compared with StemPro-34 (33,339 ± 3,931; $P<.05$). On the other hand, TSC-associated *STAR* expression was significantly reduced in DMEM-F12 compared with StemPro-34 (8,953 ± 7,527 vs. 24,064 ± 21,943 reads, respectively; $P<.05$).

Similarly to the NGS gene expressions found above after P1, somatic and mesenchymal cell markers alpha smooth muscle actin and vimentin (evaluated with the use of immunocytochemistry) were highly expressed in the majority of cells under both culture conditions, whereas Sertoli cell–associated FSHR and human SSC–associated DAZL were not detected in either condition (Fig. 3).

FIGURE 2



RNA sequencing of CD90-positive cells. (**A**) Although samples from different patients varied from each other after DP, RNA expression pattern became similar after P1 in StemPro-34 and DMEM-F12. (**B**) However, higher-resolution principle component analysis mapping revealed minor RNA expression changes between StemPro-34 and DMEM-F12. (**C**) Representative RNA markers for separate cell subpopulations demonstrate the elimination of germ cell markers throughout culture in both media, accompanied by increased somatic and mesenchymal marker expression after P1. SSC = spermatogonial stem cell. (**D**) Next-generation sequencing for RNA specific Sertoli cell markers demonstrated similar expression of *SOX9* among adherent CD90-positive cells after DP compared with after P1 in both StemPro-34 and DMEM-F12. On the other hand, antimüllerian hormone (*AMH*) expression declined significantly after culture in both media.

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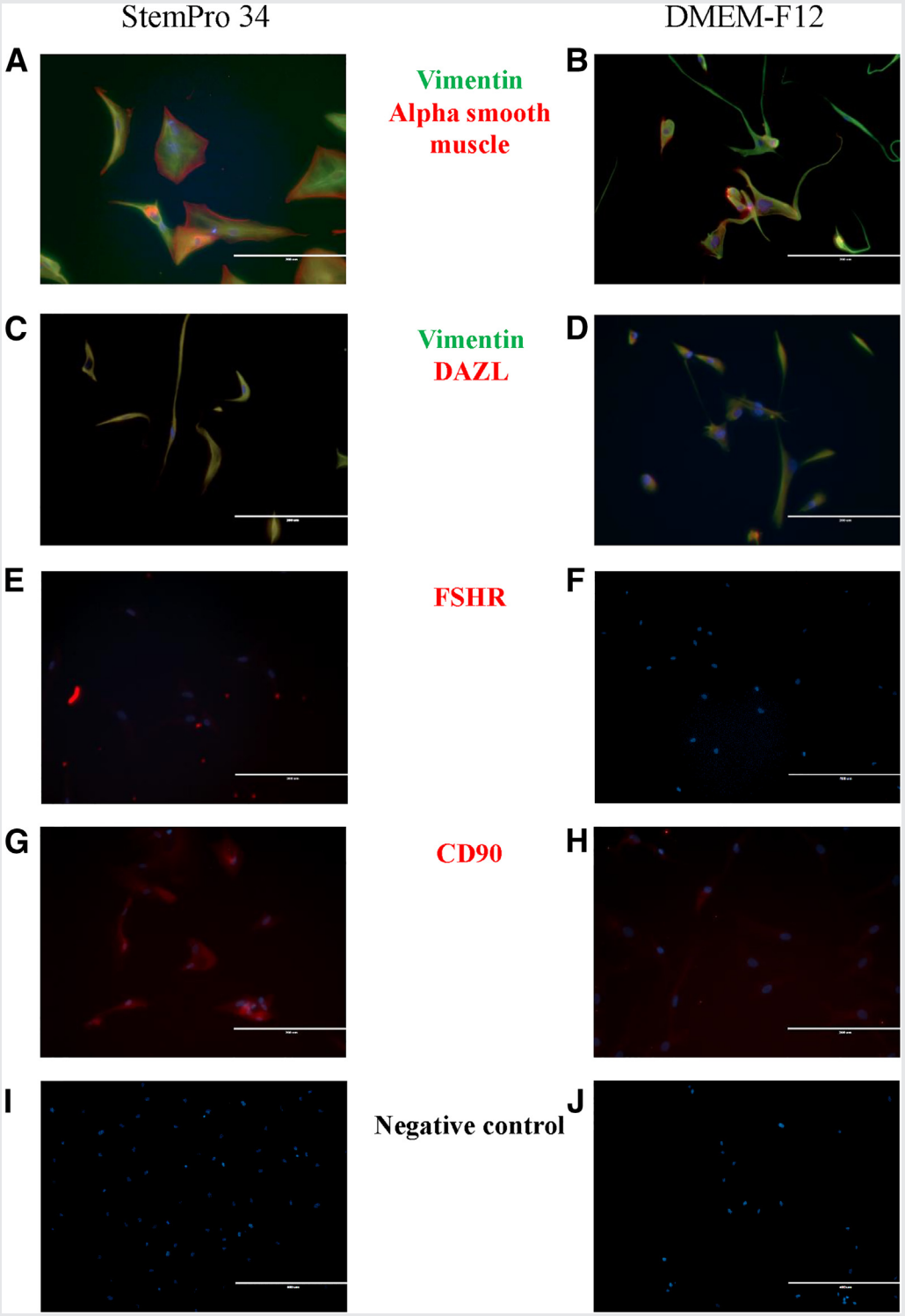
FIGURE 3



Immunocytochemistry for CD90-positive cells after P1 in StemPro-34 and DMEM-F12. (**A** and **B**) Cells stained positive for both somatic cell markers vimentin (*green*) and alpha smooth muscle (*red*) in both media. (**C** and **D**) Somatic cell origin is supported by positive staining for vimentin (*green*) compared with negative spermatogonial stem cell marker DAZL staining (*red*). (**E** and **F**) FSH receptor (*red*) was not detected after P1, whereas (**G** and **H**) cells were positive for CD90 in both media, which was consistent with fluorescence-activated cell sorting results. (**I** and **J**) Negative control.

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Testicular Somatic Cells Show Similar Paracrine Expression Profiles in DMEM-F12 and StemPro-34

Cells obtained from both StemPro-34 and DMEM-F12 conditions displayed similar levels of amplicon reads for *FGF2* (5,858 ± 1,279 and 7,506 ± 1,208, respectively), *BMP4* (717 ± 216 and 470 ± 196, respectively), *LIF* (1,740 ± 634 and 1,415 ± 72, respectively), and very low levels of *FGF1* (230 ± 111 and 222 ± 55, respectively). With the use of ELISA, only FGF2 and BMP4 were detected in both conditioned media, with no significant differences between the media. LIF, FGF1, BMP6, and GDNF were not detected in either media (Supplemental Fig. 4, available online at www.fertstert.org).

Germ Cell Survival Is Increased in DMEM-F12–Based Compared with StemPro-34-Based Culture Conditions

Because somatic cell overgrowth remains a major challenge for the ex vivo expansion of human SSCs, we assumed that somatic cell expansion differences between StemPro-34 and DMEM-F12 (Fig. 1B) could affect germ cell survival. We also hypothesized that germ cell survival would be affected by the ratio of germ cells to somatic cells plated at the start of culture. Although germ cell characterization was beyond the scope of the current research, we assessed human SSC-like aggregates and transcriptional levels of germ cell–associated markers after 12 days in culture as preliminary assessments of germ cell survival.

The highest number of SSC-like aggregates was found in the R25 (125,000 floating to 5,000 attached cells) conditions in DMEM-F12, with 108 ± 39 aggregates compared with 44 ± 32 in the parallel StemPro-34 culture and 45 ± 40 aggregates in the 125,000 germ cells–only DMEM-F12 condition ($P=.002$; Fig. 4A and 4B). All cultures containing 10,000 germ cells alone resulted in 100% cell death, with no aggregates observed at the end of the culture period. Overall, comparisons among all cell ratios for each medium demonstrated a significantly higher number of germ cell aggregates in DMEM-F12 compared with StemPro-34 (59 ± 39 vs. 28 ± 17, respectively; $P=.01$; Fig. 4C). Adding a small amount of attached somatic cells (5,000) resulted in a significantly increased number of SSC-like aggregates compared with floating germ cells–only cultures (55 ± 34 compared with 32 ± 18, respectively; $P=.03$; Fig. 4D), emphasizing the importance of somatic cell support for human SSC survival.

We performed targeted RNA sequencing analysis of cells following co-culture at R2, R13 and R25 to investigate whether germ cell markers, specifically those associated with SSC, were upregulated in the optimal (R25, R13) ratio *DAZL* vs. suboptimal ratio (R2) cultures. We found a significant upregulation of several SSC (ex. DAZL, *ITGA6*) and meiotic cell- (ex. *SYCP3*) associated amplicons in co-cultures where maximal colonies were observed when compared to those where no colonies were observed (Supplemental Fig. 5). Unfortunately we did not get adequate RNA for NGS from the samples containing floating cells only.

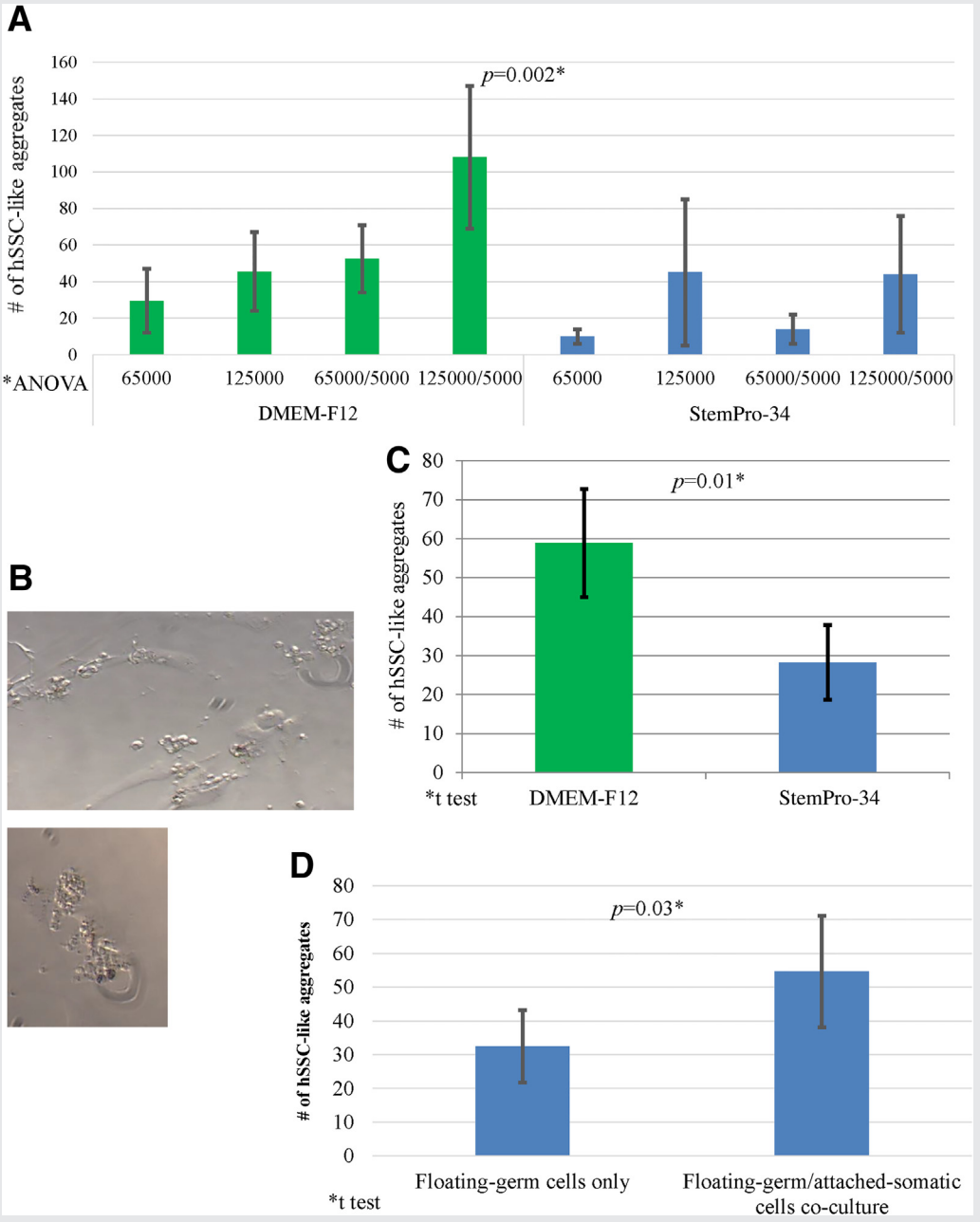
DISCUSSION

Optimizing human SSC proliferation in vitro remains a key challenge in fertility preservation for pre-pubertal boys exposed to gonadotoxic treatments and for men with NOA. SSCs undergo either self-renewal or differentiation within the testicular microenvironment through architectural support and growth factor stimulation (24). In spite of the challenge to identify the essential extrinsic factors necessary for culturing SSCs efficiently (25), some factors appear to have significant impact on this process in vitro. These include: the use of feeder cells (26), specific growth factors (27), culture dish coating materials (28) and others. Since the introduction of StemPro-34-based media in culture systems to expand murine germline stem cells (22), this has become the most commonly used media for human SSC cultures. Although the effects of media on other human adult stem cells have been investigated (29), the impact of media choice on testicular SSC expansion has not been evaluated. Considering that somatic cell overgrowth remains a critical obstacle to successful long-term human SSC expansion (12, 15), the importance of such investigation is obvious.

In the present study, we assessed the impact of StemPro-34 and DMEM-F12 media on the growth and gene expression profiles of human TSCs after expansion in culture for two passages. Our results suggest that DMEM-F12 lowers the PDT of somatic cells compared with StemPro-34, without significantly altering their phenotype. A PCA demonstrated that despite heterogeneous RNA expression between samples after DP, samples after P1 in both media had similar targeted gene expression profiles. CD90-positive cells after DP expressed transcripts associated with multiple stages of germ cell differentiation in addition to somatic and mesenchymal markers. However, after P1, the expression of germ cell–associated markers was eliminated almost completely whereas somatic/mesenchymal marker expression showed somatic cell lineage–specific changes. Interestingly, there were significant differences between media after P1, as "zoom-in" PCA revealed separation between media samples (Fig. 3B).

The gene expression profile data for TSCs isolated after P1 in both media was supported at the protein level. Although the somatic markers alpha smooth muscle (derived from *ACTA2* mRNA), vimentin, and CD90 were detected with the use of both immunocytochemistry and FACS, SSC- and Sertoli cell–specific markers (DAZL and FSHR, respectively) were not identified (Fig. 3). Moreover, TSCs expanded in either StemPro-34 or DMEM-F12 displayed similar paracrine properties, including the secretion of FGF2 and BMP4 and gene expression of LIF and FGF1. Such properties may be important in regulating the ability of TSCs to support SSC expansion, and they appear to be unaltered by medium choice. The overall similar phenotypes but significantly different growth rates between cells cultured in the two media suggest that specific factors or metabolites within each medium may differentially regulate cell development, survival, and/or proliferation. However, because both commercial StemPro-34 and DMEM-F12 include various trademarked components, we can not speculate about the factors responsible for these differences.

FIGURE 4



Human spermatogonial stem cell (SSC)–like aggregates in StemPro-34 and DMEM-F12 with versus without addition of attached somatic cells. (**A**) The highest number of aggregates was found in the 125,000:5,000 floating germ cells to attached somatic cells co-culture in DMEM-F12 (*P*=.002). (**B**) Representative pictures of SSC-like aggregates of small round cells (possibly SSCs) without confluency of somatic cells. (**C**) Overall, cultures of DMEM-F12 had significantly higher numbers of SSC-like aggregates compared with StemPro-34 (*P*=.01). (**D**) Co-cultures of floating germ and attached somatic cells contained significantly higher numbers of SSC-like aggregates compared with floating germ cells alone (*P*=.03), demonstrating the importance of the somatic cell support.

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To isolate somatic cells from germ cells, we used double-sorting methodologies. First, we performed DP, which enables the simple separation of germ cell from somatic cell subpopulations, as reported decades ago (30). DP is easy to use and renders a higher cell survival rate compared with other sorting methodologies (especially FACS, which has limited capacity and necessitates specialized equipment [6]). However, because DP may not be specific enough for research purposes, we performed FACS for CD90 to characterize a purified subpopulation of somatic cells. We found that CD90 was expressed by the majority of attached cells after DP and to a greater extent (although this did not reach significance) after P1 in both

media (Fig. 2). Therefore, we assumed that characterization of the CD90-positive cells population reflects characteristics alterations throughout in vitro expansion. Smith et al. (2014) emphasized the differentiation between CD90-positive and SSEA4-positive cells, which represent human somatic cells and SSCs, respectively (13). However, Valli et al. (2014) had previously demonstrated heterogeneous CD90 expression among human testicular cells after separation to three subpopulations by means of FACS: $CD90^{bright}$, $CD90^{dim}$, and $CD90^{negative}$. Transplantation of $CD90^{bright}$ to nude mice resulted in depleted colonizing activity, whereas $CD90^{dim}$ transplantations had high activity (6), suggesting that CD90 is expressed by both somatic and germ cells. Similarly, diverse CD90-positive testicular populations have been detected by means of immunohistochemical staining on the basement membrane of the seminiferous tubules (31) and on germ cells toward the lumen (32). In the present study, CD90-positive cells (equivalent to the $CD90^{bright}$ population previously studied by Valli et al.) isolated immediately after DP expressed genes associated with multiple stages of germ cell differentiation in addition to somatic and mesenchymal markers. That finding supports the notion that CD90 is not entirely selective for somatic cells but is also expressed in multiple stages of differentiating germ cells. The depletion of germ cell markers after P1 is supported by a previous report regarding decreased DDX4, MAGE-4, and SALL4 (all germ cell–associated markers) expression concordant with increased expression of vimentin and Acta2 (somatic cell markers) during 11 days of marmoset attached testicular cell culture (15). These changes reaffirm the well known somatic cell overgrowth which prevents germ cell propagation ex vivo. Interestingly, *SOX9* and *AMH*, two Sertoli cell–associated markers (8), were detected in CD90-positive cells after DP. However, *AMH* transcript levels decreased significantly after P1 in both media whereas *SOX9* expression remained stable (Fig. 2D). Altogether the transcript and protein expression data suggest an overall MSC-like phenotype for TSCs at P1, as reported previously (13, 33). Therefore, the conventional classification of TSCs as Sertoli, peritubular, Leydig, etc. may not apply over time in culture owing to culture-induced changes in somatic cell gene expression.

In the next step of our experiment we evaluated the impact of media choice and use of pre-defined somatic cell ratios on germ cell survival. We found significantly higher numbers of SSC-like colonies in DMEM-F12 when compared to StemPro-34 co-cultures conditions. Based on our PDT and phenotyping data, we hypothesize that the favorable outcome with regards to the increase in SSC-like colonies in DMEM-F12 may be related to decreased somatic cell proliferation which enabled better germ cell endurance and enhanced aggregate formation. We also confirmed the expression of SSC and germ cell progenitor-associated markers in cultures where colonies were observed, suggesting that these colonies contain expanding germ cell progenitors, including SSC. To our knowledge, this is the first report of improved human germ cell survival in vitro related to the type of culture media. Interestingly, co-culture of both attached and floating cells resulted in significantly increased numbers of SSC-like colonies compared to floating cells only. These results emphasize the important role of somatic cell support for germ cell growth and survival in vitro. Altogether, our results suggest that limiting somatic cell proliferation by shifting media usage to DMEM-F12 from the common StemPro-34-based media and controlling the ratio of floating germ cells to attached somatic cells can improve *ex-vivo* germ cell expansion. This effect needs to be assessed further, namely by using the gold standard SSC transplantation assay to confirm the functional phenotype of hSSCs in DMEM-F12- compared to StemPro-34-based cultures.

Several methods have been previously suggested for overcoming somatic cell overgrowth in human SSC cultures in vitro. First, separation of germ and somatic cells by means of DP to enrich germ cells has resulted in significantly increased germ cell colonies and increased expression of SSC markers after 10–11 days in culture (14, 15). However, long-term cultures of floating cell fractions resulted in a consistent decline of human SSC markers, such as FGFR3, UTF1, DAZL, and others (12), suggesting that this enrichment step alone is not sufficient for long-term in vitro germ cell expansion. Second, SSC cultures initiated from purified SSEA4-positive cells resulted in cell death within 2 weeks, mostly owing to lack of somatic cell support (13). These findings highlight the need to co-culture both somatic and germ cells in a system where somatic cell proliferation is restricted. Exposure of CD90-positive cells to γ-irradiation to eliminate their mitotic activity and using them as feeders for SSEA4-positive sorted cells resulted in the long-term expansion of SSC colonies (13) and may be another option for in vitro expansion of SSC. Our findings provide an additional simple and effective method for controlling TSC growth by switching the commonly used StemPro-34–based SSC culture medium to DMEM-F12. Defining the optimal ratio between floating (germ) and attached (somatic) cells during the establishment of primary SSC cultures may also contribute to successful human SSC propagation, but it may vary depending on the source of samples. Whether these strategies are effective for long-term human SSC propagation is currently under study.

Our research has some limitations. First, we used heterogeneous human samples, including orchiectomy samples from oncology or testicular pain patients, as well as micro-TESE from an NOA patient. This diversity likely accounts for the lack of clustering of the primary samples (after DP) in PCA. However, these differences were reduced following in vitro expansion. Second, the present study investigated only the effect of media and cell ratios on short-term human TSC and SSC cultures. Although this time course is similar to or longer than previous reports (6, 13), the effect of culture parameters over time needs to be further investigated. Finally, although we noticed significantly increased human SSC-like aggregate formation in DMEM-F12 compared with StemPro-34, cell characterization is required to confirm SSC propagation, and should include a functional assay, such as the xenotransplantation assay.

In conclusion, the present study compared the proliferation and characterization of human TSCs in two well known media. Our results suggest that limiting somatic cell proliferation by shifting from the common StemPro-34 to DMEM-F12 medium may result in improved germ cell survival.

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SUPPLEMENTAL FIGURE 1

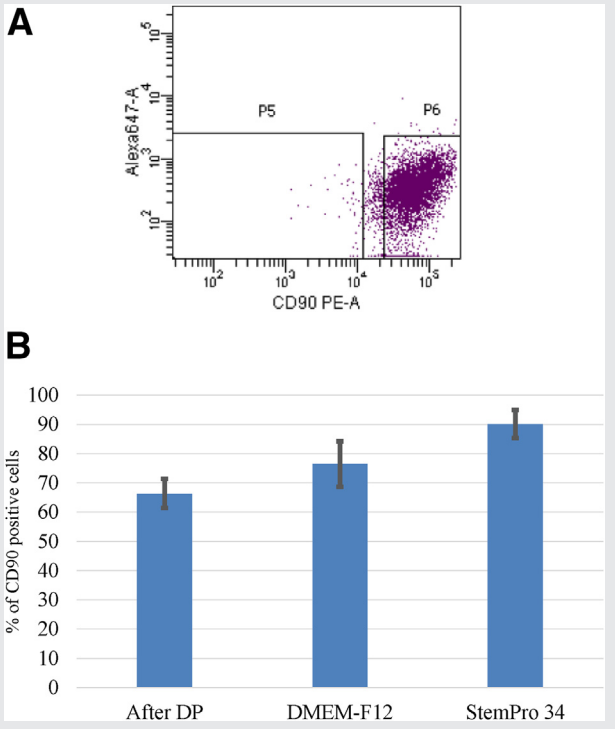
Cell Lineage- or Biological Process-associated	Gene
SSC (intracellular)	DAZL
SSC (intracellular)	MAGEA4
SSC (intracellular)	NANOS2
SSC (intracellular)	ZBTB16
SSC (intracellular)	SALL4
SSC (intracellular)	DNMT3B
SSC (intracellular)	UCHL1
SSC (self-renewal)	PIWIL2
SSC (self-renewal)	RET
SSC (self-renewal)	GFRA1
SSC (self-renewal)	ETV5
SSC (cell surface)	EPCAM
SSC (cell surface)	FGFR3
SSC (cell surface)	GPR125
SSC (cell surface)	ITGA6
SSC (cell surface)	KIT
SSC (cell surface)	ITGB1
Germ cell progenitor	TEX101
Germ cell progenitor	DPPA3
Germ cell progenitor	PHF13
Spermatid	ACRV1
Spermatid	PROM1
Spermatid	ACR
Spermatid	ODF1
Spermatid	PRM1
Spermatid	PRM2
Spermatid	ODF2
Meiotic marker	BOLL
Meiotic marker	DDX4
Meiotic marker	SPO11
Meiotic marker	TEX11
Meiotic marker	SLC26A8
Meiotic marker	STAG3
Meiotic marker	SYCP3
Meiotic marker	DMC1
Meiotic marker	FBXO5
Pluripotency marker	LIN28B
Pluripotency marker	SOX2
Pluripotency marker	TERT
Pluripotency marker	POU5F1
Pluripotency marker	KLF4
Pluripotency marker	B3GALT5
Pluripotency marker	ST3GAL2
Pluripotency marker	NANOG

Cell Lineage- or Biological Process-associated	Gene
Somatic cell	CD34
Somatic cell	INSL3
Somatic cell	SNAI2
Somatic cell	ICAM1
Somatic cell	GATA4
Somatic cell	NT5E
Somatic cell	ENG
Somatic cell	ACTA2
Somatic cell	WT1
Somatic cell	AR
Somatic cell	STAR
Somatic cell	SOX9
Sertoli cell marker	AMH
Sertoli cell marker	FSHR
Sertoli cell marker	AMHR2
Leydig cell	LHCGR
Mesenchymal stromal cell	CD44
Mesenchymal stromal cell	THY1
Cell cycle and senescence	MAP3K12
Cell cycle and senescence	CDKN2A
Cell cycle and senescence	CDKN1A
Cell cycle and senescence	H2AFX
Cell cycle and senescence	TP53
Extracellular matrix	LAMB1
Extracellular matrix	LAMA1
Extracellular matrix	COL4A1
Extracellular matrix	LAMB2
Extracellular matrix	COL4A2
Extracellular matrix	COL1A2
Extracellular matrix	COL1A1
Secreted factor	TDGF1
Secreted factor	GDNF
Secreted factor	BMP6
Secreted factor	FGF1
Secreted factor	GDF3
Secreted factor	LIF
Secreted factor	BMP4
Secreted factor	FGF2
Male Fertility	SRY
Male germ-line imprinted	H19
Male germ-line imprinted	RASGRF1
Housekeeping	LRP1
Houskeeping	MRPL13
Houskeeping	JUN

Transcript targets on custom targeted RNAseq amplicon panel.

Gat. Optimizing testicular cell culture conditions. Fertil Steril 2017.

SUPPLEMENTAL FIGURE 2



Fluorescence-activated cell sorting (FACS) for CD90 among attached cells after differential plating and after P1 in both media. (**A**) Representative plot represents the majority of cells as CD90 positive. (**B**) CD90-positive cell fraction increased significantly ($P<.05$) during culture in both media. DP = differential plating.

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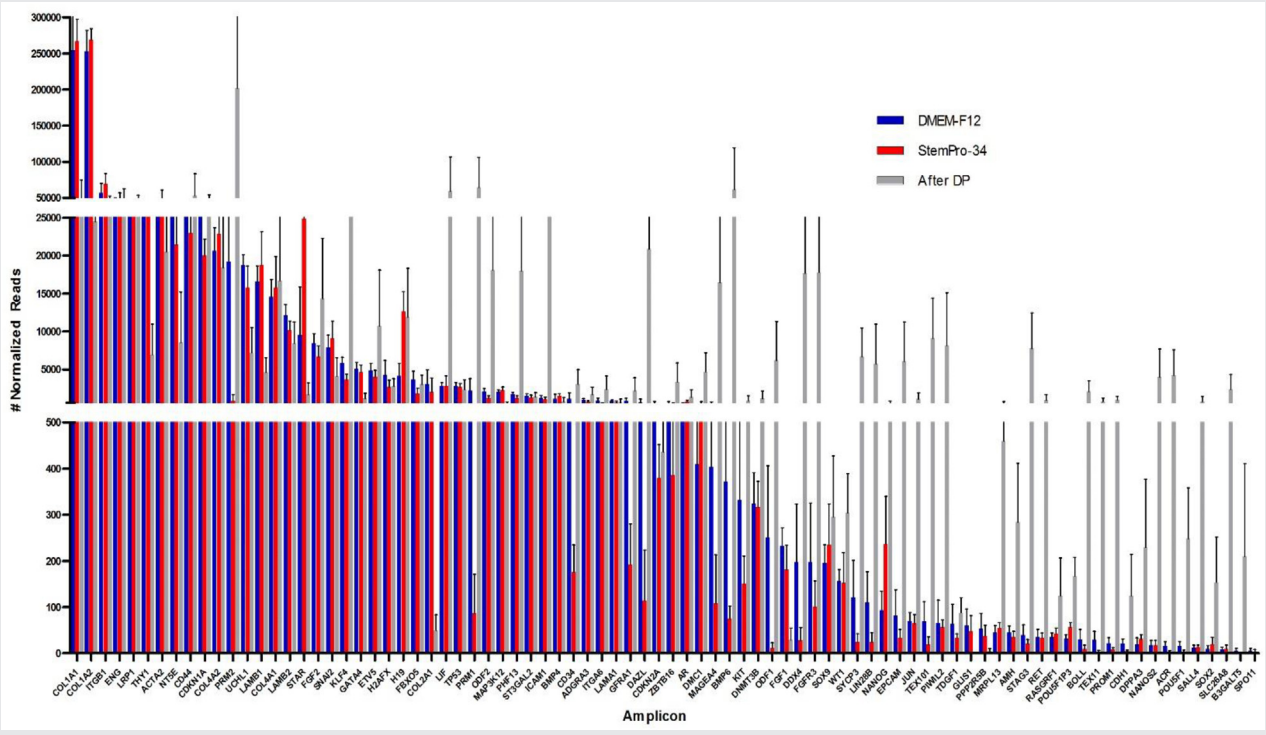
SUPPLEMENTAL FIGURE 3



RNAseq analysis showing 89 target gene amplicons. The amplicon enabled direct comparisons of numerous markers after differential plating (DP) versus first passage (P1) and demonstrated similar RNA expression after P1 in both media.

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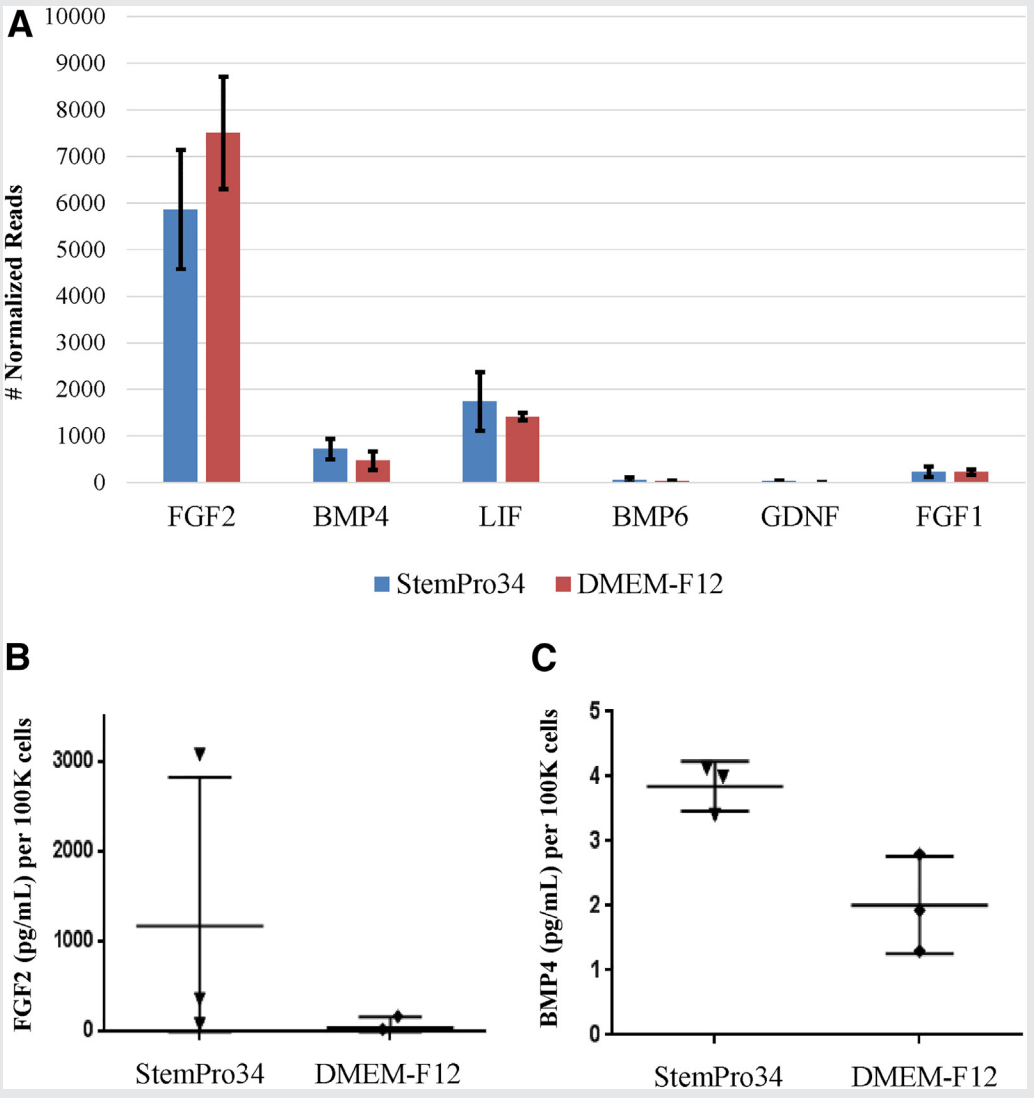
SUPPLEMENTAL FIGURE 4



Expression and secretion of growth factors by testicular somatic cells cultured in StemPro-34 and DMEM-F12. (**A**) Similar RNA expression according to next-generation sequencing between each media: FGF2, LIF, and BMP4 had the highest expression levels, compared with very low observed expression of BMP6 and GDNF. (**B** and **C**) Only FGF2 and BMP4 were detected in conditioned media by means of ELISA.

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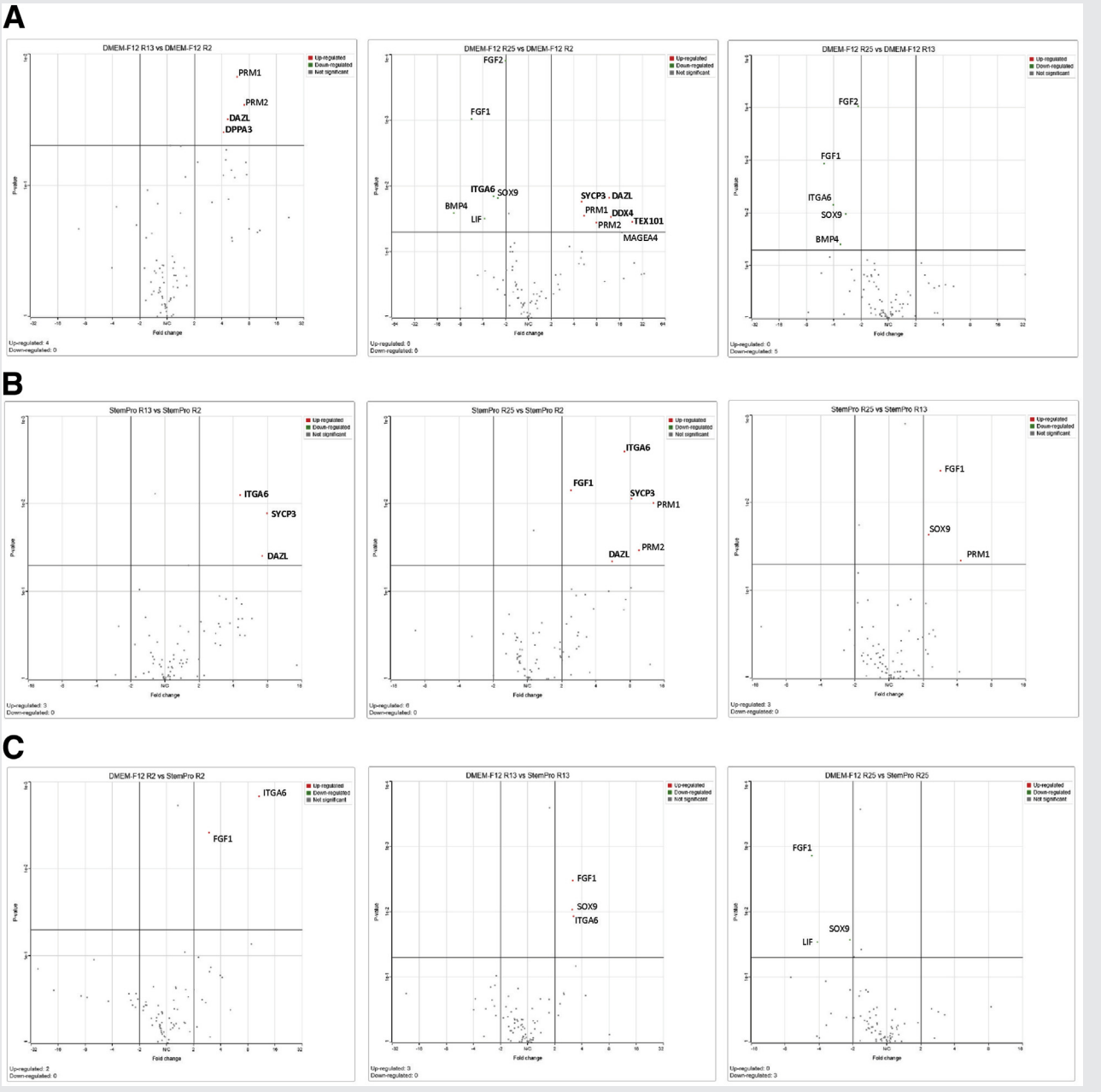
SUPPLEMENTAL FIGURE 5



Volcano plots depicting targeted RNA sequencing analysis after co-culture at R2, R13, and R25 in StemPro-34 and DMEM-F12. Germ cell markers, especially spermatogonial stem and meiotic markers, were up-regulated in R13 and R25 conditions compared with R2 in (**A**) DMEM-F12 and (**B**) StemPro-34 without significantly different expression between media (**C**). R2 = 10,000 floating germ cells/5,000 attached somatic cells; R13 = 65,000 floating germ cells/5,000 attached somatic cells; R25 = 125,000 floating germ cells/5,000 attached somatic cells.

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SUPPLEMENTAL TABLE 1

Patient data.

Patient	Tissue	Age (y)	Clinical diagnosis	Pathologic diagnosis	Sperm found?
1	MicroTESE	30	Azoospermia	Hypospermatogenesis	Yes
2	Fresh orchiectomy	32	Testicular mass	Seminoma; maturation arrest	No
3	Fresh orchiectomy	29	Testicular mass	Seminoma	Yes
4	Frozen orchiectomy	25	Testicular mass	Seminoma	Yes
5	Fresh orchiectomy	56	Testicular mass	Seminoma	Yes
6	Frozen orchiectomy	32	Testicular pain	Varicocele; epididimal cyst	Yes
7	Fresh orchiectomy	49	Testicular pain	Active spermatogenesis	Yes
8	Fresh orchiectomy	59	Testicular pain	Active spermatogenesis	Yes

Note: MicroTESE = microscopic testicular sperm extraction.

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SUPPLEMENTAL TABLE 2

Cell densities in 12 cultures initiated after differential plating.

Variable	StemPro-34	DMEM-F12
Floating germ cells only	10,000	10,000
	65,000	65,000
	125,000	125,000
Floating germ cells/attached somatic cells co-culture	10,000:5,000	10,000:5,000
	65,000:5,000	65,000:5,000
	125,000:5,000	125,000:5,000

Gat. Optimizing testicular cell culture conditions. Fertil Steril 2017.