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In vitro production of haploid cells after coculture of CD49f+ with Sertoli cells from testicular sperm extraction in nonobstructive azoospermic patients

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Objective: To isolate CD49f+ cells from testicular sperm extraction (TESE) samples of azoospermic patients and induce meiosis by coculturing these cells with Sertoli cells.
Design: Prospective analysis.
Setting: Research center.
Patient(s): Obstructive azoospermic (OA) and nonobstructive azoospermic (NOA) patients.
Intervention(s): TESE, with enzymatic dissociation of samples to obtain a cell suspension, which was cultured for 4 days with 4 ng/mL GDNF. The CD49f+ cells were sorted using fluorescence-activated cell sorting (FACS) as a marker to identify spermatogonial stem cells (SSCs), which were cocultured with Sertoli cells expressing red fluorescent protein (RFP) in knockout serum replacement (KSR) media with addition of 1,000 IU/mL of follicle-stimulating hormone (FSH), 1 μM testosterone, 40 ng/mL of GDNF, and 2 μM retinoic acid (RA) for 15 days in culture at 37°C and 5% CO_2 to induce meiotic progression. Cells were collected and analyzed by immunofluorescence for meiosis progression with specific markers SCP3 and CREST, and they were confirmed by fluorescence in situ hybridization (FISH).
Main Outcome Measure(s): Isolation of CD49f+ cells and coculture with Sertoli cells, meiosis progression in vitro, assessment of SSCs and meiotic markers real-time polymerase chain reaction (RT-PCR), immunohistochemical analysis, and FISH.
Result(s): The CD49f+ isolated from the of total cell count in the TESE samples of azoospermic patients varied from 5.45% in OA to 2.36% in NOA. Sertoli cells were obtained from the same TESE samples, and established protocols were used to characterize them as positive for SCF, rGDNF, WT1, GATA-4, and vimentin, with the presence of tight junctions and lipid droplets shown by oil red staining. After isolation, the CD49f+ cells were cocultured with RFP Sertoli cells in a 15-day time-course experiment. Positive immunostaining for meiosis markers SCP3 and CREST on days 3 to 5 was noted in the samples obtained from one NOA patient. A FISH analysis for chromosomes 13, 18, 21, X, and Y confirmed the presence of haploid cells on day 5 of the coculture.
Conclusion(s): In vitro coculture of SSCs from TESE samples of NOA patients along with Sertoli cells promoted meiosis induction and resulted in haploid cell generation. These results improve the existing protocols to generate spermatogenesis in vitro and open new avenues for clinical translation in azoospermic patients. (Fertil Steril® 2012;■:■–■. ©2012 by American Society for Reproductive Medicine.)
Key Words: CD49f+ cells, coculture, gametes, germ line, in vitro, meiosis, Sertoli cells, spermatogenesis, spermatogonial stem cell

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Obstructive (OA) and nonobstructive (NOA) azoospermia are major causes of male infertility. Testicular sperm extraction (TESE) is usually performed to search for sperm that will be later used in the intracytoplasmic sperm injection (ICSI) technique (1). Spermatogenesis is normal

in OA patients, so TESE usually solves the problem (2). However, in patients whose NOA is caused by a variety of pathologies that lead to spermatogenesis arrest, their only chance for paternity lies in sperm banks with donor sperm (3).

Essentially, spermatogenesis is a process by which germ cells (GCs) proliferate and differentiate into haploid sperm. Germ cells initially migrate to the basement membrane of the seminiferous tubules of the testis, where they maintain their ability to proliferate and differentiate to sperm under the influence of Sertoli cells throughout a lifetime (4–6).

Accumulated evidence demonstrates the existence of a stem cell population in human adult testes (7–12). Spermatogonial stem cells (SSCs) are the adult stem cell population that renews itself and produces spermatogenic cells after transplantation into the seminiferous tubules of infertile recipient males, constituting a model system to understand spermatogenesis (13). Isolation of the SSC population can be achieved by sorting them by the presence of the cell surface markers CD49f, GPR-125, and GFRA-1 (7, 11, 14, 15) and by further culturing them with the addition of growth factors such as GDNF, LIF, EGF, GFRA-1, or bFGF (11).

In humans, in vitro spermatogenesis from SSCs to obtain haploid sperm has yet to be achieved. Researchers have investigated this process via various strategies such as combinations of growth factors or hormones or/and coculture of germ cells with testicular tissue (16–22). Recently, important advances in the in vivo production of sperm in mice that have resulted in healthy offspring have used two different approaches: the first consists of starting with an epiblast-like cell intermediate that is transplanted into mouse testes (23); the second comprises an organ culture system of SSCs in prepubertal gonadal tissue (24). Our study isolated SSCs from the TESE samples of azoospermic men to induce the meiosis process in vitro by use of a coculture system with Sertoli cells.

MATERIALS AND METHODS

Human Samples

Our study was approved by the institutional review board of the Instituto Valenciano de Infertilidad (IVI) in accordance with Spanish legislation (NCT: 01375662). The samples were obtained after signed, informed consent from the patients. The samples, composed of seminiferous tubules and interstitial tissue, were obtained from 20 azoospermic patients (OA, n = 9; NOA, n = 11) as part of their diagnosis/therapeutic workup. The sperm obtained from the TESE samples was frozen for subsequent use in ICSI, and the residual tissue was transported in basic media and maintained on ice for 60 to 90 minutes after surgery. The tissue was washed three times in Hank's balanced salt solution 1x (HBSS; PAA) with penicillin/streptomycin (Invitrogen); the samples were mechanically dissected out into small pieces (1 mm^3) and enzymatically dissociated with collagenase type IA (1,000 IU/mL; Sigma) for 20 minutes at 37°C on a shaker. Then the samples were incubated with TrypLE Select (Invitrogen) for 10 minutes at 37°C on a shaker, filtered through a 50-μm mesh (Partec), and centrifuged at 1,000 rpm for 5 minutes. The cell pellet was seeded on a plate (maximum of 2×10^6 cell/cm^2 for a culture dish) containing media supplemented with 20% embryonic stem-cell qualified fetal bovine serum (ES-qualified FBS; Invitrogen), plus 4 to 40 ng/mL of GDNF (Sigma); this was incubated at 32°C in 5% CO_2 for 4 days.

CD49f+ Cell Isolation

After 4 days of culture, the media were collected, and the cultured cells were gently washed twice with Dulbecco's modified Eagle medium (DMEM; Invitrogen). They were then washed once with phosphate-buffered saline (PBS; PAA) to recover all the unattached cells and to discard the monolayer adherent cells. The collected cells were centrifuged at 1,000 rpm for 5 minutes, suspended in basic media, and filtered through a 30-μm filter (Partec). The single-cell suspension obtained was then incubated with 5% bovine serum albumin (BSA; Sigma) in PBS for 30 minutes at 4°C, washed with 0.1% BSA in PBS, and incubated with an Alexa Fluor 488 1:100 conjugated antibody against the CD49f surface marker (α6-integrin; Biolegend) on ice for 45 minutes. Peripheral blood lymphocytes were used as positive controls for CD49f, while human skin fibroblast feeder cells were employed as the negative control. The cells that expressed positive for CD49f were separated from the whole population by fluorescence-activated cell-sorting (FACS) (MoFlo Modular Flow Cytometer; Beckman Coulter).

Sertoli Cell Purification

After we had obtained the cell suspension from the TESE samples, the cells were resuspended in Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F-12; GIBCO, Invitrogen) supplemented with 10% FBS; these were seeded into culture plates and incubated at 37°C in 5% CO_2 for 10 minutes. After incubation, the media containing different spermatogenic cell types were removed, and the attached cells were cultured for approximately 1 week; the media were changed every other day until reaching confluence. The cell monolayer was trypsinized with TrypLE Select diluted (1:1) in PBS (PAA) for 5 minutes at 37°C (25). The cell number was calculated, and 1.10^6 cells were then incubated with 0.5 μg/mL Nile red (Sigma) for 15 minutes in PBS. The cells were filtered through a 50-μm sieve (Partec), and cell purification was performed using a cell sorter (MoFlo; Beckman Coulter). The Sertoli cells positive for Nile red staining were seeded at a density of 50,000 cells/well with a collagen matrix (Biocoat Collagen I; Becton Dickinson) in DMEM/F-12 supplemented with 10% FBS, and were cultured for 10 days at 37°C in 5% CO_2. Another demonstration of their nature was performed by oil red staining, electron microscopy for lipid droplet and tight junctions, and polymerase chain reaction for specific markers such as the GDNF receptor (GAF), stem cell factors (SCF), and transcription factors GATA-4 and Wilms tumor (WT-1).

Viral Transduction of Sertoli Cells with Red Fluorescent Protein (RFP)

Sertoli cells were transfected with a lentiviral vector coding for red fluorescent protein (RFP), as previously described by our group (26). Briefly, lentiviral stocks were obtained using the packaging cell line 293-T (American Type Culture Collection,

Manassas, Virginia) by the transient cotransfection of 1 μg of plasmids A179-Helix-UBC-RFP (kindly provided by the Genomics Unit, Spanish National Cancer Centre, CNIO), 1 μg of GAG POL-BAM HI (virus survival), and 1 μg of VSVG-ECORT (encapsulation virus) plasmids using Fugene HD (Roche Diagnostics). The lentiviral supernatant was harvested at 48 hours after transfection and used to infect Sertoli cells for 12 hours in the presence of 10 μg/mL of polybrene (Sigma-Aldrich), which was then incubated at 37°C in 5% CO_2. After 48 hours, the cells were analyzed under a fluorescence microscope to observe the transduced cells' efficiency to express RFP.

Coculture System of CD49f+ and Sertoli-RFP Cells

Sertoli-RFP cells were seeded on 12-well plates with a collagen matrix (Biocoat Collagen I; Becton Dickinson) at a density of 10,000 cells/well with DMEM/F-12 (GIBCO, Invitrogen) supplemented with 10% FBS. The plates were incubated for 24 hours at 37°C in 5% CO_2 to allow for cell attachment. The CD49f+-sorted cells were directly seeded onto a Sertoli-RFP feeder layer at a density of 3,000 cells/well and were grown in knockout D-MEM (Ko-DMEM; GIBCO, Invitrogen) supplemented with 20% knockout serum replacement) (KSR; GIBCO, Invitrogen), 1,000 IU/mL of follicle-stimulating hormone (FSH; Sigma), 1 μM testosterone (Qpharma), 40 ng/mL of GDNF (Sigma), and 2 μM retinoic acid (RA) (Sigma) at 37°C in 5% CO_2. The cells were collected at 48 hours for further analysis.

RNA Extraction and RT-PCR Analysis

The RNA extraction was performed using the Mini RNA Isolation I Kit (Zymo Research) according to the manufacturer's protocol, and the integrity was analyzed in a NanoDrop Spectrophotometer (NanoDrop Technologies) for RNA quantification and purity. Complementary DNA (cDNA) was synthesized by use of real-time polymerase chain reaction (RT-PCR) with an MMLV enzyme (Clontech; BD Biosciences) and 500 ng of RNA per sample. The RT-PCR analysis of CD49f+ cells was performed using the Advantage RT-PCR Kit (Clontech; BD Biosciences) following the manufacturer's protocol. The cDNA from each sample was used as a PCR template to detect the expression of those genes with the primer sequences presented in Supplemental Table 1 (available online). The program used was 95°C denaturation for 10 minutes, 35 cycles in 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 1 minute. The positive control was human testis tissue, and the negative control was human foreskin, which were included in each reaction. The PCR products were resolved on 2% agarose gels, stained with ethidium bromide and visualized in a transilluminator (BioRad).

Immunocytochemistry

The CD49f+ and Sertoli cells were seeded on a chamber slide, cultured for 3 days, fixed with 4% paraformaldehyde for 20 minutes, and washed with PBS. Two protocols were followed for staining. For the membrane surface markers, PBS was discarded, and the cells were washed three times with rinse buffer 1X—20 mM Tris (Fluka), HCl (J.T. Baker), 0.15 NaCl (Sigma-Aldrich), 0.05% Tween-20 (Sigma-Aldrich)—with PBS for permeabilization. Cells were then incubated for 10 minutes with Triton X100 0.1% (Sigma-Aldrich) and blocked with 4% normal goat serum (NGS; Sigma-Aldrich) for 30 minutes. Next, the primary antibodies CD49f (1:100) (Alexa Fluor 488 anti-human/mouse CD49f; Biolegend), GPR125 (1:200) (rabbit polyclonal GPR125; Abcam), THY-1 (1:100) (mouse anti-human THY-1 monoclonal antibody R-PE; Chemicon), and Vimentin (1:200) (mouse monoclonal vimentin antibody; Abcam) were added for 1 hour at room temperature; after incubation, this was washed three times with rinse buffer 1X and then was incubated for 1 hour with the corresponding secondary antibody (1:500) (goat anti-rabbit IgG Alexa Fluor 488; Molecular Probes/goat anti-mouse IgG1-Alexa Fluor 568; Molecular Probes). Finally, the cells were washed with rinse buffer 1X and Prolong Gold Antifade Reagent with DAPI (Molecular Probes). For the nuclear markers, the PBS was discarded, and the cells were washed three times with BSA 0.1% (Sigma-Aldrich) for permeabilization and blocked by incubating for 45 minutes at room temperature with 0.1% Triton X100 (Sigma-Aldrich), 10% normal donkey serum (NDS; Sigma-Aldrich), or normal goat serum (NGS; Sigma-Aldrich) in 1% BSA (Sigma-Aldrich). After incubation, the cells were washed three times with BSA 0.1% and incubated overnight at 4°C with primary Gata-4 antibody (1:200) (rabbit polyclonal Gata-4 antibody; Santa Cruz Biotechnology). The next day, cells were washed three times with BSA 0.1% and incubated with the corresponding secondary antibody (1:500) (rabbit anti-goat IgG-Alexa Fluor 568; Molecular Probes) for 1 hour at room temperature. Finally, they were washed with BSA 0.1%, and Prolong Gold Antifade Reagent with DAPI (Molecular Probes) was added.

Oil Red Staining

Sertoli cells were seeded in six-well plates, cultured for 5 days, fixed with 4% paraformaldehyde (PFA) (Electron Microscopy Sciences) for 20 minutes, and then washed with PBS (PAA). After the first wash, 0.5% oil red solution (Sigma) in isopropanol 100% (J.T. Baker) was prepared and filtered twice through a 0.45-μm pore mesh (Partec); then the stock solution was diluted (3:2) to be filtered again twice. For staining, Sertoli cells were incubated for 5 minutes at 150 rpm, washed three times with PBS (PAA) at 4°C, counterstained with hematoxylin-filtered samples for 3 minutes, and washed with H_2O. Finally, cells were observed under the microscope.

Electron Microscopy

For the electron microscopy analysis, cells were washed three times with 0.1 M phosphate buffer (PB; Electron Microscopy Sciences), fixed with 3.5% glutaraldehyde (Electron Microscopy Sciences) for 45 minutes at 37°C, and subsequently washed with 0.1 M PB. Samples were then post-fixed with 0.5% osmium, rinsed, dehydrated, and embedded in Araldite (Durcupan; Fluka). Semithin sections (1.5 μm) were cut with a diamond knife and stained lightly with 1% toluidine blue. Semithin sections were reembedded in an Araldite block

and detached from the glass slide by repeated freezing with liquid nitrogen and thawing. The block with the semithin sections was cut into ultrathin (0.05 μm) sections with a diamond knife, stained with lead citrate, and examined under an electron microscope (Jeol 100CX).

Analysis of Meiotic Markers *SCP3, MLH1,* and *CREST*

Cells from the coculture were harvested every 2 to 3 days by collecting the supernatant and trypsinizing the adherent cells. Cells were then centrifuged for 5 minutes at 12,000 rpm, and the supernatant was discarded. The pellet was then resuspended in 700 μL of prewarmed 0.075 mol/L potassium chloride solution (KCl) (GIBCO) and incubated for 15 minutes at 37°C. Cells were fixed with 700 μL of 1% paraformaldehyde (PFA; Electron Microscopy Sciences) for preparation on slides (Thermo Scientific Superfrost Slides). Slides were dried for approximately 24 hours at 37°C in a humid chamber and washed for 2 minutes in 0.4% Photo-Flo (Kodak). Cells were subjected to immunofluorescence following the protocol described by Sun et al. (27) using antibodies against SCP3 (lateral elements of the SC marker) (Novus Biologicals), MLH1 (Becton Dickinson), and CREST (centromere marker) (Fisher Scientific) to identify the meiosis stage of the cells at each time point. As internal controls, both the CD49f+-sorted cells and the Sertoli-RFP cells were analyzed separately before starting the coculture with the indicated antibodies.

Fluorescence in Situ Hybridization Technique (FISH)

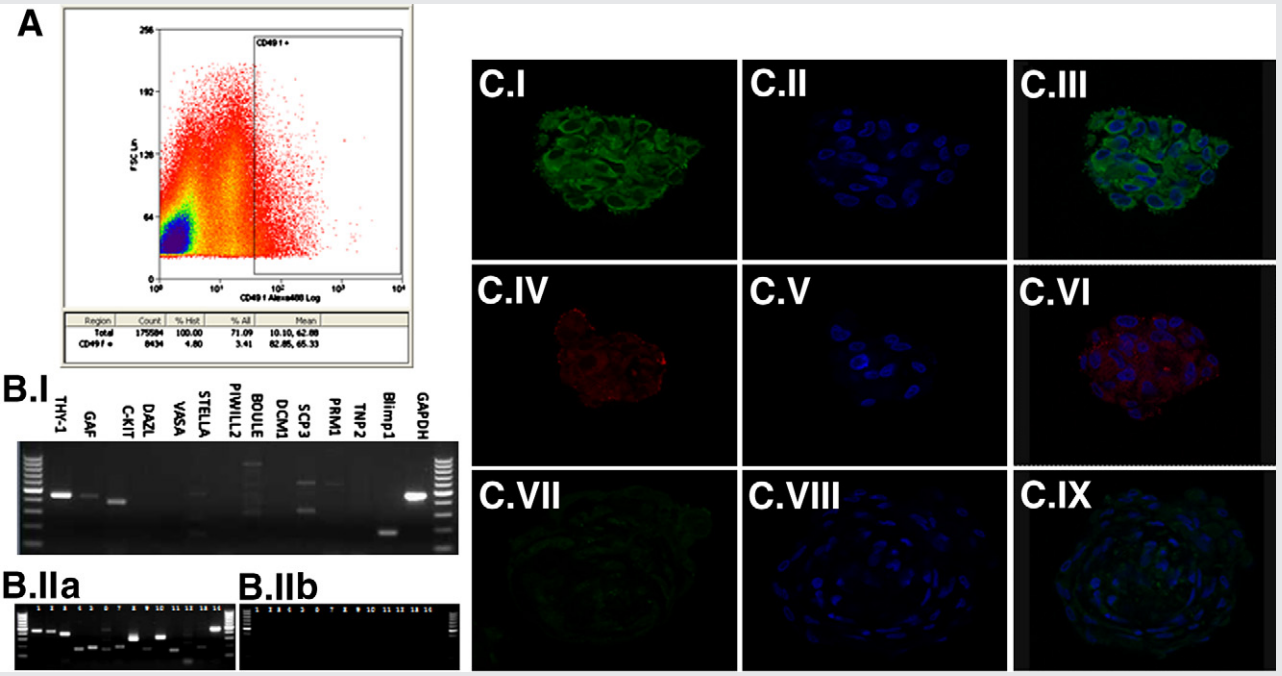
Cocultured cells were analyzed by fluorescence in situ hybridization (FISH) every 48 hours. For this purpose, cells were collected from both the supernatant and the monolayer by trypsinization and were then centrifuged for 5 minutes at 12,000 rpm; the supernatant was discarded. The pellet was then resuspended and incubated in 500 μL of prewarmed 0.075 mol/L potassium chloride solution (KCl; GIBCO) for 15 minutes at 37°C. Cells were prefixed with 500 μL of Carnoy fixative solution at −20°C. Finally, the sample was centrifuged, the supernatant was discarded, and the cells were resuspended in 500 μL of Carnoy for preparation on slides (Thermo Scientific Superfrost Slides) for the analysis. The FISH analysis was assessed for the signals of chromosomes 18, X, and Y (ADN Poseidon; Kreatech Diagnostics), and 13 and 21 (Vysis Inc.) (28). As an internal experiment control, 3,000 CD49f+ freshly isolated cells were FISH analyzed before the coculture started, as were 10,000 RFP-Sertoli cells.

RESULTS

Isolation of CD49f+ Cells from TESE

Enzymatic digestion of the TESE samples yielded 75% of viable cells after disaggregation, and they were cultured for 4 days in the media containing 20% ES cell-qualified FBS

FIGURE 1



(A) Flow cytometry isolation of CD49f cells. (B) (*I*) Real-time polymerase chain reaction (RT-PCR) for THY-1, GAF, C-KIT, DAZL, VASA, STELLA, PIWILL2, BOULE, DCM1, SCP3, PRM1, TNP2, and Blimp1 in CD49f+ cells. (*IIa*) RT-PCR for positive control. (*IIb*) RT-PCR for negative control. (C) Immunocytochemistry in CD49f positive cells: (*I*) CD49f, (*II*) dapi, and (*III*) merge; (*IV*) THY-1, (*V*) dapi, and (*VI*) merge; (*VII*) GPR125, (*VIII*) dapi, and (*IX*) merge.

Riboldi. Haploid cells after coculture of CD49f+ with Sertoli cells. Fertil Steril 2012.

with 40 ng/mL of GDNF. To isolate the SSC population, nonadherent cells were FACS-sorted for CD49f+ selection (Fig. 1A), resulting in the separation of a mean of 5.45% CD49f+ in the OA patients, and of 2.36% in the NOA patients (Supplemental Table 2, available online).

Molecular characterization of CD49f+ cells was performed by RT-PCR and immunofluorescence. Their transcriptional profile revealed a positive expression for the genes involved in the germ cell lineage, such as Thy-1 (a spermatogonial stem cell marker), Blimp-1 (a primordial germ cell marker), c-Kit (a marker for spermatogonia), GAF (a GDNF receptor), BOULE and Stella (as germ cell markers), as well as the meiotic gene SCP3 (Fig. 1B). At the protein level, immunofluorescence showed the cytoplasmic localization of CD49f, Thy-1, and GPR125 in the sorted population (Fig. 1C). These molecular results indicate that the isolated CD49f+ cells are compatible with the SSC population as they displayed specific surface markers and expressed a genetic pattern that was compatible with germ line progenitor cells, as previously demonstrated (7–9).

Isolation of Sertoli Cells and Transfection with RFP

After monolayer formation, Sertoli cells were isolated by flow cytometry by use of a lipid-droplet marker identified by Nile red (Fig. 2A). Isolated cells showed the typical structural features of Sertoli cells, such as lipid droplets and tight junctions, which were further assessed by oil red staining and electron microscopy (Fig. 2B). With RT-PCR, we detected the expression of specific markers of Sertoli cells, such as GDNF receptor (GAF), stem cell factor (SCF), transcription factors GATA-4 and Wilms tumor (WT-1) (Fig. 2C). Immunocytochemistry studies further showed the expression of GATA-4 and vimentin by fluorescence in the putative Sertoli cells (Fig. 2D), as previously demonstrated elsewhere (29).

Because Sertoli cells are used as feeders for the SSC coculture, we decided to transfect the RFP protein into Sertoli cells to differentiate them from the germinal population. Sertoli cells were transfected with 1 μg of lentiviral vector A179-Helix-UBC-RFP, 1 μg of GAG POL-BAM HI (virus survival), and 1 μg of VSVG-ECORT (encapsulation virus) plasmids. The mixture was gently shaken and incubated for 10 minutes. The DNA mixture was then gently applied in the 100 μL of Optimen medium (Invitrogen) with 10 μL of Fugene HD to be subsequently incubated for 30 minutes. The 293-T packaging cells, cultured in 60 mm diameter dishes (Falcon; BD Biosciences Discovery Labware) at 70% confluents in 2 mL of fresh warm DMEM medium, were cotransfected by dropping the entire Fugene-DNA mixture into the dishes. Afterward, the dishes were slowly shaken. The levels of transfection in the 293-T cells were analyzed after 24 hours by immunofluorescence microscopy for RFP expression. The lentiviral supernatant was recovered at 48 hours after cotransfection. For viral transduction, Sertoli cells (50,000 cells/well in six-well plates; Falcon) were incubated at 37°C in a humidified 5% CO_2 atmosphere for 12 hours. The transfection cells were analyzed under a fluorescence microscope to observe the efficiency of the method to express the RFP protein (Fig. 1E). These Sertoli-RFP cells were used as feeders for the SSC coculture.

Coculture of CD49f+ Cells with RFP-Sertoli Cells to Promote Meiosis

The CD49f+ cells were seeded directly onto the Sertoli-RFP monolayer in collagen matrix-coated plates at a proportion of 3,000 CD49f+ cells/10,000 Sertoli-RFP cells. Cocultures were allowed to grow in the presence of 1,000 IU/mL of FSH, 1 μM testosterone, 40 ng/mL GDNF, and 2 μM RA at 37°C in 5% CO_2. Finally, the cells from all the cocultures were collected at 48 hours in a time course experiment lasting 15 days, and they were subjected to a immunocytochemical meiosis stage analysis to check their meiosis progression and FISH.

The CD49f+ and Sertoli-RFP cells were interrogated separately for their meiotic status before we initiated the coculture. In each sample, 1,500 cells were isolated for testing before the coculture; 20% to 30% of them were lost during processing, and approximately 1,000 cells per sample were analyzed for meiosis markers and FISH. In the controls, as expected, the immunocytochemical analysis of the meiosis markers showed no expression of SCP3, CREST, or MLH1, indicating that no meiosis had initiated in these cells (Fig. 3A and B). Moreover, the FISH results obtained for chromosomes 18, X, and Y further demonstrated that all the CD49f+ cells, regardless of their OA or NOA origin, and Sertoli cells were diploid (Fig. 3C and D).

With the CD49+ cocultured cells, an immunocytochemical analysis of proteins SCP3, CREST, and MLH1 demonstrated the existence of patterns that are compatible with the different meiotic stages. After 3 days of coculturing the CD49f+ cells from NOA patients with Sertoli cells, SCP3 expression was detected along with low CREST expression (Fig. 4A). This concomitant staining of SCP3 and CREST remained until day 5 of the coculture and disappeared by day 7 (Fig. 4B and C). Supplemental Figure 1 (available online) summarizes the results of the meiosis progression in CD49+ from the OA and NOA patients.

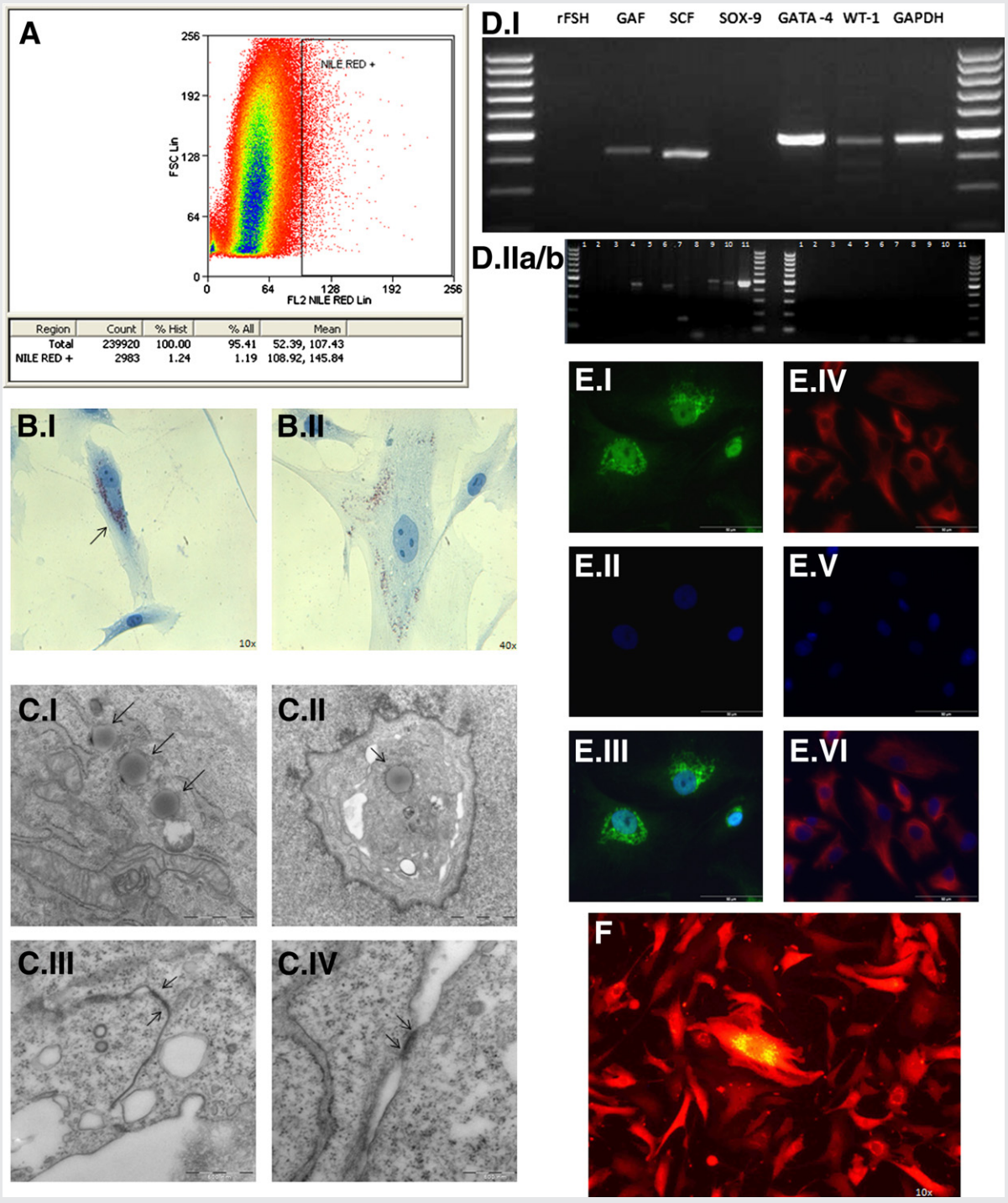
On day 5 of coculturing CD49+ cells from a NOA patient, we demonstrated the development of haploid cells, as shown by the detection of chromosomes 13, 18, 21, X, and Y via FISH (Fig. 4D). Supplemental Table 3 (available online) provides the FISH results.

DISCUSSION

Since the 1960s (30, 31), many researchers have examined and attempted meiosis progression in human spermatogenesis in vitro under various conditions. To date, complete in vitro spermatogenesis that provides human viable sperm has yet to be achieved, although different groups have studied this process in animal models, including mice and primates. Different strategies have been adopted, such as use of combinations of growth factors and hormones, the 3D agar culture system, and immature testicular tissues (16–22). Recent studies in rodents have demonstrated that a crucial prerequisite to pass the meiotic barrier is the presence of the niche, the appropriate in vivo microenvironment provided by the seminiferous tubule (32–34).

Our study isolated SSCs in TESE samples obtained from azoospermic patients with (NOA) or without (OA)

FIGURE 2



(A) Flow cytometry for Nile red-positive staining in testicular cells. (B) (*I*) Lipid droplet by oil red in Sertoli cells (magnification: ×10). (*II*) Lipid droplet by oil red in Sertoli cells (magnification: ×40). (C) (*I*) Lipid droplet by electron microscopy in Sertoli cells. (*II*) Lipid droplet by electron microscopy in Sertoli cells. (*III*) Tight junctions by electron microscopy in Sertoli cells. (*IV*) Tight junctions by electron microscopy in Sertoli cells. (D) (*I*) Real-time polymerase chain reaction (RT-PCR) for GAF, SCF, GATA-4, and WT-1 in Sertoli cells. (*IIa*) RT-PCR for positive control. (*IIb*) RT-PCR for negative control. (E) Immunocytochemistry in Sertoli cells for (*I*) GATA-4, (*II*) dapi, (*III*) merge; (*IV*) vimentin, (*V*) dapi, and (*VI*) merge. (F) Sertoli cells express red fluorescent protein (RFP).

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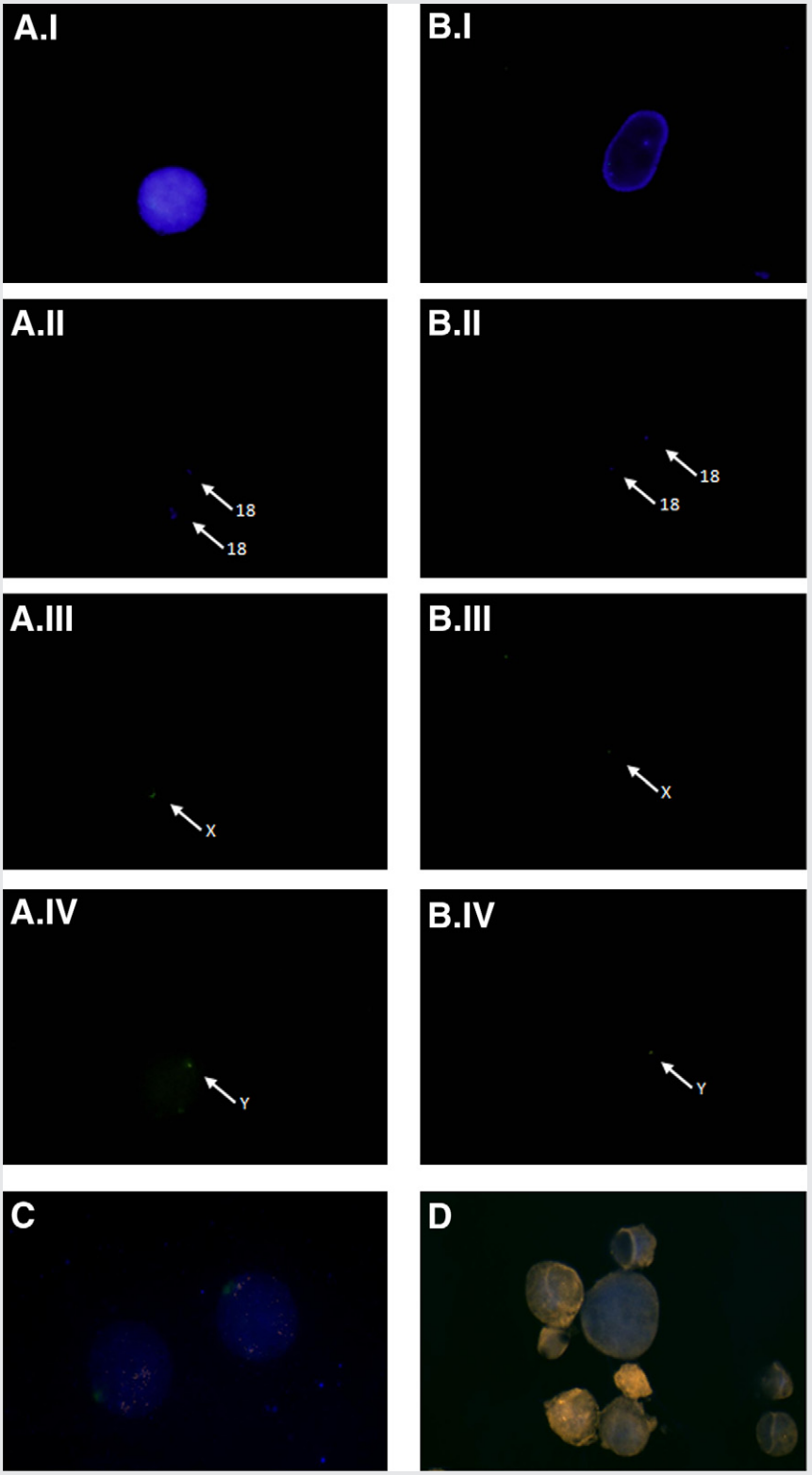
FIGURE 3



(A) Fluorescence in situ hybridization (FISH) in CD49f+ cells: (*I*) dapi, (*II*) chromosome 18, (*III*) chromosome X, and (*IV*) chromosome Y. (B) FISH in Sertoli cells: (*I*) dapi, (*II*) chromosome 18, (*III*) chromosome X, and (*IV*) chromosome Y. (C) Immunocytochemistry for meiosis in CD49f+ cells (staining: negative for SCP3, CREST, and MLH1; positive for DAPI). (D) Immunocytochemistry for meiosis in Sertoli cells red fluorescent protein (RFP) (staining: negative for SCP3, CREST, and MLH1; positive for DAPI).

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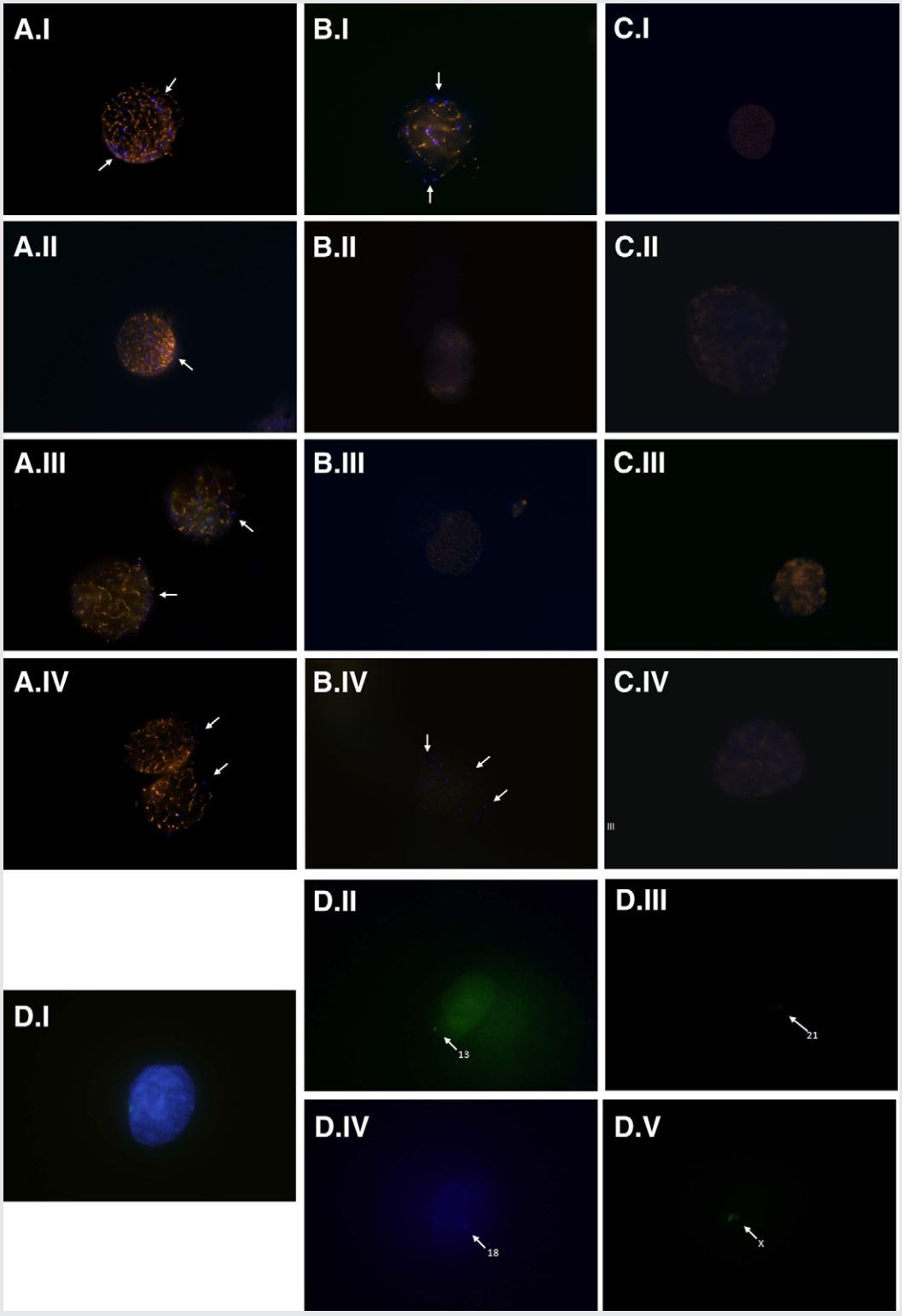
FIGURE 4



(A) Immunocytochemistry for meiosis in nonobstructive azoospermic (NOA) coculture on day 3. (*I–IV*) Positive staining for SCP3 and CREST. (B) Immunocytochemistry for meiosis in NOA coculture on day 5. (*I*) Positive for SCP3 and CREST. (*II*) Negative for SCP3, CREST, and MLH1. (*III*) Positive for SCP3. (*IV*) Positive for SCP3 and CREST. (C) Immunocytochemistry for meiosis in NOA coculture on day 7. (*I*) Positive for SCP3. (*II*) Negative for SCP3, CREST, and MLH1. (*III*) Positive for SCP3. (*IV*) Positive for SCP3. (D) FISH for the four chromosomes in NOA by CD49f+ cells: (*I*) dapi, (*II*) chromosome 13, (*III*) chromosome 21, (*IV*) chromosome 18, and (*V*) chromosome X.

Riboldi. Haploid cells after coculture of CD49f+ with Sertoli cells. Fertil Steril 2012.

spermatogenic arrest (diagnosis proved by a pathologist) and cocultured them in vitro with RFP-Sertoli cells to mimic the seminiferous tubule microenvironment that results in haploid cell generation. Testicular size varied among the samples (~1 mm^3) and was composed of seminiferous tubules and interstitial tissue. After mechanical dissection into small pieces, the samples were enzymatically dissociated with collagenase type IA and selected on the basis of previous reports (35, 36). The concentration and time were defined via dose-response and time-course preview experiments. The total number of isolated cells from the testicular samples varied from 250,000 to 7,200,000 (see Supplemental Table 2). Depending on the number of cells collected, they were distributed in a varying number of wells to a maximum of 2.10^6 cells per well. Our strategy is based on simulating the proper niche for SSC differentiation to lead to spermatogenesis.

The putative spermatogonial stem cells were separated from the TESE samples by isolating the cells that tested positive for surface marker CD49f, which is expressed in the spermatogonia and primary spermatocytes attached to the basal membrane of seminiferous tubules (9). Once the cells were characterized, their transcriptional profile revealed positive expression for the genes involved in germ-cell lineage such as Thy-1 (a spermatogonial stem cell marker), Blimp-1 (a primordial germ cell marker), c-Kit (a marker for spermatogonia), GAF (a GDNF receptor), BOULE, Stella (germ cell markers), and the meiotic gene SCP3. This transcriptional profile clearly demonstrates no cross-reactivity with Sertoli cells. It is interesting that the percentage of SSCs in the TESE samples from OA patients was twice that of the NOA patients (5.45% vs. 2.36%, respectively), indicating the existence of this germinal primordial population in all the azoospermic patients, even those with Sertoli cell-only syndrome (see Supplemental Table 2).

Sertoli cells cultured on collagen matrix after growth were sorted by Nile red staining, which identified the existence of characteristic lipid droplets that were further confirmed by the expression of GATA-4, vimentin, WT-1, SCF, and GAF as well as by the presence of tight junctions, thus confirming that they match the characteristics of functional human Sertoli cells (29). We then cocultured the SSC population onto a monolayer of Sertoli cells and collagen matrix components of the basal layer of seminiferous tubules, which provided structural support to SSCs. In addition, the Sertoli cells were stimulated with FSH (30, 37) and testosterone, the two main players in the differentiation and maturation of spermatogonia toward sperm (38–40). Because the presence of Leydig cells was demonstrated to not be mandatory in a bovine coculture SSC system, Aponte et al. (41) grew SSCs in a mix of somatic cells (e.g., Sertoli, Leydig, peritubular), and the presence of Leydig cells was anecdotal and not stimulated by luteinizing hormone (LH). The absence of Leydig cells was compensated for by the direct addition of testosterone. Sertoli cells based in collagen matrix were provided as coculture, and they were sufficient for meiotic differentiation of the SSCs with the addition of growth factors and hormones. The role of RA in the regulatory mechanisms implicated in gametogenesis has been extensively demonstrated (42–44), as has the important role of GDNF in maintaining the testicular niche (45). The aim of adding specific hormones (FSH and testosterone) and factors (GDNF and RA) to the coculture is to confer the microenvironment conditions of the testicular niche and to contribute to the SSCs' maturation and meiosis progression.

In the first part of the meiotic process, a primary spermatocyte divides to produce two haploid cells; the chromosomes pair up in a synaptic process to form a structure known as a bivalent (two chromosomes) or a tetrad (four chromatids). Prophase I is the meiosis stage in which the homologous chromosomes pair up and exchange their DNA by genetic recombination. This step comprises four different subphases: leptotene, where chromosomes begin to condense and positive staining for SCP3 can be observed; zygotene, where chromosomes become closely paired and can be identified by positive staining for SCP3 with low CREST expression; pachytene, when crossing over occurs, as evidenced by positive staining for SCP3 and CREST with low MLH1 expression; and diplotene, where homologous chromosomes begin to separate yet remain attached by the chiasmata, with positive staining for SCP3, CREST, and MLH1. On day 3 of the coculture, the SSCs from NOA patients presented SCP3 staining along with low CREST expression, suggesting that they had entered meiosis and progressed through the leptotene phase. On day 5 after coculture, SCP3 staining was maintained in the SSCs from the NOA patients along with stronger CREST staining, suggesting continuation to the zygotene meiosis stage. No staining was detected from day 7 onward, implying that meiosis had stopped in those cells after zygotene for some reason. This observation is coincident in time with the detection of haploid cells on day 5, which is expected to occur just after the zygotene phase. It is interesting that progress through the zygotene phase and generation of haploid cells have been observed in the SSCs isolated from the TESE samples of NOA patients.

Other researchers have already reported on the usefulness of coculture systems together with the addition of specific hormones and factors to attempt human spermatogenesis in vitro (16–20). Our work presents evidence for the first time that SSCs isolated from TESE samples of NOA patients can enter meiosis and produce haploid cells in vitro under the indicated culture conditions. Thus, our study incorporates new perspectives in the field of male infertility. Although more studies with tissues, stem cells, or induced cells are still necessary before any application in assisted reproduction, the maturation of germ cells could allow the acquisition of sperm, and genetic and epigenetic characteristics (46).

It is important to recall that the spontaneous onset of meiosis was determined by positive staining for SCP3 in the CD49f+ cells obtained from the OA patients. The cells obtained from the NOA patients could not initiate meiosis spontaneously, as demonstrated by the absence of SCP3, CREST, and MLH1 under noninducing conditions. It is clear that the efficiency of spermatogenesis progression is very low, and the haploid cell generation demonstrated by FISH occurred in only one of the NOA samples on day 5 of coculture. Further work is needed to ascertain the proper culture conditions that avoid meiosis arrest.

The putative clinical strategy that emanates from this work involves the use of autologous CD49f+ and Sertoli cells in TESE samples of NOA patients in which no sperm is found for in vitro maturation of their own SSC. The same alternative can be offered to patients with other types of spermatogenic arrest and those who are undergoing chemotherapy during childhood, at which time TESE can be performed to preserve testicular tissue in the prepubertal stage so that they can latter attempt to have their own biological offspring. To develop the technique in the future, the safety of CD49f+ antibodies on sperm function must be properly tested.

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SUPPLEMENTAL FIGURE 1

	Before co-culture										% co-culture															
											D3					D5					D7					D10				
	L	Z	P	D	T	L	Z	P	D	T	L	Z	P	D	T	L	Z	P	D	T	L	Z	P	D	T	L	Z	P	D	T
NOA	2.7%		0		97.3%	0				100%	7.9%		0.3%		91.8%	5.8%		0.9%		93.3%	0.1%		0		99.9%	0				100%
OA	0.2%		1.8%		98%	0				100%	0				100%	0				100%	0				100%	NA				
	CD49f+					Sertoli RFP																								

Mean estimate of positive staining for meiosis markers (SCP3, CREST, MLH1). ''Before coculture'' is the CD49f+ and Sertoli red fluorescent protein (RFP) control before the start of coculture. ''Percentage coculture'' is the time course of the cells in coculture (day 3 to day 10). D = diplotene (positive staining for SCP3, CREST, and MLH1); L = leptotene (positive staining for SCP3); P = pachytene (positive staining for SCP3, CREST, and sometimes MLH1); NA = not available (data not shown); NOA = nonobstructive azoospermia; OA = obstructive azoospermia; Z = zygotene (positive staining for SCP3 and sometimes CREST).

Riboldi. Haploid cells after coculture of CD49f+ with Sertoli cells. Fertil Steril 2012.

SUPPLEMENTAL TABLE 1

Primers sequence for real-time polymerase chain reaction.

Gene	Primer sequence 5′–3′	Base pair
THY1	*TGCCGCCATGAGAATACCA* *TCAGAGAAGTAGGATCTCTGCA*	439
GAF	*AAGCACAGCTACGGAATGCT* *ATTGCCAAAGGCTTGAATTG*	426
C-KIT	*GCACGGTTGAATGTAAGGCT* *TCATGGCCGCATCTGACTTA*	396
STELLA	*GTTACTGGGCGGAGTTCGTA* *TGAAGTGGCTTGGTGTCTTG*	174
DAZL	*ATGTTAGGATGGATGAAACTGAGATTA* *CCATGGAAATTTATCTGTGATTCTACT*	178
VASA	*AGAAAGTAGTGATACTCAAGGACCAA* *TGACAGAGATTAGCTTCTTCAAAAGT*	199
PIWIL2	*TCTATGGGGCCATCAAGAAG* *CCATCCCGATCACCATTAAC*	195
BOULE	*ATGTAGCTCCCCTGTGATGG* *GTGATGGCACTTGGAGCATA*	300
DCM1	*CTTTCAGGCAGATCCCAAAA* *CCCAATTCCTCCAGCAGTTA*	172
SCP3	*GCCGTCTGTGGAAGATCAGT* *TGGTTAAGCTTCTGCCTTTGA*	327
PRM1	*CACCATGGCCAGGTACAGAT* *GTCTTCTACATCGCGGTCTG*	155
TNP2	*CACAGGCAAGAAGGAAGAGG* *AGCCAATGCATTCTTCCAAC*	235
BLIMP1	*GCCAAGTTCACCCAGTTTGT* *GATTCGGGTCAGATCTTCCA*	183
GATA4	*AGACATCGCACTGACTGAGAAC* *GACGGGTCACTATCTGTGCAAC*	475
WT1	*TCCTTCATCAAACAGGAGCCGAGC* *CTGTAGGGCGTCCTCAGCAGCAAAG*	450
rFSH	*CACAGTCCCCAGGTTCCTTA* *ATGCTGCTGGCTTTTTCACT*	166
SCF	*GCTCCAGAACAGCTAAACGG* *TCTTTGACGCACTCCACAAG*	417
SOX9	*GAGGAAGTCGGTGAAGAACG* *AGACAGCCCCCTATCGACTT*	276
GAPDH	*TGAGCTGAACGGGAAGCTCA* *GTCTACATGGCAACTGTGAGGA*	470

Riboldi. Haploid cells after coculture of CD49f+ with Sertoli cells. Fertil Steril 2012.

SUPPLEMENTAL TABLE 2

Total number of cells isolated from testicular sperm extraction and number of CD49f+ isolated, percentage (%), and mean.

	Total cells	CD49f+	% CD49f+	Mean
OA	250,000	16,300	0.5%	5.45%
	7,200,000	500,000	6.25%	
	1,750,000	235,000	15%	
	700,000	17,500	3%	
	730,000	19,400	3.6%	
	13,920,000	20,000	1.04%	
	2,250,000	28,000	2%	
	9,600,000	50,845	3%	
	1,080,000	33,000	1.8%	
NOA	4,600,000	5,000	2.5%	2.36%
	3,200,000	13,000	4.35%	
	850,000	8,000	1.30%	
	3,000,000	25,000	2.6%	
	6,000,000	193,000	10.19%	
	2,000,000	32,000	2.66%	
	485,000	7,000	1.10%	
	270,000	400	0.19%	
	735,000	850	0.2%	
	250,000	795	0.7%	
	525,000	1,200	0.16%	

Note: NOA = nonobstructive azoospermia (11); OA = obstructive azoospermia (9).

Riboldi. Haploid cells after coculture of CD49f+ with Sertoli cells. Fertil Steril 2012.

SUPPLEMENTAL TABLE 3

Average estimate of cell ploidy from fluorescence in situ hybridization analysis for chromosomes 18, X, and Y.

	Before coculture				Percentage coculture											
	CD49f+		Sertoli RFP		D3		D5		D7		D10		D12		D15	
	2n	1n	2n	1n	2n	1n	2n	1n	2n	1n	2n	1n	2n	1n	2n	1n
NOA	100%	0	100%	0	100%	0	99.3%	0.57%	100%	0	NA		NA		100%	0
OA	100%	0	100%	0	100%	0	100%	0	100%	0	100%	0	100%	0	100%	0

Note: "Before coculture" is the CD49f+ and Sertoli red fluorescent protein (RFP) control. "Percentage coculture" is the time course of the cells in coculture (day 3 to day 15). NA = not available (data not shown); NOA = nonobstructive azoospermia; OA = obstructive azoospermia.

Riboldi. Haploid cells after coculture of CD49f+ with Sertoli cells. Fertil Steril 2012.