Stem cell based therapeutical approach of male infertility by teratocarcinoma derived germ cells

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Infertility affects 13–18% of couples and growing evidence from clinical and epidemiological studies suggests an increasing incidence of male reproductive problems. There is a male factor involved in up to half of all infertile couples. The pathogenesis of male infertility can be reflected by defective spermatogenesis due to failure in germ cell proliferation and differentiation. We report here in vitro generation of a germ cell line (SSC1) from the pluripotent teratocarcinoma cells by a novel promoter-based sequential selection strategy and show that the SSC1 cell line form mature seminiferous tubule structures, and support spermatogenesis after transplantation into recipient testes. To select differentiated germ cell population, we generated a fusion construct (Stra8-EGFP) harbouring the 1.4 kb promoter region of germ line specific gene Stra8 and coding region of enhanced green fluorescence protein. This region was sufficient to direct gene expression to the germinal stem cells in testis of transgenic mice. The purified cells expressed the known molecular markers of spermatogonia Rbm, cyclin A2, Tex18, Stra8 and Dazl and the beta1- and alpha6-integrins characteristic of the stem cell fraction. This cell line undergoes meiosis and can develop into sperm when transplanted into germ cell depleted testicular tubules. Sperm were viable and functional, as shown by fertilization after intra-cytoplasmic injection into mouse oocytes. This approach provides the basis that is essential for studying the development and differentiation of male germ line stem cell, as well as for developing new approaches to reproductive engineering and infertility treatment.

INTRODUCTION

Stem cells exist in many tissues and are responsible for generating differentiated cells during an animal’s lifetime (1,2). Understanding the cellular and molecular mechanisms controlling stem cell function is crucial to the future use of stem cells in regenerative medicine, as well as in understanding aging, tumor formation and gametogenesis (3–5). Recent advances in the establishment of male germ line stem cells provided researchers with the ability to identify, isolate, maintain, expand and differentiate the spermatogonia, the primitive male germ cells, as cell lines under in vitro conditions. The ability to culture and manipulate stem cell lines from male germ cells has gradually facilitated research into spermatogenesis and male infertility, to an extent beyond that facilitated by the use of somatic stem cells. Germ line cells are responsible for transmitting genetic information and for reproducing totipotency from generation to generation. In mouse, germ cell competence is induced at embryonic day (E) 6.5 in proximal epiblast cells by signals emanating from the extraembryonic ectoderm (6). During the specification period, precursor cells give rise to primordial germ cells (PGCs) at the base of the allantoid in gastrulating embryos at E7.5. PGCs migrate through the dorsal mesentery and enter the developing fetal gonad, the genital ridge, between E10.5 and E12.5. Once they arrive in the genital ridge, the PGCs are enclosed by somatic Sertoli cells and become gonocytes (3). The gonocytes proliferate for a few days and then arrest in G0/G1 phase until birth. Within a few days after birth, the gonocytes resume proliferation to initiate spermatogenesis.

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By day 6 postpartum, these cells migrate to the basement membrane of seminiferous tubules and become undifferentiated type A spermatogonia, the spermatogonial stem cells (SSCs). SSCs normally divide asymmetrically, giving rise to one stem cell and one spermatogonia, which initiates differentiation to produce spermatoozoa (7,8). SSCs maintain populations of highly differentiated but short-lived cells, sperm, through a critical balance between alternate fates: daughter cells either maintain stem cell identity or initiate differentiation (7). The potential of embryonic stem (ES) cells, embryonic germ (EG) cells and teratocarcinoma stem (TC) cells to generate all lineages of embryo in vivo has been widely reported in the literature (9–12). It is now thought that for most stem cell types the extracellular environment, niche, provides signals necessary for self-renewal and differentiation (13). Given the evidence that transplantation of ES, EG and TC into seminiferous tubules of testis results in tumor formation (14,15), it was suggested that these stem cells may have a high proliferative activity and the differentiation pathway is suppressed.

In the present study, we used F9 teratocarcinoma stem cells as a model system and developed a directed sequential selection (DSS) strategy to isolate selectively a differentiated germ cell population.

RESULTS

Derivation of differentiated germ cells from TC cells

To select a differentiated germ cell population, we generated a fusion construct (Stra8-EGFP) harbouring the 1.4 kb promoter region of germ line specific gene Stra8 and coding region of enhanced green fluorescence protein (Fig. 1A). Expression of mouse Stra8 gene is restricted to the male germ cells from E14.5 to spermatogonia (16). Previously, it was shown that the 400 bp promoter region directs transgene expression to testis and brain (17). Therefore, we used a prolonged 1.4 kb promoter region and generated transgenic mice to examine the specificity of transgene expression. RT–PCR expression analysis using EGFP specific primers demonstrated a testis specific expression in two independently established transgenic lines (Fig. 1B). In two transgenic lines analyzed, a limited number of GFP positive cells (11% per tubuli) were detected (Fig. 1C) in the periphery of the seminiferous tubules. A minor fraction contained more than four cells (up to 10), often located close to each other, a pattern evocative of sister cells generated by successive cell division. The profile of the transgene expression appeared to be restricted to a subpopulation of spermatogonia (Fig. 1C).

Teratocarcinoma F9 cell line was transfected with Stra8-EGFP and cultured for 2 months in the presence of G418. Culture, transfection and selection of cells were performed according to previously described protocols (18). No expression of Stra8-EGFP was observed (Fig. 2A and D). After retinoic acid (RA) induction (final concentration 10⁻⁶ M), expression of Stra8-EGFP was detected in ~42% of all cells (Fig. 2B and E). The positive cells were selected using fluorescence-activated cell sorting (FACS) and cultured for a further 2 months in non-induced condition. To examine whether the selected cells maintain the differentiation properties, after RA induction, the cells were subjected to the second sorting.
the expression of Oct4 is maintained until the beginning of spermatogenesis and its expression is restricted to undifferentiated spermatogonia (24).

In the SSC1 cell culture, cell types of variable size had formed by day 10 after sorting (data not shown). Relative to non-sorted TC cells, a reduction in Oct4-expressing cells (from 81 to 32%) and an increase in c-kit-expressing cells (from 12 to 66%) was observed (n > 300, Fig. 3E). Within the GFP positive cells, three distinct types of cells were identified: (i) cells only expressing Oct4; (ii) cells expressing both Oct4 and c-kit; and (iii) cells only expressing c-kit. This is an indication that SSC1 cells contained cell populations (GFP+/Oct4+/c-kit−, GFP+/Oct4+/c-kit+, GFP+/-Oct4−/c-kit+) at different stages of male germ cell development. In consideration of the expression pattern of Oct4 and c-kit in male germ cells, these results suggested that the first fraction (GFP+/Oct4+/c-kit−) correspond to spermatogonial stem cells, the cells expressing both Oct4 and c-kit (GFP+/Oct4+/ c-kit+) may represent type A spermatogonia and the cells (GFP+/Oct4−/c-kit+) which did not express Oct4 but c-kit likely represent differentiated spermatogonia (from Aal to B spermatogonia).

**TC-derived germ cells can differentiate into sperm**

Testis cell transplantation technique provides access to the mammalian germ line and has been used in experimental animal models to study stem cell/niche biology and germ
line development (25). To investigate spermatogonial stem cell capacity and further development of SSC1 cell line in vivo, cells were transplanted into one of the testes of germ cell depleted recipient mice (26). The other testis served as an internal control. After 3 weeks, proliferation of EGFP positive cells was observed in seminiferous tubules of transplanted testes (Fig. 4A). After 8 weeks, SSC1 cells were able to migrate to the basement membrane and colonize the tubules (Fig. 4B). Histological analysis of testes after 3 and 7 months showed the appearance of germ cells with nuclear condensation in the inner cell layer of the tubules (Fig. 4D) and sperm in the lumen (Fig. 4F). No regeneration of spermatogenesis was observed in non-transplanted control testes (Fig. 4C and E) controlled by serial histological analysis and PCR analysis (Fig. 4I). A fraction of GFP positive spermatogonia were observed in the periphery of seminiferous tubules (Fig. 4G and H). Colonization of SSC1 was evaluated by histological sectioning. Each slide was viewed at a magnification of $\times 400$ for the analysis. To assess the level of spermatogenesis in the recipient testes, the numbers of tubule cross-sections with evidence of spermatogenesis (defined as the presence of multiple layers of germ cells in the entire circumference of the seminiferous tubule) or lacking evidence of spermatogenesis were recorded for three sections from each testis, and at least 85 seminiferous tubules were counted. In the sections of treated non-transplanted testes, no spermatogenesis was observed. In transplanted testis, 3.0 colonies per $1 \times 10^7$ SSC1 cells were found. This finding indicates that SSC1 cells can be differentiated into postmeiotic germ cells and can be developed to sperm.

Formation of the male gamete occurs in sequential mitotic, meiotic and postmeiotic phases. Many germ cell specific transcripts are produced during this process. Their expression is developmentally regulated and stage specific. To further substantiate postmeiotic differentiation on the molecular level, we examined expression of different stage-specific genes in transplanted testes. In testes of adult mouse, expression of Stra8 gene was restricted to the premeiotic germ cells (16). The synaptomal complex protein 3 (SCP3) is a part of the axio-lateral element of the synaptonemal complex to which the chromatin loops are attached and is an excellent marker for detection of the meiotic transition in mammals because its expression is required for the onset of the first meiotic division (27). The mRNA of testis-specific isozyme of phosphoglycerate kinase 2 (Pgk2) first appears in spermatogenic cells during the prophase of meiosis and increases in amount after meiosis (28). Transition protein 2 is a basic chromosomal protein which functions as an intermediate in the replacement of histones by protamines and its mRNA is first detectable in step 7 round spermatids (29). As shown by RT–PCR, the transplanted testes were positive for all these genes (Fig. 4I) providing additional evidence for postmeiotic differentiation of SSC1 cells.

DNA image cytometry was carried out to examine the ploidy in the cells of the inner cell layer and in lumen of tubules. Measurements of DNA content confirmed the
Male germ cell development consists of an assortment of unique processes, including formation of the acrosome and flagellum, and remodeling and condensation of the chromatin (30). The acrosome is a unique membranous organelle that is formed during spermiogenesis through an integrated process of transport vesicle production, trafficking and fusion. The acrosome contains different enzymes which are required during the fertilization process. Nuclear elongation and chromatin condensation are concomitant with modifications in the basic protein complement associated with DNA. A number of biochemical events accompany the displacement of histones and the appearance of transition proteins and protamines in spermatids (29). The formation of acrosome and nuclear condensation in the SSC1-derived postmeiotic cells was revealed by immunocytochemical analysis with two specific antibodies to outer acrosomal membrane protein (OAM) and to transition protein 2 (Tp2), respectively (Fig. 4L and M).

Analysis of TC-derived sperm
We demonstrate that SSC1 cells are able to develop to sperm when transplanted to the seminiferous tubules of germ cell depleted mice. Seven months after transplantation, the recipient males were mated with wild-type females over a period of 2 months to examine whether transplantation of SSC1 cells

Figure 4. Analysis of differentiation of SSC1 cells in vivo. (A) A fluorescent microscopic picture of a section of a 3 week transplanted testis showed proliferation of GFP positive cells (2.1% of tubuli). (B) Eight weeks after transplantation, GFP positive cells migrated to the basement membrane and colonized the tubules (3.8 colonies per 10^7 cells). These cells were able to initiate spermatogenesis and to differentiate into spermatids after 3 months (D) and into mature sperm after 7 months (F) of transplantation (3.0 colonies per 10^7 cells). A higher magnification of a spermatid and a sperm cell are shown at the right corner of the pictures. The other non-transplanted testis served as an internal control (C and E), no regeneration of spermatogenesis was observed. Hemalaun-eosin staining (G) and the corresponding fluorescence picture (H) showed the GFP positive cells in the periphery of seminiferous tubules (arrow) and their differentiation into sperm (arrow). A higher magnification of a sperm cell is shown at the right corner of the picture. (I) RT–PCR analysis of transplanted testis (TR) 3 months after transplantation, compared with a germ cell depleted but non-transplanted testis (NTR). Expression of genes specific for premeiotic (EGFP, Stra8), meiotic (SCP3, Pgk2) and postmeiotic (Tp2) stages shows that SSC1 cells were differentiated into postmeiotic cells. For control, RNA from SSC1 cells and testicular RNA (T) were used. GAPDH served as an internal standard. (J) DNA image cytometry analysis. The DNA contents were quantified by assigning an optical density to each pixel in the image and summing the optical density values for each nucleus. One hundred cells in luminal layer of the same sections in (G) and (H) were evaluated for DNA ploidy analysis. The presence of a haploid cell population (arrow) was confirmed. (K) As reference cells, mouse epidermis and lymphocytes were measured. (L and M) Characterization of differentiated cells. Testis sections were subjected to indirect immunofluorescence with antibodies to outer acrosomal membrane protein (L) and transition protein 2 (M) as primary antibodies and Cy3-conjugated (red) secondary IgG antibodies. Positive cells are shown (arrows).
was able to restore fertility in recipient males. No pregnancy was observed in female mice mated with recipient males. To analyze infertility in recipient males, they were mated with wild-type female mice and sperm number was determined in uterus and oviduct. A strongly reduced number of sperm was found in uterus (823 ± 221 as compared to the wild-types 2 ± 0.6 × 10^6) and no sperm were detected in the oviduct. The recovered sperm showed increased morphological abnormalities (43% as compared to the wild-type sperm 6–8%) in head (Fig. 5A) and in tail (Fig. 5B). Sperm with normal morphology were also observed (Fig. 5C). The normal sperm showed no structural abnormalities examined by electron microscopy (data not shown). Sperm flushed from uteri were subjected to three separate PCR assays to detect Stra8-EGFP transgene. This analysis revealed the presence of sperm derived from SSC1 cells.

Figure 5. Characterization of SSC1-derived sperm. (A–C) Morphology of sperm isolated from epididymis showed about 48% abnormalities in head (A) and tail (B). About 52% of sperm showed normal morphology (C). (D) Detection of transgene allele in sperm recovered from uteri of wild-type females inseminated by transplanted males from three independent mating (U1, U2 and U3). (E) Sperm (S1, S2, S3, S4) were isolated from epididymis and subjected to PCR using GFP primers. Transgene allele could be detected in sperm, indicating the derivation of sperm from SSC1 cells. (F–L) Oocyte-activating competence of SSC1-derived sperm. (F) Sperm with normal morphology from epididymis were injected into cytoplasm of metaphase II-arrested oocytes of wild-type females using a micro-manipulator. Injected oocytes were transferred into M16 medium and monitored for early embryonic development. After injection (8 h), polar pody extrusion (arrow) and pronuclear formation can be seen in phase contrast (G) and fluorescent image of embryo stained with DAPI [blue, (H)]. Injected oocytes were developed into the 2-cell (I), 4-cell (J), 8-cell (K) and morula (L) stages. (M) After 3.5 days, embryos were subjected to PCR using GFP primers to exclude parthenogenetic activation of oocytes and to detect transgene allele (E1–E8).
(Fig. 5D). To further analyze the sperm parameter of recipient males, sperm were isolated from epididymis. A strongly reduced velocity (8% as compared to 76% in wild-type) and a high rate of morphological abnormalities (48% as compared to 6% in wild-type) were detected which could explain defect of migration of sperm to oviduct. In control transplantation experiments using normal germ cells, no increased sperm abnormalities were observed (data not shown). Transmission of transgene allele by sperm from epidiymis was confirmed by PCR analysis using GFP specific primers (Fig. 5E).

Intracytoplasmic injection of sperm isolated from epididymis into unfertilized oocytes of wild-type females (Fig. 5F) was carried out to examine the oocyte-activating competence of sperm and development of preimplantation embryos. Polar body extrusion and pronuclear formation (Fig. 5G and H), and normal features of preimplantation embryos (Fig. 5I–L) indicate that SSC1-derived sperm are viable and functional. Detection of transgene allele in produced embryos by this system excludes parthenogenetic activation of oocytes and revealed transmission of transgene to embryos (Fig. 5M).

DISCUSSION

Malignant germ cell tumors are classified into seminomatous and non-seminomatous germ cell tumors. Non-seminomatous germ cell tumors comprise immature teratoma, teratocarcinoma (malignant teratoma), embryonal carcinoma, yolk sac (endodermal sinus) tumor and choriocarcinoma. Although the potential of teratocarcinoma cells to differentiate into a wide range of tissues is now well attested, little is understood of the key regulatory mechanisms that control their differentiation. Apart from the intrinsic biological interest in elucidating these mechanisms, a clear understanding of the molecular process involved will be essential if the clinical potential of these cells is to be realized. We show that F9 TC stem cells are able to differentiate into spermatogonial stem cells and spermatagonia in vitro and that these cells can initiate spermatogenesis in vivo and can produce mature sperm. However, the derived sperm show higher abnormalities and reduced motility. These could be due to the mutations which occurred during the in vitro culture of TC cell. The impaired motility of sperm affect the migration in the female genital tract and penetration of zona pellucida during fertilization process. To overcome this barrier, we used the sperm for microinjection into oocyte cytoplasm. As can be observed in Figure 5F and E, the sperm are able to fertilize the oocyte and can produce embryos.

It was shown that TC is able to differentiate into derivatives of all three germ layers (11,12). Although TC cell lines have been established in vitro from transplantable testicular teratomas which contain PGC, it has not been clear whether spermatogonia can be derived from TC cells in vitro. We used a novel strategy (DSS) which is based on directed sequential selection of differentiated cells by RA. To select a differentiated germ cell population, we generated a fusion construct (Stra8-EGFP) harboring the 1.4 kb promoter region of germ line specific gene Stra8 and coding region of enhanced green fluorescence protein. During mouse embryogenesis, Stra8 expression was restricted to the male developing gonads, and in adult mice, the expression of Stra8 was restricted to the premeiotic germ cells (16). Using this strategy, differentiated male germ cells were sorted which have no more tumorigenic activity indicating the suppression of high proliferation capacity and initiate directed differentiation of TC cells. Given the evidence that human TC cells are also pluripotent, DSS strategy offers the prospect of fertility treatment in oncological patients and infertile men.

It was previously reported that germ cells can be derived from stem cells. Hubner et al. (31) were able to produce oocytes from mouse embryonic stem cells. It was demonstrated that ES cells can also generate male germline cells in vitro (32,33). Toyooka et al. (33) used a knock-in in ES cells at Mvh (mouse vasa homolog) locus to detect the emergence of PGCs from ES cells in vitro. Therefore, sperm derived from these ES cells contain also disruption of the Mvh locus. In contrast to this approach, in our strategy, endogenous genes remain intact. From these results, it can be concluded that ES and TC cells are capable of differentiating into meiosis-competent cells and can be developed to mature germ cells. Thus, these in vitro culture systems can contribute to various areas, including analysis of the gene function involved in segregation of the germ cell and somatic cell lineages and germ cell and somatic cell interaction and differentiation, development of novel reproductive engineering approaches and advanced studies on fertility treatment.

MATERIALS AND METHODS

Construction of the germ cell-specific Stra8-GFP reporter gene

The −1400/+7 fragment (+1 indicates the start of transcription) (17) amplified from Stra8 genomic DNA (primers gStra8-F1: and gStra8-R1.) was inserted in the Scal/HindIII site of pEGFP-1 vector (BD Biosciences).

Generation of transgenic mice

Transgenic lines were generated by microinjection in FVB fertilized eggs according to standard procedures (34). For microinjection, the Stra8-GFP fragment was isolated free of vector sequences by digestion with Scal and purified after gel electrophoresis by binding to glass beads (BIO 101, Inc., La Jolla). The fragment was quantified and diluted to a concentration of 3 µg/ml in injection buffer consisting of 10 mmol/l Tris, pH 7.4 and 0.2 mmol/l EDTA as previously described (34). The DNA was injected into the pronuclei of fertilized 1 cell mouse embryos. The injected embryos were transferred into FVB pseudopregnant hosts. Transgenic mice harbouring Stra8-GFP sequences were identified by PCR and Southern blot analyses using a GFP cDNA probe on DNA extracted from mouse tail biopsies as previously described (34). Positive founder animals were bred with non-transgenic FVB mice. Transgenic progeny of such crosses were identified and bred together to produce homozygous animals.
Culture of teratocarcinoma stem cell

TC stem cell F9 was grown on 0.1% gelatine coated tissue culture plates and in DMEM medium containing 4.5 g/l glucose and supplemented with 15% FCS (PAN), 2 mM 1-glutamine (PAN), 100 μM non-essential amino acids (Invitrogen), and 50 μg/ml each penicillin and streptomycin (PAN). Stra8-EGFP TC cells were generated as follows: TC cells were suspended to a concentration of 2 × 10^6 cells/ml in serum-free DMEM containing the lipofection reagent Lipofectin (Qiagen) and 10 μg construct DNA (Stra8-EGFP, Fig. 1A) in a 4:1 v/v ratio. The cell suspension was then transferred to a 60 mm culture flask, and after 2 h, medium was added that was supplemented as described above. Cells were cultivated for 3 days at 37°C and in 5% CO_2, then trypsinized and selected by G418 (Geneticin, Gibco/BRL) at a concentration of 350 μg/ml. Use of the selective medium was continued for 1–2 weeks, with frequent changes of medium, until distinct colonies could be visualized. Individual colonies were then trypsinized and transferred to multiwell plates for further propagation in the presence of selective medium. Two weeks after selection of resistant clones, a PCR analysis was done with EGFP-specific primers (5'-CTG AAG TTC ATC TGC TGC ACC AAA-3', 5'-GGT GGT GCA GAT GAA CTT CAG-3') and Stra8-EGFP primers (gStra8-F2: 5'-AGT TGA TCT TCT CCC -3').

Derivation of germ cells from TC cells

Stra8-EGFP TC cells were grown in tissue culture plates in TC cell medium at a density of ~1–2 × 10^4 cells per cm^2. Non-adherent cells were removed after 3–4 days and medium was replaced. Cultures were maintained in the above medium for an additional 3 days and then medium was changed by TC cell medium containing retinoic acid (Sigma) at a final concentration of 10^{-6} M. After 12 h, GFP expression was monitored. Approximately 40% of all cells showed GFP expression at that time. Positive cells were sorted by FACS. Briefly, cells were dissociated with 0.25% trypsin/EDTA, neutralized with DMEM with 10% FCS, washed twice with PBS and then resuspended in PBS containing 0.5% BSA. Approximately 2 × 10^6 cells/ml in PBS/BSA were used for sorting. The flow cytometry was performed on a FACStar Plus (Becton Dickinson) equipped with dual 488 nm argon and 633 nm helium neon lasers. Sorted cells were cultured in retinoic acid free medium. After 8–10 weeks, medium was changed with medium supplemented with retinoic acid (10^{-6} M) and after 12 h, GFP positive cells were sorted by FACs. Approximately 86% of all cells showed GFP expression. These cells were given fresh medium every 2–3 days and designated as SSC1 cells.

Transplantation into seminiferous tubules of testes

Recipients used were 129/Sv mice. At 6–8 weeks of age, the recipient mice were injected i.p. with busulfan (40 mg/kg body weight), which destroys endogenous spermatogenesis. Recipients were used for transplantation ≥4 weeks after injection. Cultured SSC1 cells were harvested using 0.25% trypsin plus 1 mM EDTA, and the cells were suspended in DMEM culture medium. Cell concentration for transplantation was 10–30 × 10^6 cells/ml. Approximately 10 μl of cell suspension was transplanted via rete testis into seminiferous tubules of one testis. Other testes served as internal control. Thirteen animals were transplanted and 10 animals were used as controls. As external control, busulfan treated non-transplanted males were used. After 3, 8 and 12 weeks, recipient mice were killed and testes were examined by immunocytochemistry, image cytometry and RT–PCR analysis. Fertility of a group of recipient and control mice was examined 7 months after transplantation. All of the experimental procedures complied with National Regulations for the Care and Use of Laboratory Animals (similar to the US National Research Council guidelines).

DNA image cytometry

After deparaffinization, 5 μm thick paraffin sections were placed in 5 N hydrochloric acid for 60 min and stained with Feulgen stain for 1 h (CAS DNA staining kit by Cell Analysis System, Pharmingen-Becton Dickinson, Hamburg, Germany). The slides were then rinsed in acid alcohol, cleaned in xylene and covered with synthetic medium. Rat hepatocytes stained with Feulgen were used as control cells. Slides prepared in this way were evaluated with a CAS200 image analyzer (Pharmingen-Becton Dickinson, Hamburg, Germany) and quantitative DNA analysis software. The DNA contents were quantified by assigning an optical density to each pixel in the image and summing the optical density values for each nucleus. The image system was calibrated by measuring the DNA contents of rat hepatocytes. One hundred cells were evaluated for DNA ploidy analysis.

Immunocytochemistry and fluorescence microscopy

Adherent cells were fixed in 4% paraformaldehyde (PFA) in PBS, pH 7.4; for 1 h at ambient temperature. Fixed cells were rinsed in PBS, pH 7.4, and subsequently incubated overnight with primary antibody specific to integrin α6, integrin β1, heat shock protein 90α (Hsp90α), c-kit, Oct4, transition protein 2 (Tp2) or outer acrosomal membrane (OAM) protein. All primary antibodies except that for Tp2 and OAM, were purchased from Santa Cruz. Cells were rinsed three times and incubated in the appropriate Cy3 or FITC conjugated secondary antibody (Sigma). All incubations were in PBS, pH 7.4, 5% BSA and 0.1% Triton-X100. For nuclear stainings, fixed cells were incubated for 5 min with DAPI (4', 6'-diamidino-2-phenylindole) (Vector) dye. Fluorescence microscopy was performed on a Zeiss fluorescence microscope.

RNA isolation and RT–PCR

Total RNA was isolated and cDNA synthesis was carried out with oligo-tT primers as described (35). RT–PCR amplification was performed using specific primers for EGFP, Stra8, Tex18, Dazl, Rbm, Cyclin A2, Oct4, c-kit, Sep3, Pgk2 and Tp2. RT–PCR was achieved after 35–40 cycles of 94°C, 30 s; 50–62°C, 30 s; 72°C, 45 s, depending on the primer sets. Following primers were used for RT–PCR analysis: EGFP (5'-CTG AAG TTC ATC TGC TGC ACC AAA-3', 5'-GGT GGT GCA GAT GAA CTT CAG-3').
5′-GGT GGT GCA GAT GAA CTT CAG-3′, Stra8 (5′-TCA CAG CCT CAA AGT GGC TGG AGG AGT AGT-3′, 5′-GCA ACA GAG TGG ACC AGG AGT-3′), Dazl (5′-TTC AGG CAT ATC TTC ATC TTC-3′, 5′-ATG CTC ACC CAC ACC-3′), Cyclin A2 (5′-CAC CTC GAC TCA TTC GGG-3′, 5′-CGG GTC ACC TTT GCT CTG CTC-3′), C-kit (5′-CAA CAG CAA TGG CCT CAG GAG T-3′, 5′-GTC GTG ACC TTC GGG-3′), Oct4 (5′-TCT CAT GAG TCA CGG TCG-3′, 5′-AAT CGG TAT GCC GAT GTG CAG-3′), Tp2 (5′-GGG CCT CAA AGT CAC ACC AGT-3′, 5′-AGT CCG TTT CCG CCT CAG GAC-3′).

Western blot analysis

Protein was extracted from cells according to published protocol and western blot was performed with antibody to c-kit (35).

Analysis of fertility and sperm parameter

Analysis of fertility and determination of sperm parameters were performed as previously described (36).

Intracytoplasmic sperm injection and analysis of embryos

Female 129/Sv wild-type mice were superovulated as described before (36) and oocytes were collected 10–12 h after hCG administration. The cumulus cells were removed by hyaluronidase treatment and the oocytes were washed and subsequently cultured in fertilization medium (MediCult, Jyllinge, Denmark). Spermatozoa were isolated from the cauda epididymis and capacitated in IVF medium at 37°C for 1.5 h. Sperm with normal morphology were injected into the oocytes using a micromanipulator (Fig. 5F). After injection, oocytes were cultured in M16 (Sigma) at 37°C and 5% CO2 and monitored for embryonic development.

Electron microscopy

Testes and epididymides were fixed with 5% glutaraldehyde in 0.2 M phosphate buffer, postfixed with 2% osmium tetroxide, and embedded in epoxy (Epon) resin. Selected areas were sectioned and examined by electron microscopy.

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