A combined approach facilitates the reliable detection of human spermatogonia in vitro

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Submitted on February 8, 2013; resubmitted on July 16, 2013; accepted on July 26, 2013

STUDY QUESTION: Does a combined approach allow for the unequivocal detection of human germ cells and particularly of spermatogonia in vitro?

SUMMARY ANSWER: Based on our findings, we conclude that an approach comprising: (i) the detailed characterization of patients and tissue samples prior to the selection of biopsies, (ii) the use of unambiguous markers for the characterization of cultures and (iii) the use of biopsies lacking the germ cell population as a negative control is the prerequisite for the establishment of human germ cell cultures.

WHAT IS KNOWN ALREADY: The use of non-specific marker genes and the failure to assess the presence of testicular somatic cell types in germ cell cultures may have led to a misinterpretation of results and the erroneous description of germ cells in previous studies.

STUDY DESIGN, SIZE, DURATION: Testicular biopsies were selected from a pool of 264 consecutively obtained biopsies. Based on the histological diagnosis, biopsies with distinct histological phenotypes were selected (n = 35) to analyze the expression of germ cell and somatic cell markers. For germ cell culture experiments, gonadotrophin levels and clinical data were used as selection criteria resulting in the following two groups: (i) biopsies with qualitatively intact spermatogenesis (n = 4) and (ii) biopsies from Klinefelter syndrome Klinefelter patients lacking the germ cell population (n = 3).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Quantitative real-time PCR analyses were performed to evaluate the specificity of 18 selected germ cell and 3 somatic marker genes. Cell specificity of individual markers was subsequently validated using immunohistochemistry. Finally, testicular cell cultures were established and were analyzed after 10 days for the expression of germ cell- (UTF1, FGFR3, MAGE A4, DDX4) and somatic cell-specific markers (SMA, VIM, LHCGR) at the RNA and the protein levels.

MAIN RESULTS AND THE ROLE OF CHANCE: Interestingly, only 9 out of 18 marker genes reflected the presence of germ cells and cell specificity could be validated using immunohistochemistry. Furthermore, VIM, SMA and LHCGR were found to reflect the presence of testicular somatic cells at the RNA and the protein levels. Using this validated marker panel and biopsies lacking the germ cell population (n = 3) as a negative control, we demonstrated that germ cell cultures containing spermatogonia can be established from biopsies with normal spermatogenesis (n = 4) and that these cultures can be maintained for the period of 10 days. However, marker profiling has to be performed at regular time points as the composition of testicular cell types may continuously change under longer term culture conditions.

LIMITATIONS, REASONS FOR CAUTION: There are significant differences regarding the spermatogonial stem cell (SSC) system and spermatogenesis between rodents and primates. It is therefore possible that marker genes that do not reflect the presence of spermatogonia in the human are specific for spermatogonia in other animal models.

WIDER IMPLICATIONS OF THE FINDINGS: While some studies have reported that human SSCs can be maintained in vitro and show characteristics of pluripotency, the germ cell origin and the differentiation potential of these cells were subsequently called into question. This study provides critical insights into possible sources for the misinterpretation of results regarding the presence of germ cells in human testicular cell cultures and our findings can therefore help to avoid conflicting reports in the future.
Spermatogonial stem cells (SSCs) form the basis for spermatogenesis throughout male life. In vitro culture systems turned out to be highly beneficial for the thorough characterization of these cells. The first organ culture systems for testicular tissues were established by Trowell in 1959. These systems have been modified since and have been used for rodent as well as human testicular tissues (Trowell, 1959; Steinberger, 1967, 1975; Sato et al., 2011). The main advantage of testicular organ culture is that the structure of the seminiferous tubules is maintained. However, despite the small size of testicular fragments the supply of nutrients and oxygen seems to be impaired (Trowell, 1959; Steinberger, 1975). Nonetheless, it was demonstrated in a recent study that all cell types were still detectable after 12 days of human testicular organ culture (Roulet et al., 2006). Moreover, morphologically normal spermatogonia could even be maintained for a period of 16 days (Roulet et al., 2006). With regard to the propagation and characterization of selected testicular cell types though, two-dimensional cell cultures have a clear advantage over the organ culture approach. Furthermore, a sufficient supply of nutrients and oxygen can be ensured. Despite the fact that two-dimensional cell culture cannot mimic the structural conditions of the testis (Reuter et al., 2012), it was reported in 1966 that spermatogonia from rodent testes can be maintained for a period of 4 weeks (Steinberger and Steinberger, 1966). Since then, the culture conditions have been improved and in the meantime, a number of research groups have reported the isolation and long-term culture of mouse SSCs. The germ cell origin of cultured cells was conclusively demonstrated performing germ cell transplantation assays which resulted in the reestablishment of spermatogenesis in recipient mouse testes (Nagano et al., 1998; Kanatsu-Shinohara et al., 2003). In addition to that, it was shown that unipotent SSCs from neonatal and adult mouse testes can be reprogrammed to a pluripotent state under certain culture conditions. These cells were termed germline-derived pluripotent stem (gPS) cells and closely resemble mouse embryonic stem cells (ESCs) with regard to their differentiation potential (Kanatsu-Shinohara et al., 2004; Guan et al., 2006; Ko et al., 2009).

The possibility to propagate unipotent human SSCs as well as pluripotent human gPS cells in vitro would be of great clinical value. While the culture of human SSCs would provide the basis for autologous spermatogonial transplantations and for the in vitro derivation of sperm, human gPS cells would be the only source of adult patient-specific pluripotent stem cells that do not require genetic modification. However, there are remarkable differences concerning the SSC system between rodents and primates (Ehmcke et al., 2006) that hamper the direct transfer of protocols. In rodent spermatogenesis A

In contrast to that, the SSC system in the human includes rarely dividing reserve stem cells termed A

Despite these differences, a number of research groups reported that the culture of human testicular cells under ESC conditions resulted in the formation of SSC colonies. Further characterization of these colonies revealed the expression of pluripotency markers and the ability to differentiate into cell types of the three germ layers in vitro (Conrad et al., 2008; Golestanian et al., 2009; Kossack et al., 2009; Mizrak et al., 2010). However, in three out of these four publications the cells failed to induce the formation of teratomas following their injection into immuno-deficient mice and it was speculated that the SSCs had not been sufficiently reprogrammed (Golestanian et al., 2009; Kossack et al., 2009; Mizrak et al., 2010). These conflicting reports provoked a debate regarding the pluripotency of the so-called human adult germline stem cells (GSCs). This debate was further fuelled by the re-evaluation of the microarray data published by Conrad et al. by an independent research group (Conrad et al., 2008; Ko et al., 2010). For this comparison, Ko et al. repeated the procedure that had been described for the derivation of human adult GSCs (Conrad et al., 2008; Ko et al., 2010). However, they omitted the selection procedures including magnetic-activated cell separation and matrix selection. This modified approach resulted in the derivation of human testicular fibroblast cells (Ko et al., 2010). Subsequently, the expression profiles of human ESCs, human testicular fibroblast cells and the previously published gene array data of the human adult GSCs were compared (Conrad et al., 2008; Ko et al., 2010). These analyses revealed that the gene expression profile of human adult GSCs was similar to that of fibroblasts and clearly differed from human ESCs, particularly with regard to the expression of the pluripotency marker genes octamer-binding transcription factor 4 (OCT4, also POU5F1) and NANOG (Ko et al., 2010). These new insights prompted another research group to re-evaluate the characteristics and the differentiation potential of their previously published human testis-derived ES-like cells (hES-like cells) (Mizrak et al., 2010; Chikhovskaya et al., 2012). Interestingly, microarray analysis performed in this study demonstrated that the expression profile of the hES-like cells was similar to mesenchymal stem cells but distinct from ESCs as well as fibroblasts (Chikhovskaya et al., 2012). Based on these recent findings, the differentiation potential as well as the germ cell origin of published human GSCs has been challenged. Considering the data that is available today, there appear to be several causes for the conflicting reports regarding the culture of human adult SSCs and GSCs.

Most importantly, there is no functional assay available to validate the germ cell origin of human cells. For this reason, research groups strongly...
relied on the use of germ cell markers for the characterization of testicular cell cultures (Conrad et al., 2008; Golestaneh et al., 2009; Kossack et al., 2009; Sadri-Ardekani et al., 2009, 2011). Indeed, a number of studies performing microarray analyses using testicular tissues from patients with different levels of spermatogenic damage found that it is feasible to distinguish tissues with normal spermatogenesis from those with defective spermatogenesis based on the gene expression signature (Fox et al., 2003; Spiess et al., 2007; Chalmeil et al., 2012). Moreover, comparing testicular tissue samples with a Sertoli cell-only (SCO) syndrome from those containing spermatogonia as the only germ cell type facilitated the identification of genes expressed specifically in spermatogonia (von Kopylow et al., 2010). However, it has recently been shown using a non-human primate model that testicular multipotent stromal cells and spermatogonia show a high degree of overlap regarding the expression of markers (Eldermann et al., 2012). Consequently, the use of non-specific marker genes and the failure to assess the presence of testicular somatic cell types in germ cell cultures may have led to a misinterpretation of results with regard to the presence of germ cells in previous studies.

Finally, in contrast to the mouse, human testicular tissue is highly heterogeneous, particularly when it is obtained in the frame of fertility treatment procedures. While some patients present with an obstruction and normal spermatogenesis, others present with an SCO syndrome and are lacking the germ cell population entirely (Bergmann and Kliesch, 2010). While biopsies from the former patient group are suitable for the derivation of germ cells and specifically SSC cultures, this is impossible using biopsies with a true SCO syndrome. Working with human testicular tissue, it is therefore essential to perform a histological analysis of the starting material. However, while sparse histological information was included in some reports (Kossack et al., 2009; Sadri-Ardekani et al., 2009; He et al., 2010), it is completely missing from others (Ko et al., 2010; Sadri-Ardekani et al., 2011) hindering the direct comparison of results.

Seeking to establish SSC cultures and to avoid conflicting reports in the future, we aimed to establish an approach for the reliable detection of human germ cells in vitro. Following the selection of testicular biopsies based on an in-depth clinical and histological evaluation, the first aim of this study was to identify informative markers that facilitate the detection of human germ cells and testicular somatic cells at the RNA and the protein levels. Based on this combined setup, our second aim was to establish culture conditions for human spermatogonia and to validate their germ cell origin.

Materials and Methods

Ethical approval

Testicular biopsies were taken from male infertility patients presenting with obstructive azoospermia or non-obstructive hypergonadotropic azoospermia at the Department of Clinical Andrology, University Hospital Münster, Germany. Ethical approval was obtained to ask patients for one additional testicular biopsy during routine surgical procedure (Ethics Committee of the Medical Faculty of Münster and the State Medical Board Nr. 2008-090-f-5). Following written informed consent for the use of the testicular tissue for this research project, research biopsies were obtained at the same time as biopsies for therapeutic testicular sperm extraction (TESE) and histological analysis.

Hormone measurements and histological evaluation of testicular biopsies

Hormonal parameters were determined in the frame of the routine clinical examination. While follicle stimulating hormone (FSH) and luteinizing hormone (LH) serum concentrations were measured employing immuno-fluorometric assays (Autodelfia; Perkin-Elmer, Freiburg, Germany), serum testosterone concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) (DRG AURICA ELISA Testosterone Kit; DRG Instruments, Marburg, Germany). These assays are continuously validated by internal and external quality controls and the cut-off values for FSH, LH and testosterone are 0.23 U/l, 0.12 U/l and 0.69 nmol/l, respectively. In addition, intra-assay coefficient of variation was below 5% and inter-assay coefficient of variation was below 10%.

For routine histological analysis two biopsies were obtained from each testicle, they were fixed overnight in Bouin’s solution, were washed with 70% ethanol and embedded in paraffin prior to sectioning. Subsequently, sections were stained with periodic acid-Schiff/hematoxylin following dewaxing and rehydration. The detailed protocol has previously been published (Brinkworth et al., 1995). Two entire sections obtained from the two independent biopsies were evaluated. For this evaluation, every tubule was analyzed with regard to the presence of the different germ cell types, an SCO appearance or the presence of tubular shadows. The total number of tubules was then summarized and the percentage of tubules with a specific phenotype was determined. Furthermore, the percentage of tubules with elongated spermatids was calculated according to Bergmann and Kliesch (2010), using a score from 0 (no elongating spermatids in the tubules) to 10 (100% of tubules contain elongating spermatids). The number of Leydig cells was visually evaluated using a patient with normal spermatogenesis as a reference. Based on these findings, the relative number of Leydig cells was classified as normal, slightly increased or increased. Finally, testicular tissue sections were routinely stained for placental alkaline phosphatase (M7191; Dako-Cytomation, Hamburg, Germany; dilution 1:20 and 1:40) to exclude testicular intraepithelial neoplasia. Human placenta and a confirmed testicular tumour served as positive controls, whereas the omission of the antibody served as a negative control.

Selection of testicular biopsies for expression analyses and cell culture experiments

For expression analyses, testicular biopsies were selected from a pool of 264 consecutively obtained biopsies. Selection was based on the histological findings obtained from the same testis. In addition, TESE outcome was taken into account to ensure a high homogeneity of testicular tissues. Based on these criteria, testicular biopsies were assigned to the following groups: (i) qualitatively normal spermatogenesis (n = 10, score >8, sperm in TESE samples), (ii) arrest of germ cell differentiation at the stage of spermatogonia or spermatocytes (each n = 3, score = 0, no sperm in TESE samples, with the exception of one patient with a germ cell arrest at the stage of spermatogonia who had sperm in seven out of nine TESE samples), (iii) SCO biopsies (n = 10, score = 0, seminiferous tubules devoid of germ cells, no sperm in TESE samples) and (iv) biopsies from patients with Klinfelter syndrome (47,XXX), which constitutes one of the most frequent chromosomal male disorders (n = 9, score = 0, no sperm in TESE samples, with the exception of one patient who had sperm in one out of two TESE samples).

For cell culture experiments, testicular tissue was immediately processed and the histological analysis could therefore not be employed as a selection criterion. Instead, gonadotrophin levels and clinical data were used as an indication for the status of spermatogenesis, and histological findings and TESE outcome were analyzed retrospectively, resulting in the following groups:
(i) biopsies with at least partially and only qualitatively intact spermatogenesis (normogonadotropic, n = 4, scores 5, 6, 7 and 9; sperm in TESE samples) and (ii) biopsies from 47,XXY patients (hypergonadotropic, n = 3, score 0, no sperm in TESE samples).

**Processing of testicular tissue for RNA expression analysis and immunohistochemistry**

All research biopsies were immediately transferred into MEMx (Life Technologies GmbH, Gibco, Darmstadt, Germany) and were maintained on ice prior to processing. For expression analysis, ~70% of each biopsy was snap frozen and maintained at ~80 °C, whereas 30% was fixed in Bouin’s solution or 4% paraformaldehyde (PFA) for immunohistochemical analyses.

**Immunohistochemistry and periodic acid-Schiff staining on testicular tissue sections**

Processing of testicular tissue for histological evaluation and the protocol for immunohistochemistry were as previously described (Albert et al., 2012). Primary antibodies included fibroblast growth factor receptor three (FGFR3, sc-13121; Santa Cruz Biotechnology, Inc., Heidelberg, Germany; dilution 1:25), undifferentiated embryonic cell transcription factor 1 (UTFI, MAB4337; Millipore, Schwalbach, Germany; dilution 1:50), melanoma antigen family A4, (MAGE A4, provided by Prof. G. C. Spagnoli from the University Hospital of Basel Switzerland; dilution 1:20), boule-like (BOLL, sc.16660; Santa Cruz Biotechnology, Inc.; dilution 1:50), DEAD (Asp-Glu-Ala-Asp) box polypeptide 4, (DDX4, also VASA, ab13840; Abcam, Cambridge, UK; dilution 1:100), luteinizing hormone chorionic gonadotrophin receptor (LHCGR, NB1-04718; Novus Biologicals, Cambridge, UK; dilution 1:400), vimentin (VIM, sc-5565, Santa Cruz Biotechnology, Inc.; dilution 1:50) and smooth muscle actin (SMA, A2547; Sigma-Aldrich Biochemie GmbH, Hamburg, Germany; dilution 1:1000). Omission of primary antibodies and incubation with unspecific immunoglobulin G (IgG) served as negative controls. Following overnight incubation, the sections were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody solution (dilution 1:100). Finally, diaminobenzidine (Sigma-Aldrich) treatment was performed and sections were briefly counterstained with hematoxylin.

**RNA isolation and quantitative real-time PCR analysis of testicular tissue**

Ultraspec™ (Biotec Laboratories, Inc., Houston, TX, USA) and the DNA-free™ Kit (Life Technologies GmbH, Ambion) were used for RNA isolation and subsequent DNase digestion. cDNA was then generated with SuperScript II Reverse Transcriptase (Life Technologies GmbH, Invitrogen) using 2 μg of total RNA and random hexamer primers. For relative quantification analysis, we selected the following previously published germ cell marker genes: acrosomal vesicle protein (ACRV1; SP100), BOLL, DDX4, deleted in azoosperma-like (DAZL), developmental pluripotency associated 3 pseudogene 2 (DPPA3P2; STELLAR), FGFR3, GDFN family receptor alpha 1 (FGRA1), G protein-coupled receptor 125 (GPR125), integrin, alpha 6 (ITGA6), lactate dehydrogenase (LDHC), MAGE A4, proteamine 2 (PRM2), synaptoplasmic complex protein 3 (SYCP3), Thy-1 cell surface antigen (THY1), ubiquin carboxyterminal esterase L1 (UCHL1; PGP9.5), UTF1, v-kt Hardy-Zuckerma 4 fetal sarcoma viral oncogene homolog (KIT), zinc finger and BTB domain containing 16 (ZBTB16; PLZF). In addition, the three somatic cell marker genes were analyzed: actin, alpha 2, smooth muscle, aorta (ACTA2, the gene for SMA), LHCGR, VIM. Primer sequences, product sizes, the location of protein expression and the respective references are summarized in Supplementary data, Tables SI and SII. For the relative quantification of these marker genes quantitative real-time PCR (qPCR) analyses were performed using SYBR® Green technology. For primer design, primer optimization and the evaluation of primer specificity the SYBR® Green PCR protocol provided by the manufacturer was followed (Life Technologies GmbH, Applied Biosystems).

For qPCR analyses, cDNA was diluted 1:8 and 2 μl were used for each 20 μl PCR reaction with Power SYBR® Green Mastermix (Life Technologies GmbH, Applied Biosystems). The PCR programme consisted of one cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. qPCRs were run on the StepOnePlus™ and were subsequently analyzed using the StepOne™ software 2.2 (Life Technologies GmbH, Applied Biosystems). Results were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene and are shown as 2^{-ΔΔCt} values according to Schmittgen and Livak (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

**Statistical analyses**

Analyses were performed using GraphPad Prism® Version 5.0 (GraphPad Software, Inc., San Diego, USA). Statistical differences between the groups were evaluated using the non-parametric Kruskal – Wallis test, followed by a pairwise Mann–Whitney U-test to analyze inter-group differences. Significant differences between the groups are marked with *P < 0.05, **P < 0.01 and ***P < 0.001.

**Digestion of testicular tissue and cell culture**

Prior to the digestion of testicular biopsies, ~20% of each biopsy was snap frozen and served as tissue control for qPCR analyses. The remaining tissue was mechanically dissected and was incubated in MEMx containing 1 mg/ml collagenase IA (Sigma-Aldrich), at 37 °C for 30 min. Subsequently, the reaction was stopped by addition of MEMx with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (Pen/Strep) and the cells were pelleted at 438 g for 5 min. Then, the supernatant was removed and the tissue was incubated in 5 ml Hank's balanced salt solution containing 20 mg trypsin (Life Technologies GmbH, Gibco) and 11 mg of DNAse (Sigma-Aldrich) for 10 min at 37 °C. Single cells were obtained by strong pipetting and the reaction was stopped as outlined above. Cells were washed three times with MEMx and were resuspended in 2 ml prior to counting using trypan blue staining. A mean ± SD of 2.91 ± 1.206 million cells was obtained from testicular biopsies with qualitatively normal spermatogenesis and 2.87 ± 2.030 million cells from the three biopsies obtained from 47,XXY patients. Cells were plated at a density of <50.000 cells/cm² onto uncoated cell culture dishes. In preliminary experiments two conditions had been compared. For condition I: cells were cultured in MEMx with 10% FCS and 1% Pen/Strep and for condition II: in KnockOut™ DMEM (Life Technologies GmbH, Gibco) supplemented with 10% FCS, 1 mM l-glutamine, 0.1 mM non-essential amino acids, 2-Mercaptoethanol and 1% Pen/Strep. These experiments revealed a decreased expression of germ cell markers and an increased expression of somatic markers in the latter medium following 10 days of culture. Based on these results, cells were cultured under condition I at 35 °C and 5% CO₂ for subsequent experiments. In order to achieve an enrichment of the germ cell population, the cells from the supernatant were separated from attached cells 18 h after plating. Cell numbers were determined and cells from the supernatant were plated into separate culture plates. The medium was changed every third day until cells were collected for qPCR and for immunofluorescence analysis.

**Immunofluorescence staining of testicular cells in culture**

Cells were plated onto 8-well chamber slides (Becton Dickinson GmbH, Heidelberg, Germany) for immunofluorescence staining. As the attached cells reached confluency after 7 days on this untreated glass surface, cultures were stopped 3 days prior to the gene expression analysis to avoid the
overgrowth of testicular somatic cells. However, comparing the morphological characteristics of cells on chamber slides on Day 7 to those on cell culture plastic on Day 10, no apparent differences could be observed. For immunofluorescence staining, cells were fixed in 4% (w/v) PFA in phosphate-buffered saline for 15 min at room temperature. Primary antibody dilutions for UTF1, MAGE A4, DDX4, VIM and SMA as well as negative controls were as outlined above and the staining protocol was as previously published (Kossack et al., 2009). For co-staining, cells were incubated with the corresponding combinations of primary antibodies at 4°C overnight. Goat anti-mouse Alexa 488 (Life Technologies GmbH, Invitrogen; dilution 1:100) was employed for the detection of UTF1, MAGE A4 and SMA staining and goat anti-rabbit Alexa 546 (Life Technologies GmbH, Invitrogen; dilution 1:100) for the detection of DDX4 and VIM. Slides were mounted with Vectashield Mounting Media with 4’,6-diamidino-2-phenylindole as nuclear counterstain (Vector Laboratories, Inc., Burlingame, CA, USA).

RNA isolation and qPCR analysis of cell cultures

Cells were collected by trypsinization after 10 days of culture for RNA expression analysis. RNA isolation and DNase digestion were performed using the RNeasy Micro Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. cDNA was generated with SuperScript II Reverse Transcriptase (Life Technologies GmbH, Invitrogen) using 100 ng of total RNA and random hexamer primers. For qPCR analyses, cDNA was diluted 1:2 with Nuclease-Free Water (Qiagen), using 2 μl for each 20 μl PCR reaction with Power SYBR® Green Mastermix. The PCR programme, data analysis and statistical analyses were performed as outlined above.

Results

Identification of informative germ cell- and somatic cell-specific marker genes

In order to evaluate the specificity of published marker genes, testicular biopsies from patients with qualitatively normal spermatogenesis and from patients with an SCO and a 47,XXY syndrome were selected based on their distinct histological characteristics. Importantly, all patients were negative for atypical spermatogenesis such as testicular intraepithelial neoplasia (placental alkaline phosphatase stainings are not shown). In-depth evaluation of the qualitatively normal biopsies revealed that elongated spermatids were present in the majority of seminiferous tubules (scores 8–9) and that spermatozoa were found in TESE samples. Furthermore, normal gonadotrophin (FSH and LH) and testosterone levels characterized this patient group. The histological analysis of SCO and 47,XXY biopsies revealed that germ cells including elongated spermatids were absent from seminiferous tubules (score 0) and evaluation of TESE samples confirmed the absence of spermatids in all biopsies, with the exception of one Klinefelter patient who had sperm in one TESE sample. Apart from these similarities, these latter two patient groups differed with regard to the composition of somatic cell types. While 91.4 versus 27.6% of the tubules showed an SCO syndrome, 8.6 versus 72.4% presented as tubular shadows in SCO and 47,XXY patients, respectively. In addition to the decreased proportion of Sertoli cells in testicular biopsies from 47,XXY patients the testicular tissue also contained an increased number of Leydig cells. Regarding hormonal parameters, SCO and 47,XXY patients were hypergonadotropic with significantly increased FSH and LH levels. While testosterone levels were within the normal range in SCO patients, they were significantly reduced in the 47,XXY group compared with normal patients (Table 1).

Relative quantification of 18 previously published germ cell marker genes in testicular biopsies from selected patient groups revealed that the expression of only nine of them was significantly higher in normal biopsies compared with those obtained from SCO and 47,XXY patients, indicating that they reflect the presence of germ cells. These marker genes were UTF1, FGR3, MAGE A4, SYP3, BOLL, LDHC, SP10, PRM2 and DDX4 (Fig. 1A–I). Remaining marker genes were divided into the following three groups based on their expression pattern. The first group of genes (ITGA6, PLZF and THY1) either showed significantly higher expression levels only in SCO samples or in SCO as well as 47,XXY samples compared with normal testicular tissues (Supplementary data, Fig. S1A–C). The second group (KIT, GPR125 and UCHL1) revealed comparable expression levels in all three patient groups (Supplementary data, Fig. S1D–F). Finally, the expression levels of DAZL, GFRα1 and STELLAR were significantly increased in normal patients compared with either the SCO or the 47,XXY patient group (Supplementary data, Fig. S1G–I). As the testicular biopsies from SCO and 47,XXY patients were devoid of germ cells, our findings indicate that those genes summarized in Supplementary data, Fig. S1 are also expressed by testicular somatic cells, they were therefore not considered to be germ cell-specific and were not used in subsequent experiments. In addition, we found that the expression of somatic marker genes VIM, ACTA2 and the LHCG was...

**Table 1** Hormonal and histological parameters of those patient groups that were selected for expression analyses.

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<thead>
<tr>
<th>Patient groups</th>
<th>Hormonal parameters (normal range)</th>
<th>Histological parameters of tubules</th>
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<tr>
<td></td>
<td>FSH (1–7 UI)</td>
<td>LH (2–10 UI)</td>
</tr>
<tr>
<td>Normal (n = 9)</td>
<td>6.4 (± 7.7)</td>
<td>3.4 (± 1.7)</td>
</tr>
<tr>
<td>SCO (n = 7)</td>
<td>24.0 (± 11.1)</td>
<td>7.6 (± 3.1)</td>
</tr>
<tr>
<td>47,XXY (n = 9)</td>
<td>35.3 (± 14.6)</td>
<td>19.0 (± 5.9)</td>
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Normal hormone values were provided by Autodelfia, Perkin-Elmer, Freiburg, Germany (FSH, LH) or DRG Instruments, Marburg, Germany (T) and are in agreement with common standards defined by the European Association of Urology. To compare hormone levels among groups, the Kruskal–Wallis test was performed, followed by the Mann–Whitney test to analyze inter-group differences. Data are presented as mean ± SD. FSH, follicle stimulating hormone; LH, luteinizing hormone; LC#, relative Leydig cell number; % SCO, percentage of tubules with Sertoli cell only (SCO) syndrome; % TS, percentage of tubules with tubular shadows; score, refers to the Bergmann and Kliesch score (Bergmann and Kliesch, 2010); T, testosterone; + = regular number of Leydig cells; +++ = relative increase in the number of Leydig cells (Leydig cell hyperplasia).

Compared with hormone values in the normal patient group a, b, c, and d represent: *P < 0.05, **P < 0.01, ***P < 0.001 and compared with values of SCO patients ****P < 0.001.
significantly increased in SCO and 47,XXY patients compared with normal controls. While the highest expression of \textit{ACTA2} was detected in SCO samples, the highest \textit{LHCGR} expression was found in biopsies from 47,XXY patients (Fig. 1J–L).

**Evaluation of the stage-specific expression of germ cell-specific marker genes**

Aiming to evaluate whether the expression of germ cell-specific marker genes \textit{UTF1}, \textit{FGFR3}, \textit{MAGE A4} and \textit{DDX4} reflects the presence of certain germ cell stages, biopsies with a spermatogenic arrest at the level of spermatogonia (in 44.3\% of the seminiferous tubules), at the level of spermatocytes (in 96.3\% of the seminiferous tubules), with qualitatively intact spermatogenesis (score 6–8) and an SCO phenotype (in 99.3\% of seminiferous tubules) were selected (Supplementary data, Table SIII). We found that the expression of \textit{UTF1} and \textit{FGFR3} was highest in patients with a spermatogonial arrest (Fig. 2A and B), whereas patients with a spermatogonial and spermatocyte arrest showed an increased expression of \textit{MAGE A4} compared with biopsies with normal spermatogenesis or SCO syndrome (Fig. 2C). Finally, the expression of \textit{DDX4} was highest in biopsies with a spermatocyte arrest and normal spermatogenesis compared with the other patient groups (Fig. 2D).

\textbf{Figure 1} mRNA levels of germ cell- and somatic cell-specific marker genes in human testicular biopsies. White bars show the results for biopsies with qualitatively normal spermatogenesis (\(n = 9\)), black bars for biopsies with SCO syndrome (\(n = 7\)) and grey bars for biopsies obtained from Klinefelter syndrome (47,XXY) patients (\(n = 10\)). Data are shown as mean with SEM and significant differences between the groups are marked with *\(P < 0.05\); **\(P < 0.01\) and ***\(P < 0.001\).
Identification of germ cell- and somatic cell-specific markers using immunohistochemistry

Spermatogonia, located at the basement membrane of the seminiferous tubules stained positive for UTF1 (nuclear) and FGFR3 (cytoplasm and cell membrane) (Fig. 3A and B), which is in agreement with previous studies (Kristensen et al., 2008; von Kopylow et al., 2010). Also, the cytoplasm of spermatogonia and spermatocytes was immunopositive for MAGE A4 (Fig. 3C). While spermatogonia were strongly immunopositive, spermatocytes showed a weaker staining. In addition to that, spermatocytes stained positive for BOLL (Fig. 3D). Finally, the expression of DDX4 was detected in all germ cells, including spermatogonia, spermatocytes as well as round spermatids (Fig. 3E). These results for the latter three markers are also supported by previous studies performing immunohistochemistry (Castrillon et al., 2000; Aubry et al., 2001; Xu et al., 2001). As no positive cells were detected screening testicular sections from SCO patients (Supplementary data, Fig. S2A–E), these markers were subsequently regarded as germ cell specific. In contrast to that, the expression of the somatic markers VIM, SMA and LHCGR was detected in testicular sections from normal and SCO patients. While Sertoli-, peritubular- and interstitial cells were positive for VIM (Fig. 3F, and Supplementary data, Fig. S2F), SMA-stained peritubular- and -vascular cells (Fig. 3G, Supplementary data, Fig. S2G) and Leydig cells were immunopositive for the LHCGR (Fig. 3H, Supplementary data, Fig. S2H). The expression of these somatic markers has been described in previous studies, yielding comparable results (Rogatsch et al., 1996; Themmen and Huhtaniemi, 2000; Albrecht et al., 2006). No staining was detected in the corresponding IgG controls (Fig. 3I and Supplementary data, Fig. S2I, respectively).

Figure 2 Relative quantification of four germ cell-specific marker genes in testicular biopsies. Mean (± SEM) values for the germ cell-specific marker genes UTF1 (A), FGFR3 (B), MAGE A4 (C) and DDX4 (D) are shown. The tissue was obtained from patients with normal spermatogenesis (n = 4), spermatogonial arrest (SPG; n = 3), spermatocyte arrest (SPC; n = 3) and an SCO syndrome (SCO; n = 3).

Enrichment of germ cells using cell culture approaches

For cell culture experiments, biopsies were processed immediately and were therefore selected based on clinical diagnosis, including hormone levels. Patients who were categorized to be normal (n = 4) had FSH levels within the normal range (2.9 ± 0.6 U/l), elongated spermatids in the majority of seminiferous tubules (score 5–9) and sperm in all TESE samples (Supplementary data, Table SIV and Fig. 4A–D). In contrast, 47,XXY patients (n = 3) had greatly increased FSH levels (39.9 ± 29.7 U/l, Supplementary data, Table SIV) and germ cells were absent from the testicular tissue (Fig. 4E and F). Histological analysis further revealed that 40.7% of the tubules showed an SCO syndrome, whereas 59.3% showed tubular shadows (Supplementary data, Table SIV). Finally, no sperm were detected in TESE samples.

In order to enrich the germ cell population, the supernatant and the attached cell fraction was separated 18 h after the initial plating and were cultured separately. After 7 days of culture, the supernatant fraction derived from normal testicular tissues was enriched in putative germ cell clusters, which consisted of compact round cells of different sizes. Apart from these clusters, the supernatant fraction contained a few putative somatic cells, which were attached to the culture plate and had a spindle-shaped flat morphology (Fig. 5A). Cultures obtained from testicular tissues from 47,XXY patients, putative germ cell clusters were absent from the supernatant fraction (Supplementary data, Fig. S3A). The attached cell fraction, however, showed a similar morphology to that from patients with normal spermatogenesis (Supplementary data, Fig. S3E).
Characterization of testicular cell cultures using immunofluorescence analysis

Co-stainings showed that the round compact cells present in the supernatant were immunopositive for the spermatogonial marker UTF1, whereas spindle shaped cells were positive for the somatic marker VIM (Fig. 5B). A similar staining pattern was observed performing co-stainings for MAGE A4 and VIM (Fig. 5C). Interestingly, co-staining experiments showed that those germ cells, which are attached to the somatic cells or the culture plate, are strongly positive for MAGE A4, whereas the remaining cells in the cluster only stained weakly for MAGE A4 and were strongly positive for DDX4 (Fig. 5D). This staining pattern therefore indicates that the cell clusters do not consist of a homogenous germ cell population. It rather suggests that spermatogonia (strongly MAGE A4+) are attached to the culture plate or the somatic cells and that these spermatogonia are physically attached to differentiating germ cells, putatively spermatocytes (weakly MAGE A4+ and strongly positive for DDX4), that remain in the supernatant.

While the attached cell fraction contained cells positive for either SMA, VIM or both, cells negative for both markers could also be detected (Fig. 5F). Negative controls using corresponding IgG antibodies remained negative (a representative image is shown in Supplementary data, Fig S3D). In testicular cell cultures that were established using biopsies...
from 47,XXY patients, we could not detect cells positive for UTF1, MAGE A4 or DDX4 (Supplementary data, Fig. S3B and C) and attached cells were immunopositive for the somatic markers SMA, VIM or both (Supplementary data, Fig. S3E).

**Molecular characterization of germ cell enriched cultures by expression profiling**

To monitor the proportion of testicular cell types in culture, expression levels were compared between the initial testicular tissue, the supernatant and the attached cell fraction after 10 days of culture. In cell cultures established from patients with normal spermatogenesis, these analyses revealed similar expression levels of the germ cell-specific marker genes (UTF1, FGFR3, MAGE A4 and DDX4) in the initial tissues and the supernatant fraction. Furthermore, the expression of FGFR3, MAGE A4 and DDX4 was significantly higher in the supernatant compared with the attached cell fraction, indicating the enrichment of the germ cell population in the supernatant fraction (Fig. 6A–D). However, the expression of somatic marker genes VIM and ACTA2 showed no significant differences among the three cell fractions analyzed (Fig. 6E and F). Finally,
the expression of the LHCGR was low in initial tissues and could not be detected following 10 days of culture. These findings indicate that the Leydig cells constitute a small proportion of the testicular cell types in culture or lose their LHCGR expression in the course of the culture period (data not shown).

In contrast to the cell cultures established from biopsies with normal spermatogenesis, the expression of the germ cell-specific marker genes UTF1, FGFR3, MAGE A4 and DDX4 was barely detectable in the initial tissues and cell cultures established from 47,XXY patients (Fig. 6A–D).

None of those biopsies that were obtained for histological analysis or TESE contained germ cells or sperm, respectively. Despite this fact, it is possible that the tissue that was used for cell culture experiments contained small areas of active spermatogenesis, which would account for the very low expression levels of FGFR3. In contrast to the germ cell-specific marker genes, high expression levels of VIM and ACTA2 were detected in the tissue, the supernatant and the attached cell fraction (Fig. 6E and F), demonstrating that these cultures primarily consisted of somatic cells.

**Figure 5** Phase contrast micrographic images of testicular cell cultures obtained from a patient with qualitatively normal spermatogenesis after 7 days of culture. Representative bright field images of cells from the supernatant (SN, A) and the attached cell fraction (AT, E) are shown. Arrowheads indicate germ cell clusters. Microscopic images showing immunofluorescence stainings of cells obtained from the supernatant (B–D) and the attached cell fraction (F). Arrowheads indicate cells that are positive for germ cell markers, whereas arrows indicate those cells positive for the somatic marker VIM (B, C, F). In figure D, arrowheads indicate cells strongly positive for MAGE A4, arrows indicate unstained cells (putative somatic cells) and arrowheads indicate those cells positive for the general germ cell marker DDX4. In figure (F) unstained cells are indicated by arrowheads, whereas arrows and asterisks indicate cells positive for VIM and SMA or both. Incubation with corresponding immunoglobulin G antibodies was used as a negative control and a representative image is shown in Supplementary data, Fig. S3D. Scale bars represent 100 μm.
Discussion

The survival of human testicular tissue in an organ culture system was recently re-evaluated and it was shown that morphologically normal spermatogonia can be maintained in vitro for a period of 16 days (Roulet et al., 2006). Apart from that, a number of studies have reported the culture of human spermatogonia, including SSCs, in a two-dimensional culture system (Conrad et al., 2008; Golestaneh et al., 2009; Kossack et al., 2009; Sadri-Ardekani et al., 2009, 2011). However, conflicting results gave rise to a debate regarding the cellular origin of the alleged SSCs (Ko et al., 2010; Chikhovskaya et al., 2012). In this study, we therefore aimed to establish a combined approach for the reliable detection of human germ cells in vitro.

We found that gonadotrophin levels, which are often available before the biopsy is taken, provided important information for the selection of testicular biopsies suitable for the establishment of human germ cell cultures. Abnormally high FSH levels were indicative of severely disturbed spermatogenesis, an observation that was corroborated by histological findings and by previous publications reporting that high FSH levels are often associated with impaired spermatogenesis (Bergmann et al., 1994; Chen et al., 2010). However, results from the histological analysis and TESE outcome were found to be indispensable as they provide

Figure 6  Expression of germ cell- and somatic cell-specific marker genes in testicular cell cultures. Quantitative real-time PCR results are shown for the germ cell marker genes UTF1 (A), FGFR3 (B), MAGE A4 (C) and DDX4 (D), as well as for the somatic marker genes VIM (E) and ACTA2 (F) in the initial testicular tissues (light grey bars), the supernatant (SN, dark grey bars) and the attached (AT, white bars) cell fraction after 10 days of culture. While the three columns on the left represent the data obtained from testicular biopsies with normal spermatogenesis (n = 4), the three columns on the right represent data obtained from patients with 47,XXY syndrome (n = 3, hatched bars). Data are shown as mean with SEM and statistical differences are marked with *P < 0.05.
The highest expression levels of samples containing spermatogonia as the only germ cell type showed the markers of human spermatogonia. In agreement with this, we found that in their conclusion that the expression of these genes reflects the presence of human spermatogonia (Kopylow et al., 2010). Evaluating significant differences in resulting gene expression levels, they found that UTF1 and FGFR3 are specific biomarkers of human spermatogonia. In agreement with this, we found that samples containing spermatogonia as the only germ cell type showed the highest expression levels of UTF1 and FGFR3 supporting previous studies in their conclusion that the expression of these genes reflects the presence of human spermatogonia (Kristensen et al., 2008; von Kopylow et al., 2010). In addition, our analyses revealed that MAGE A4 reflects the presence of spermatogonia and spermatocytes and that DDX4 can be considered a general germ cell marker, which is also corroborated by previous findings (Aubry et al., 2001; Medrano et al., 2010). The specificity of selected marker genes at the protein level was subsequently demonstrated performing immunohistochemistry. As the expression of UTF1, FGFR3, MAGE A4, BOLL and DDX4 could only be detected in testicular tissues with normal spermatogenesis but not in those with an SCO phenotype, they were considered to be germ cell specific. Moreover, the localization of these markers within the germ cells was in accordance with previous publications (Castirion et al., 2000; Aubry et al., 2001; Luetjens et al., 2004; Kostova et al., 2007; von Kopylow et al., 2010; Van Saen et al., 2012a,b; von Kopylow et al., 2012).

In contrast to the germ cell specific marker genes, we found that the expression of nine marker genes could also be detected in biopsies lacking the germ cell population. This finding was initially unexpected as markers such as ITGA6, UCLH1, KIT, PLZF and DAZL are widely used to identify human spermatogonia in culture (Conrad et al., 2008; Sadri-Ardekani et al., 2009, 2011). For the first three marker genes our RNA expression results are supported by published protein data as it was shown that ITGA6 is expressed by all cells located at the basal membrane of the seminiferous tubules, including the somatic Sertoli- and fibroblast cells (Conrad et al., 2008; Tapia et al., 2011). Furthermore, it has been demonstrated that spermatogonia but also somatic Leydig cells and nerve fibers are immunopositive for UCLH1 (von Kopylow et al., 2010) and that the expression of KIT can be detected in germ cells as well as in cells located in the interstitial space of the testis (Umini et al., 2009; Medrano et al., 2010). In contrast to these three marker genes, the expression of PLZF and DAZL is restricted to the germ cell population at the protein level. While PLZF is expressed in spermatogonia (He et al., 2010), DAZL can be detected in spermatogonia, spermatocytes as well as round and elongating spermatids of the human testis (Reijo et al., 2000). One possible reason for these inconsistent findings regarding our RNA (Supplementary data, Fig. S1) and published protein data is an apparent discrepancy between transcript and protein levels, which has been reported for a substantial number of markers (Ghazalpour et al., 2011). As the same testicular tissues were used to evaluate the specificity of all marker genes included into this manuscript (Fig. I, and Supplementary data, Fig. S1) it is, however, unlikely that small areas of germ cells within the SCO samples are responsible for these results. Our findings therefore highlight the necessity to validate the germ cell specificity of marker genes in order to avoid the misinterpretation of expression data regarding the presence of germ cells in testicular cell cultures, a finding which is consistent with data from a recent non-human primate study (Eldermann et al., 2012). It is of importance to note though, that there are species-specific differences regarding the SSC system. As our study was limited to the analysis of human testicular tissues, it is possible that marker genes that do not reflect the presence of spermatogonia in the human facilitate the reliable detection of spermatogonia in other animal models.

Aiming to exclude further sources of misinterpretation, we also strove to identify marker genes that reflect the presence of testicular somatic cell types. As the proportion of RNA obtained from somatic cells is higher in testicular tissue lacking the germ cell population compared with normal controls, the significantly increased expression levels of the somatic marker genes VIM, ACTA2 and LHCGR in SCO and 47,XXY patient biopsies was in accordance with the histological findings. Furthermore, performing immunohistochemical analyses, the expression of SMA, VIM and the LHCGR was detected in the somatic cells of normal and SCO samples and their localization was in line with previous studies (Rogatsch et al., 1996; Themmen and Huhtaniemi, 2000; Albrecht et al., 2006). Based on these results we are convinced that qPCR analysis for validated candidate genes in combination with immunocytochemistry provides reliable information with regard to the presence of germ cells, including spermatogonia and of testicular somatic cells in culture. As relatively few cells are needed to perform qPCR analyses, it is the method of choice that can be employed to regularly monitor the composition of testicular cell types in vitro.

An approach for the enrichment of human germ cells was first established in the 1970s and was based on the separation of the germ cell-enriched supernatant fraction from the attached somatic cells (Steinberger, 1975). This strategy has been successfully used since for the enrichment of murine as well as human spermatogonia (Kanatsu-Shinohara et al., 2003; Sadri-Ardekani et al., 2009). Using our validated marker panel and biopsies lacking the germ cell population as negative controls, we were able to demonstrate in this study that germ cells can indeed be enriched via the separation of the supernatant from the attached cell fraction and that characteristic germ cell clusters, including spermatogonia, can be maintained in culture for at least 10 days. The germ cell origin of cultured cells was verified performing qPCR analysis, which revealed increased expression levels of UTF1, FGFR3, MAGE A4 and DDX4 in the germ cell-enriched supernatant fraction. Furthermore, these results were supported by immunofluorescence analyses, showing that compact round cells of the cell clusters are spermatogonia as they are positive for UTF1 and MAGE A4. In contrast to other publications (Conrad et al., 2008; He et al., 2010), however, we found that the germ cell clusters did not consist of a homogeneous cell population. Co-stainings for MAGE A4 and DDX4 rather revealed that those single cells that are in close contact with the somatic cells or the culture plastic are the least differentiated, whereas the cells in the supernatant appeared to consist mostly of differentiating germ cells. Most importantly, our analyses showed that testicular somatic cells were still present in the germ cell-enriched fraction as was demonstrated by the expression of somatic marker genes (VIM and ACTA2). This finding is in agreement with a previous publication reporting that repeated differential passaging was necessary in order to separate somatic cells from the germ cell fraction (Sadri-Ardekani et al., 2009) and highlights the
importance of regularly monitoring germ cell cultures for the presence of somatic cell types. Based on our data, the enrichment and short-term culture of human germ cells, including spermatagonia, is feasible. Consequently, these cells may have been present in initial studies reporting the culture of human SSCs (Conrad et al., 2008; Golestaneh et al., 2009; Kossack et al., 2009). However, as the proportion of testicular somatic cells was not monitored over the course of the culture period, the possibility that other somatic cell types were present and eventually outgrew the germ cells cannot be excluded. This would also explain why the overall expression profile after a number of passages rather resembled that of fibroblast or mesenchymal stem cells (Ko et al., 2010; Chikhovskaya et al., 2012). The strengths of this study is therefore that it provides tools for the reliable identification of spermatagonia and testicular somatic cells in culture and thereby provides the basis for the establishment of true SSC cultures and the evaluation of their differentiation potential in future studies.

In conclusion, to avoid conflicting data regarding human SSC cultures in the future, we suggest a combined approach. The selection of suitable testicular biopsies should be based on clinical information including hormone values and detailed histological information. Apart from this, validated markers should be employed for the detection of germ cells and somatic cells in testicular cell cultures. Finally, as it is highly unlikely to establish germ cell cultures from biopsies with a true SCO syndrome, those biopsies constitute a valuable negative control for human SSC culture experiments.

**Supplementary data**

Supplementary data are available at http://humrep.oxfordjournals.org/.

**Acknowledgements**

We thank Daniela Hanke, Jolanta Körber, Raphaele Kürten, Heidi Kersebom, Jutta Salzig, Monika Tümler and Benedikte Twardy for technical support and we thank Prof. Giulio C. Spagnoli, University Hospital of Basel Switzerland for providing the monoclonal αMAGE A4 antibody 57B.

**Authors’ roles**

N.K. was involved in conception and design, acquisition of data, analysis and interpretation of data, drafting the article, final approval of the manuscript. N.T. was involved in acquisition of data, analysis and interpretation of data, critical revision of the manuscript, final approval of the manuscript. J.W., J.E., S.S. and H.S. were involved in analysis and interpretation of data, critical revision of the manuscript, final approval of the manuscript. S.K. was involved in provision of study material and clinical data of patients, analysis and interpretation of data, critical revision of the manuscript, final approval of the manuscript. J.G. was involved in conception and design, analysis and interpretation of data, critical revision of the manuscript, final approval of the manuscript.

**Funding**

This project was supported by the Stem Cell Network North Rhine-Westphalia and the Innovative Medical Research of the University of Münster Medical School (Grant KO111014). In addition, it was funded by the DFG-Research Unit FOR 1041. Germ Cell Potential (GR 1547/11-1 and SCHL 394/11-2), the BMBF (01GN0809/10) and the IZKF (CRA 03/09).

**Conflict of interest**

The authors indicate no potential conflicts of interest.

**References**


