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ALTERED GENE EXPRESSION SIGNATURE OF EARLY STAGES OF THE GERM LINE SUPPORTS THE PREMEIOTIC ORIGIN OF HUMAN SPERMATOGENIC FAILURE

Running title: Early germ line gene expression in impaired sperm production

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ABSTRACT (300 words)

The molecular basis of spermatogenic failure (SpF) is still largely unknown. Accumulating evidence suggests that a series of specific events such as meiosis, are determined at the early stage of spermatogenesis. The present study aims to assess the expression profile of pre-meiotic genes of infertile testicular biopsies that might help to define the molecular phenotype associated with human deficiency of sperm production. An accurate quantification of testicular mRNA levels of genes expressed in spermatogonia was carried out by RT-qPCR in individuals showing SpF due to germ cell maturation defects, Sertoli cell-only syndrome (SCO) or conserved spermatogenesis (CS). Additionally, the gene expression profile of SpF was compared with that of testicular tumour (GCT), which is considered to be a severe developmental disease of germ cell differentiation. Protein expression from selected genes was evaluated by immunohistochemistry.

Our results indicate that SpF is accompanied by differences in expression of certain genes associated with spermatogonia in the absence of any apparent morphological and/or numerical change of this specific cell type. In SpF testicular samples, we observed down-regulation of genes involved in cell cycle *(CCNE1* and *POLD1)*, transcription and post-transcription regulation (*DAZL, RBM15* and *DICER1*), protein degradation (*FBXO32* and *TM9SF2*) and homologous recombination in meiosis (*MRE11A* and *RAD50*) which suggests that the expression of these genes is critical for a proper germ cell development. Interestingly, a decrease in the *CCNE1*, *DAZL*, *RBM15* and *STRA8* cellular transcript levels was also observed, suggesting that the gene expression capacity of spermatogonia is altered in SpF contributing to an unsuccessful sperm production. Altogether, these data point to the spermatogenic derangement being already determined at, or arising in, the initial stages of the germ line.

Key words: gene expression, testis, early stages of germ line, spermatogenic failure, male infertility

INTRODUCTION

Spermatogenesis is a highly orchestrated developmental process by which spermatogonia develop into mature spermatozoa. During the course of spermatogenesis the three major forms of cell cycle are represented: mitosis of primitive spermatogonia; two rounds of meiosis, from primary spermatocytes to haploid round spermatids; and differentiation including structural and nuclear changes to generate mature spermatids and spermatozoa. These processes are unique in male germ cell differentiation and depend on precise, developmental stage- and germ cell type- specific gene expression. However, the regulatory network that confers specific germ line gene expression in mammals is not properly understood, especially at the mitotic and meiotic stages. Understanding the regulatory step is essential for determining the molecular requirements for the progression of spermatogenesis, and thus for understanding male infertility which is often based on lack of replication of spermatogonia or meiotic blockade.

Accumulating evidence suggests that a series of specific events during spermatogenesis, such as meiosis and morphological changes, are determined at the early stage of spermatogenesis. Spermatogonia, and specifically the type B spermatogonia, should be an important preparation stage for meiosis. These data are supported by the description of activation or up-regulation of many genes during this specific germ cell stage (Guo *et al.*, 2004) and the generation of recombinant mouse models of spermatogonia-expressed genes exhibiting severe defects in meiosis (Wang *et al.*, 2001). Many of these genes codify germ cell specific proteins involved in transcriptional or post-transcriptional regulation of gene expression (Wang *et al.*, 2001).

Furthermore, cellular interactions between germ line and somatic components of the testicular seminiferous tubule, where spermatogenesis takes place, are essential to

achieve germ cell development, and thus for maintaining male fertility. The relevance of these cellular interactions is supported by physiological events (for review, see (Mclachlan *et al.*, 2007).

Some studies have used microarray technology to characterize the transcriptional profile in germ and somatic cells at different steps of testicular development (Chalmel *et al.*, 2007; Diederichs *et al.*, 2005; Namekawa *et al.*, 2006; Pang *et al.*, 2003; Schlecht *et al.*, 2004; Schultz *et al.*, 2003; Sha *et al.*, 2002; Shima *et al.*, 2004). We have used information from cDNA microarrays and mouse models to focus mainly on genes specifically or preferentially expressed in immature germ cells in mammals, to determine whether early gene expression changes are associated with subsequent spermatogenic disorders and thus, male infertility. Additionally, the gene expression profile of germ cell maturation failure was compared with that seen in testicular tumour, which is considered to be a developmental disease of germ cell differentiation, in order to give additional clues about the functional pathways involved in spermatogenic derangement.

MATERIALS AND METHODS

Subjects of study

Our study recruited nineteen patients (range 30-49 yr) infertile due to spermatogenic failure (SpF) at different germ cell stages, with a phenotype consistent with non-obstructive azoospermia or severe oligozoospermia (<5 million sperm per mL). In addition, fourteen patients with Sertoli cell-only syndrome (SCO) phenotype or germ cell aplasia, were studied as controls of somatic cells (negative controls), and seventeen infertile patients (range 32-50 yr), who were diagnosed with obstructive azoospermia (as a consequence of congenital absence of vas deferens or previous vasectomy) and showed conserved spermatogenesis (CS) were studied as gene expression controls of a complete spermatogenic process (positive controls). Patients were selected on the basis of the testicular histological pattern of >20 tubules from the same testicular section; only testicular samples with a homogeneous histological pattern were included in the study.

An extra group of individuals (n=5) diagnosed with germ cell tumour (GCT) (range 29-46 yr) were analyzed. Two GCT samples were histologically classified as carcinoma *in situ* (CIS) whereas the remaining three samples were classified as non-CIS or GCT of advanced stages: one as classic seminoma, one as embryonal carcinoma and one as mixed germ cell tumour (80% embryonal carcinoma; 20% classic seminoma).

Infertile individuals were selected from men referred for couple infertility to the Andrology Service of the Fundació Puigvert, whereas GCT samples were recruited from the Andrology Service of the Fundació Puigvert and the Urology Service of the Hospital Universitari de Bellvitge. The study was approved by the Institutional Review Board of both Centres, and all the participants signed a written informed consent.

The clinical procedures for infertile patients included medical history, physical examination, semen analyses [performed in accordance with World Health Organization guidelines (World Health Organization, 1999)] and hormonal study. The routine genetic study for all non-obstructive samples included karyotype and analysis of chromosome Y microdeletions, the latter performed according to the European guidelines (Simoni *et al.*, 1999; Simoni *et al.*, 2004). Men with a chromosomal aberration or a Y-chromosome microdeletion were not included in the study.

Testicular samples

Testicular biopsies from infertile men were obtained when necessary to confirm the clinical diagnosis and for sperm retrieval (TESE) and cryopreservation purposes. Each specimen was divided into three aliquots, one piece (≈10-20 mg) was fixed in Bouin's solution and reserved for histological analysis, a second aliquot (≈100-200 mg) processed for sperm extraction and the third (≈10 mg) was immediately transferred to liquid nitrogen and stored at –80ºC until analysis for gene expression experiments.

Referring to GCT, testicular samples were obtained directly after orchidectomy and macroscopic pathological evaluation. For gene expression studies, one tissue fragment was taken from the tumour portion of the testis and was immediately frozen at –80ºC.

Histological analysis

An assessment of spermatogenic status and the severity of the alteration were performed after hematoxylin-eosin staining of paraffin samples from infertile patients (5-µm sections) by quantification of specific germ cells (spermatogonia, spermatocytes I, round spermatids and elongated spermatids) and Sertoli cells. The average number per tubule

was calculated after analysis of at least 15-20 cross-sectioned tubules per testis. A modified Johnsen score (JS) count (Schulze *et al.*, 1999) was calculated on the basis of the number of different cell types per tubule and infertile samples were classified as CS, SpF-HS (hypospermatogenesis), SpF-MA (meiotic arrest) and SCO (Table 1).

RNA extraction and cDNA synthesis

Total RNA was obtained from the testicular biopsy using Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA), following the manufacturer's instructions. The quality of RNA [28S/18S ratio and RNA Integrity Number (RIN)] was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Testicular RNA from the five groups of study (SCO, SpF-MA, SpF-HS, GCT and CS) showed similar quality values: both 28S/18S ratio and RIN presented no significant differences among them ($p = 0.056$ and $p = 0.072$, respectively). Mean 28S/18S ratio value between all samples (mean±SD) was 1.17±0.18 and mean RIN value was 7.77±0.62.

Single-stranded cDNA was obtained by reverse transcription (RT) of 500 ng of RNA, using random primers and the High Capacity cDNA Reverse Transcription Kit (AB, Foster City, CA). The resulting cDNA solution was aliquoted and stored at –20ºC until use.

Gene expression quantification and statistical evaluation

Quantitative real-time PCR (qPCR) assays were performed by means of the application of the PCR arrays on micro fluidic cards (MFC), using 384-well TaqMan® Low Density Arrays (TLDAs) on an Applied Biosystems 7900HT Fast Real-Time PCR System (AB, Foster City, CA). The 48-gene format MFC (47 experimental assays and 1 TLDA amplification control, *18S*) allowed simultaneous measurement of 34 target

genes that were selected based on a preferential expression in spermatogonia among germ cells (n=26) and/or in Sertoli cells (n=8) -information obtained from cDNA microarrays and mouse models bibliography data- (Supplemental Table 1), three marker genes of the presence and/or function of spermatid (*PRM1*), Leydig (*INSL3*) and myoid cells (*S100A6*) and 10 potential reference genes. Genes and the corresponding assays on demand used for the setup of the TLDA are listed in Supplemental Table 2. Selected target genes are involved in different functional pathways (Supplemental Table 1 and Supplemental Table 2).

Patient and control samples were always analysed as paired samples in the same analytical run in order to exclude between-run variations. Additionally, a calibrator sample was included in all the plates to compare the change in expression of a nucleic acid sequence against the expression in all samples in the same study. Real-time PCR data (Ct values) were pre-processed and stored in SDS 2.2 software (AB, Foster City, CA).

Expression stability of the candidate reference gene/s was calculated with the GeNorm software (Vandesompele *et al.*, 2002), in order to select the most stable reference genes and improve normalization of target genes. GeNorm software calculates the gene expression stability value M of multiple candidate genes as the average pair-wise variation of a particular gene compared with all other candidate reference genes. Lower M values indicate genes with less expression variation among samples. Therefore, target gene expression was calculated relative to the expression of *PGK1* and *PGM1* reference genes for SpF and control samples, whereas *PGK1* and *PPIA* combination was selected as the most appropriate for GCT and controls. They showed no statistical differences in absolute expression levels between groups (Kruskal-Wallis

test) (Supplemental Figure 1) and low M-value (GeNorm software indicating stable expression among samples.

Thus, raw data (Ct values) were normalised to the two reference genes and relative quantification (RQ) values were calculated using the qBase program (Hellemans *et al.*, 2007). and the $2^{-\Delta\Delta Ct}$ strategy. The Mann-Whitney U test was used to evaluate differences in relative expression of target genes in each patient group or subgroup compared with controls. Multiple test adjustment was applied by using Bonferroni correction.

Pearson product-moment correlation coefficients were calculated to determine the correlation between the expression ratios of the target genes and the various histological parameters in patient groups and controls.

All statistical analyses were performed using the SPSS software version 12 (Lead Technologies, Chicago, USA)

Immunohistochemistry

Tissue sections were prepared from Bouin-fixed, paraffin-embedded fragments of testicular biopsies. For this study, the following commercially available polyclonal rabbit antibodies were used: DAZL, HPA019777, Sigma-Aldrich, Inc; CCNE1 (C-19): sc-198, Santa Cruz Biotechnology, Inc; CDKN1C (C-20):sc-1040, Santa Cruz Biotechnology, Inc; DLK1 (H-118): sc-25437, Santa Cruz Biotechnology, Inc.

Immunohistochemistry was performed using the Dako EnVision+ kit (DAKO, Hamburg, Germany) in conjunction with the Dako autostainer, according to the instructions provided by the manufacturer.

Endogenous peroxidase was quenched by incubation in 0.5% hydrogen peroxide. Dilutions of primary antibodies were adjusted at 1:300 to 1:100 to optimize

the results. Secondary goat anti-rabbit antiserum was coupled to a labelled polymer-HRP, and staining was done with DAB and haematoxylin-eosin. Incubation with non immune serum was used as a negative control (data not shown). Stained sections were evaluated in bright-field microscopy (Axioskop 40, Zeiss, Göttingen, Germany) and images captured with a Nikon Coolpix 5400 digital camera. The immunoexpression of proteins was determined on tissue samples from at least six different individuals showing each of the spermatogenic phenotypes.

RESULTS

Quantitative determination of spermatogenic status in defective spermatogenesis

Paraffin-embedded testicular specimens from infertile patients and controls were available for histological quantification. Considering the heterogeneity of human testicular pathologies, we needed a detailed definition of the spermatogenic status of each sample in order to acquire high quality data related to germ cell specific transcriptional changes. To this end, the average number of Sertoli cells and specific germ cell types per tubule were determined in haematoxylin and eosin stained testicular sections and additionally the JS value was calculated (Table 1).

Using this strategy we confirmed the diagnosis of SCO (JS score 1-2) and CS (JS score 9-10) phenotypes. With respect to SpF patients, seven of them presented maturation arrest at primary spermatocyte level (SpF-MA) (JS score 4-6) and twelve presented hypospermatogenesis (SpF-HS) (JS score 7-8) (Table 1).

Round and elongated spermatids were absent in 4 out of 7 SpF-MA samples whereas the other three had very low values: round spermatids ranged between 2-4 per tubule and elongated spermatids ranged between 1-2 per tubule suggesting a 80-90% of meiotic arrest. This result confirms that only histological phenotypes with a defined and highly homogeneous pattern of individual tubules were included in the study.

The number of Sertoli cells showed a near two-fold increase in the group SCO (21.03 ± 7.00) compared with the CS samples (13.69 ± 3.12). Interestingly, the number of spermatogonia and Sertoli cells showed no significant differences ($p = 0.318$ and $p = 0.447$ respectively) among SpF-MA, SpF-HS and CS groups (Table 1).

Gene expression related to impaired spermatogenesis

PRM1 expression confirms the histological phenotype

In order to confirm the histological quantification of samples, we first analyzed the expression of *PRM1,* the marker gene for the presence of spermatids. As expected, negligible transcript level values were found in SCO and 100% MA samples; very low values were determined in incomplete MA samples and decreased levels in HS samples when compared with CS controls, showing a fold decrease in expression of $2.24x10^4$ (SCO), 42.59 (SpF-MA) and 4.45 (SpF-HS) ($p<0.001$). The absence of quantifiable *PRM1* expression values in GCT samples confirmed the absence of mature germ cells.

Our *PRM1* gene expression results consistently agreed with the histological diagnosis of all samples included in the study.

Expression behaviour of Leydig and myoid cells in spermatogenic disorders

Referring to the expression of the marker genes for the presence and/or function of Leydig (*INSL3*) and myoid cells (*S100A6*), no statistically significant differences were found between SpF-HS and CS groups (p=0.245 and p=0.059 respectively) and between SpF-MA and CS samples (p=0.065 and p=0.075 respectively), however these genes were significantly over-expressed in SCO group when compared with CS samples (p=0.001 and p=0.000 respectively), probably attributable to the absence of germ cells; since total testis samples are analysed, the loss of germ cells enrich the relative contribution of the remaining somatic cells in SCO phenotype.

GCT samples also showed no statistically significant differences in *INSL3* and *S100A6* expression when compared with the CS control group (p=0.401 and p=0.542 respectively)

Most of the spermatogonia-transcriptionally associated genes analyzed show a preferential germ line expression

Spermatogonia-associated genes included in the study have been previously described as having a preferential gene expression in the early germ line stages among germ cells (Supplemental Table 1). The assessment of expression of these genes in the SCO phenotype would provide important new information about whether these genes can be expressed also in somatic cells of the testis.

Selective or preferential germ cell expression was confirmed for 21 out of the 26 spermatogonia-related genes of our study. First, the negligible transcript level values found for *CCNE1*, *DAZL, RBM15* and *STRA8* in complete SCO samples supported their selective germ cell lineage gene expression. Furthermore, statistically significant reduced transcript values in SCO compared with CS suggested a preferential germ cell expression: *ATM*, *BARD1*, *CCND1*, *CCNF*, *DICER1*, *E2F3*, *FBXO32*, *c-KIT*, *MRE11A*, *POLA1*, *POLD1*, *RAD50*, *SIRT6* and *TM9SF2* presented a very significant fold-change decrease in SCO ranging from 1.46 to 6.10 ($p<10^{-4} – 10^{-6}$) whereas *BAX*, *CDKN1C* and *XPA* showed less marked decrease, with a fold-change ranging from 1.22 to 1.55 ($p<0.05$) (Supplemental Table 3).

However, for the five remaining spermatogonia-associated genes studied, a preferential expression in the germ cell lineage could not be demonstrated. *CDKN1A* and *DLK1* were found significantly over-expressed in SCO samples when compared with CS controls ($p=0.003$ and $p<0.002$ respectively), showing a fold-change increase of 1.77 and 3.99 respectively. Also, *BMPR1A, SMAD3* and *VEGFA* were not differentially expressed between SCO and CS samples ($p>0.05$) (Supplemental Table 3). Taken together, these results indicate a substantial expression of these five genes in testicular somatic cells.

Differential gene expression in defective and conserved spermatogenesis

We then looked for differences in target gene expression between SpF samples and CS controls.

For the 21 spermatogonia-associated genes preferentially expressed in the germ line, we identified 13 differentially expressed genes and grouped them into three gene clusters by their expression behaviour (Figure 1). In cluster I, both SpF-MA and SpF-HS phenotypes were associated with notable significant decreases in transcript levels of *BARD1, CCNE1*, *DAZL*, *FBXO32*, *RBM15* and *TM9SF2* genes ($p<0.002$), the reduction of expression being more pronounced in the SpF-MA phenotype. Cluster II contained genes significantly decreased in the SpF-HS ($p<0.05$) but not in the SpF-MA phenotype (*CCNF* and *E2F3).* Cluster III included all those genes differentially expressed in SpF-MA but not in SpF-HS phenotype: *DICER1*, *MRE11A, POLD1* and *RAD50* expression levels were decreased whereas *CCND1* was increased in the SpF-MA ($p<0.05$) when compared with controls. *CDKN1C* presented a similar pattern of gene expression deregulation to *CCND1,* although the difference in expression was not statistically significant (CS:0.51±0.15; HS: 0.61±0.54p=0.394; MA: 1.04±0.73 p=0.172) (Supplemental Table 3).

Interestingly, significant positive correlation coefficients (Pearson's correlation $r\geq0.6$; $p<0.0001$) were found between the number of elongated spermatids and the transcription levels of genes from cluster I (Supplemental Table 4.A), suggesting that these changes in gene expression could be of physiological relevance.

In regard to the spermatogonia and somatic cell-expressed genes, *CDKN1A* was significantly increased in the SpF-MA and SCO samples ($p<0.05$). *DLK1* was found highly over-expressed in SpF samples, although the differences were only statistically significant for the SpF-HS and SCO (p=0.001) and not the SpF-MA phenotype (probably due to a high standard deviation value) when compared with controls (Figure

2; Supplemental Table 3).When considering the Sertoli cell-preferentially expressed genes, *SCIN* and *SLC4A11* were found very significantly decreased in MA and HS samples when compared with CS. SCO samples, although presenting an increased number of Sertoli cells, showed reduced levels of *SPAG7, SCIN* and *SLC4A11* compared with CS controls ($p<0.006$) (Figure 2; Supplemental Table 3). The expression of *FASLG* mRNA in SpF samples was increased compared with CS, although no statistically significant changes in the average of *FASLG* expression were observed among groups due to high standard deviation values in the infertile groups (CS control: 1.10±0.71; HS: 1.79±1.19; MA: 3.44±2.87).

Reduced cellular expression levels of germ cell–specific genes in SpF

We additionally analyzed the transcript levels per cell of spermatogonia-associated genes with a selective germ cell expression, in SpF subgroups compared to CS controls in order to exclude the differences in gene expression due to changes in testicular cellularity and to determine whether transcript level per cell is also altered in SpF. Selective germ cell expression of *CCNE1*, *DAZL*, *RBM15* and *STRA8* was previously confirmed as negligible transcript level values were found in SCO samples as previously described, furthermore, *DAZL* and *STRA8* were previously described to be expressed in spermatogonia but not in somatic tissues (Wang *et al.*, 2001). Values of transcript amount per cell, in arbitrary units, were obtained for each testicular sample by dividing the *CCNE1*, *DAZL*, *RBM15* and *STRA8* expression values by either the proportion of the spermatogonia (Figure 3e, f, g and h), as it is the germ cell stage that predominantly expresses *CCNE1*, *DAZL*, *RBM15* and *STRA8* or by the proportion of the spermatogonia plus spermatocytes (Figure 3i, j, k and l), as the meiotic germ cells potentially express these genes although at much lower levels (GermSAGE,

http://germsage.nichd.nih.gov; GermOnline http://www.germonline.org), present in a seminiferous tubule of the sample. Significant differences in cellular transcript levels were additionally found for *CCNE1*, *DAZL*, *RBM15* genes between SpF-HS patients and controls and between SpF-MA patients and controls, when considering either the proportion of spermatogonia or the proportion of spermatogonia and spermatocytes in the tubule ($p \leq 0.002$). Interestingly, cellular transcript levels for *STRA8* were found statistically decreased in SpF-MA when compared with CS when considering either the proportion of spermatogonia or the proportion of spermatogonia and spermatocytes in the tubule ($p \leq 0.002$). These results suggest that the decreased tissular expression levels in SpF are not attributable to a decreased number of spermatogonia or spermatocytes in the tubule but to a reduced number of transcripts in immature germ cells. Furthermore, the decreased cellular *STRA8* expression levels observed in SpF-MA suggest that the number of genes whose expression is altered in immature germ cells might be higher than that initially observed in the whole tissue with meiotic arrest.

Strikingly, the decrease cellular expression observed in patients was accompanied by a higher severity in spermatogenic impairment, and cellular expression levels of *CCNE1*, *DAZL*, *RBM15* and *STRA8* genes were highly significantly and positively correlated with the number of elongated spermatids in the tubule (Pearson's correlation range; r=0.68-0.87; $p<0.0001$) (Supplemental Table 4.B.)

Gene expression pattern in Germ cell tumours

Relative expression values of fourteen spermatogonia preferentially- expressed genes were found to have extremely significant differences in expression between GCT and CS samples: *ATM*, *BARD1*, *CCNE1*, *CDKN1C*, *DAZL*, *DICER1*, *E2F3*, *FBXO32*, *MRE11A*, *RAD50*, *RBM15*, *SIRT6*, *STRA8* and *TM9SF2* ($p<0.002$). Less consistent

statistical differences were found for four additional genes *BAX, CCND1, POLD1* and *XPA* ($p<0.05$). All these differentially expressed genes, with the exception of *BAX*, were found to be under-represented in GCT samples compared with controls (Table 2; Supplemental Table 3).

Regarding the somatically expressed genes; ten genes were found to have statistical differences in expression: *AMHR2*, *BCL3, SCIN*, *SMARCA1*, *SOX9, SPAG7, VEGFA* ($p<0.002$) *BMPR1A, CDKN1A* and *FASLG* ($p<0.05$). *FASLG* was found to be over-expressed, whereas the other nine genes were under-expressed, in GCT samples compared with controls (Table 2; Supplemental Table 3).

Some differences in expression behaviour were found when GCT samples were divided into CIS and non-CIS samples (Supplemental Table 3): over-expression of the *KIT* gene (an established marker for early-stage GCT) in CIS samples consistently agreed with the histological diagnosis of samples. No difference in expression was found for *BARD1*, *BMPR1A*, *CCND1*, *CDKN1A*, *CDKN1C*, *SMAD3* and *XPA* in CIS samples compared with CS controls, whereas their transcript values were found highly decreased in non-CIS samples ($p<0.002$). *DLK1* was significantly over-expressed in CIS samples ($p=0.012$), contrary to the marked under-expression found in non-CIS samples ($p<0.002$). Thus, this set of genes is, somehow, associated with different stages in tumour progression.

Expression signature by functional categories

In order to obtain some clues about the functional pathways that are affected in testis with SpF, genes were grouped into functional clusters according to the process they are involved in: cell proliferation, apoptosis/cell cycle, meiosis, DNA repair, transcription regulation, post-transcriptional regulation and degradation. The functional expression

signature was compared with that obtained from the GCT samples (Supplemental Table 3).

Some of the spermatogonia-preferential transcripts differentially represented between the SpF subgroups and controls encode proteins involved in the regulation of the mitotic and meiotic cell cycle (cyclins, cyclin –dependent-kinase-inhibitors and DNA polymerases). *CCNE1* and *POLD1* were significantly under-expressed, this being particularly noticeable for *CCNE1*, whereas *CCND1* was over-expressed in the MA samples. In GCT only the *CCNE1* transcript value of this group of genes was significantly decreased compared with controls. Furthermore, we observed decreased expression levels of several genes that encode for putative RNA-binding proteins (such as the germ cell specific *DAZL* and *RBM15*), other proteins essential for production of miRNAs (such as *DICER1)*, proteins involved in the protein degradation pathway (*FBXO32* and *TM9SF2*) and also proteins implicated in the homologous recombination in meiosis (*MRE11A* and *RAD50)* in both SpF and GCT groups.

There were no differences in expression of genes such as *BMPR1A, c-KIT* and *VEGFA* (cell population proliferation), *BAX* gene (apoptosis), *ATM*, *SIRT6* and *XPA* (DNA repair genes) observed between SpF and CS groups, in contrast to the observed expression alteration of these genes in GCT.

The expression of all the Sertoli-specific expressed genes, with the exception of *SLC4A11* was significantly affected in the GCT samples, whereas only those genes involved in cell cycle/apoptosis, *SLC4A11* and *SCIN,* were significantly decreased in the SpF meiotic altered samples.

Protein expression

We sought to determine whether the changes in transcript levels would correlate with modifications at the protein level. At the same time we aimed to determine whether the suggested alterations in gene expression affected expression levels of encoded proteins in the germ line. We focused first on robust differences in expression levels which might be discerned by immunohistochemistry and chose *CCNE1* and *DAZL* as examples of spermatogonia-associated genes specifically expressed in the germ line. Secondly, we selected proteins whose coding genes presented an upregulated expression in SpF samples such as DLK1 and CDKN1C (Figure 4).

CCNE1 immunostaining (A-D; Figure 4) in CS samples was observed mainly in both the nucleus and the cytoplasm of postmeiotic secondary spermatocytes/round spermatids, and in the in nucleus of spermatogonia. Sertoli cells showed less intense CCNE1 expression in the nucleus; some staining of the cytoplasms of interstitial Leydig cells and Sertoli cells was also seen, which tended to increase in samples with MA and SCO; in the germ line of SpF samples CCNE1 expression was exclusively detected in the spermatogonia and not detected in primary spermatocytes and elongated spermatids, when present. The DAZL protein (E-H¸ Figure 4) was almost exclusively present in the cytoplasm of primary pachytene spermatocytes in CS samples and therefore expression was negative in testis with SCO. Overall, immunoexpression in testis sections of both CCNE1 and DAZL protein decreased within seminiferous tubules, in the germ line, as spermatogenic damage progressed, showing good correlation with RNA expression.

CDKN1C (I-L; Figure 4) in CS samples was seen in the cytoplasm of Sertoli cells and Leydig cells; the immunostaining for Leydig cells was more intense in MA and SCO samples; peritubular cells were positive in SCO; however in SpF samples some spermatogonia and primary spermatocytes also exhibited moderate expression of CDKN1C, leading to a global increase in CDKN1C expression in biopsies with SpF.

The immunoreactivity of DLK1 (M-P; Figure 4) in CS samples seemed to be restricted to the cytoplasm of a few Leydig cells, which were more frequently stained in MA, and more so in SCO suggesting a relevant contribution of Leydig cells to DLK1 expression. Overall, immunoexpression of both CDKN1C and DLK1 protein in testis sections, preferentially in somatic cells for DLK1, increased as spermatogenic damage progressed, showing good correlation with RNA expression.

DISCUSSION

The aim of this study was to assess early testicular transcriptional changes that could be involved in human severe deficiency of sperm production. We focused our attention mainly on the expression profile of premeiotic germ cells as this is a key step in male germ cell maturation. The accurate quantification of testicular mRNA levels in SpF by RT-qPCR experiments led to the identification of differences in expression of certain genes associated with spermatogonia in the absence of any apparent morphological and/or numerical change of this specific cell type.

The gene expression profile in SpFcan be used as a basis for identification of candidate genes that contribute to spermatogenic impairment. SpF and GCT expression signature comparison could additionally give some clues about the molecular mechanisms underlying the origin of these alterations. Our data indicate that, in the SpF patients, a large proportion of spermatogonia-preferentially expressed genes exhibited reduced testicular expression levels when compared with CS individuals. As expected, the number of genes whose expression was altered as well as the magnitude of increase or decrease of gene expression in GCT was even higher, possibly related to the fact that the germ line in testicular tumour has undergone a dedifferentiation process representing an extreme situation of gene expression deregulation of spermatogenic impairment. Interestingly, gene expression signatures of both phenotypes, SpF and GCT, share some aberrant patterns of gene expression supporting the idea that the participation of these genes is essential for physiological germ cell development. In contrast, other genes are differentially affected in both pathological groups suggesting that they contribute to the phenotype and could be used as potential molecular markers.

The cellular complexity of the testis is an inherent problem which should be taken into account when studying gene expression profiles in this organ. As the

pathological seminiferous tubules lack germ cells to varying degrees, changes in gene expression at the tissue level can reflect changes in the capability for transcribing the mRNA in a specific cell type as well as changes in the cell type composition in pathological testis. The absence of significant differences in the spermatogonia and Sertoli cell number among the SpF-MA, SpF-HS and CS groups in our study indicates that transcript levels cannot be attributed to the presence or absence of these specific cell types. Additionally, we are aware of the fact that the levels of different mRNAs could change as the proportion of immature germ cells is different in tubules in conserved spermatogenesis, in which all stages of germ cell are present, and in those in maturation arrest, where only some stages are present. In this condition, we assume that there would be less mRNA from most spermatogonia-expressed genes in CS testis than in SpF testis. Our study shows that most of the differentially expressed genes showed higher expression in testis with conserved spermatogenesis suggesting that the reduction of target genes could not be attributable to either the spermatogonia cell number or to different proportion of this germ cell stage in the tubule, but to real differences in the expression capability of the cell. Furthermore, we describe reduced cellular expression levels of four germ line specific genes in SpF samples supporting this premise.

The spermatogonia-related genes whose transcripts were differentially represented between SpF subgroups and controls included genes involved in specific functional pathways. A first group of genes encodes proteins involved in the regulation of the mitotic and meiotic cell cycle such as cyclins, cyclin-dependent-kinase-inhibitors and DNA polymerases indicating that the regulation of this specific functional gene cluster in the initial stages of spermatogenesis is critical for further differentiation and meiosis of germ cells. Additionally, decreased expression levels of several genes encoding putative RNA-binding proteins (such as the germ cell specific *DAZL* and

RBM15), other proteins essential for production of miRNAs such as *DICER1*, and also proteins involved in the protein degradation pathway (*FBXO32* and *TM9SF2*) in both SpF and GCT groups, underlie the complexity of post-transcriptional control in proliferation and differentiation of germ cells. *MRE11A* and *RAD50,* involved in homologous recombination in meiosis, also showed altered expression in meiotic blockade. Unexpectedly, we observed no difference in testicular expression for *STRA8*, a well known gene involved in meiotic cell cycle, participating in chromosome pairing and in the process that leads to stable commitment to the meiotic cycle (Mark *et al.*, 2008), in SpF group nor in SpF-MA subtype samples when compared with controls. Interestingly, an additional statistically significant reduction in the expression levels of germ cell–specific genes per spermatogonia was observed in MA and HS when compared to CS samples, demonstrated for *CCNE1*, *DAZL* and *RBM15* genes, being more pronounced in the MA phenotype suggesting that the expression capacity in immature germ cells correlates with the severity of testicular damage. More intriguingly, a decreased cellular expression level was even observed for *STRA8* in MA pattern, suggesting that in MA phenotype, premeiotic cellular expression could be affected for a larger number of genes. The remarkable correlation coefficient between the *CCNE1*, *DAZL, RBM15* and *STRA8* transcript levels per cell and the number of elongated spermatids in the testicular tubule additionally underlines the determinant role of premeiotic *CCNE1*, *DAZL, RBM15* and *STRA8* expression in the progression of the spermatogenic process.

In previous studies, the reduction of gene expression in SpF patients has been mainly attributed to the decreased number of germ cells that specifically express the gene of interest (i.e. significantly lower *DAZL* mRNA concentrations were previously found in testes of non-obstructive azoospermic men (Lin *et al.*, 2001)), although germ

cell quantification was not properly performed. Here, we demonstrate that the changes in expression observed among groups could be not exclusively explained by the immature germ cell number but the contribution of the reduced cellular expression of i.e. *DAZL* mRNA in spermatogenic impairment should be also taken into account.

Protein data on nonobstructive testicular tissue corroborate our mRNA expression results: CCNE1 and DAZL protein decreased within seminiferous tubules, in the germ line, as spermatogenic damage progressed. The reduced transcript levels of other genes expressed in spermatogonia as the ones involved in piRNA processing machinery such as *PIWIL2* and *TDRD1* in SpF, as we recently described (Heyn *et al.*, 2012) further supports the role of a proper gene expression in early germ line stages for a successful sperm production.

The expression levels of genes participating in cell population proliferation, mitochondria-mediated apoptosis and DNA repair (assuring the maintenance of genome integrity) are in general maintained in SpF, contrary than in GCT, supporting the idea that they are similarly processed in meiotic derangement and in conserved spermatogenesis. However, there is one exception possibly due to other regulatory pathways: the levels of spermatogonia-specific full-length *BARD1* transcript (Feki *et al.*, 2004; Irminger-Finger *et al.*, 2001), involved in germ cell apoptotic events, could be repressed in SpF by the high levels of FSH in spermatogenic failure samples (Feki *et al.*, 2004).

Although the transcription profile of spermatogonia-associated genes in SpF is different from that in GCT, some functional clusters are affected in both phenotypes: genes with functions in cell cycle, transcription and post-transcriptional regulation and protein degradation. Meanwhile, other spermatogonia-expressed genes encoding proteins involved in cell proliferation, apoptosis and DNA repair pathways are not

affected in SpF, suggesting that in spermatogenic failure, although the abnormal initiation of the meiotic process is already determined in these immature germ cells, these cells maintain their activity related to mitosis and cell proliferation.

Regarding the Sertoli-specific expressed genes, the absence of differences in gene expression of six out of eight genes studied in the SpF-MA and SpF-HS phenotypes suggest that, in spermatogenic derangement, the functions of Sertoli cells are in general maintained, but not those involved in cell cycle/apoptosis (*SLC4A11* and *SCIN*) related to germ cell support. Previous studies have shown that, in mouse, chemically-induced germ cell depletion can alter expression of several Sertoli cell genes (Jonsson *et al.*, 1999; Maguire *et al.*, 1993; O'shaughnessy *et al.*, 2008) demonstrating that germ cells regulate Sertoli cell activity by means of the regulation of Sertoli cell gene expression. Here we observed a similar pattern in a pathological naturally-occurring phenotype. We observed a gradual decrease of *SLC4A11* and *SCIN* transcript levels attributable to the progressive depletion of germ cell stages (SCO>MA>HS>CS). It is noteworthy that even in the presence of a two-fold increase of the number of Sertoli cells as a compensatory phenomenon in SCO, the decrease in expression of certain Sertoli cell genes becomes much more noticeable in the total absence of germ cells.

Some recent microarray studies have assessed global gene expression analysis in testicular biopsies from infertile men in order to identify the genes critical for spermatogenesis (Chalmel *et al.*, 2012; Von Kopylow *et al.*, 2010; Ellis *et al.*, 2007; Feig *et al.*, 2007; Rockett *et al.*, 2004; Fox *et al.*, 2003). In these studies specific germ cell transcription patterns are inferred from infertile testicular phenotypes in men and a pattern of significantly decreased regulated genes has been attributed to the degree of spermatogenic failure and the loss of specific stages of germ cells. We provide data suggesting that the molecular basis for severe spermatogenic impairment is more

complex than initially proposed. In SpF the immature germ cells present an altered and decreased transcriptional pattern of certain genes, and thus the number of genes associated with these cells could be underestimated from microarray studies of infertile samples. Furthermore, our results should be helpful to better interpret microarray or future NGS transcriptome studies. Elucidation of a more extensive transcriptional profile with the detailed analysis of testicular cellular composition could be important in understanding the molecular mechanisms that underlie male infertility.

It is conspicuous that most of the altered spermatogonia-related genes are involved in essential processes during spermatogenesis and aberrant expression is often associated with spermatogenic defects. Whether the observed differential expression profiles represent the cause or consequence of maturation arrest remains to be elucidated. Considering the heterogeneous aetiologies and highly individual molecular causes which may underlie spermatogenic failure in humans, the molecular changes described here may represent common symptoms, but may also reflect early dysfunction events affecting germ cells which may causally contribute to the pathology. This data should be useful in delineating the patterns of gene expression involved in male germ cell maturation deficiency, which may contribute to understanding male infertility.

In summary, our study provides evidence that the premeiotic stage of germ cell differentiation, exhibits associated patterns of gene expression deregulation in spermatogenic impairment, which is more severe in meiotic arrest. This altered gene expression pattern is observed despite there being no apparent morphological and/or numerical change observed in this early stage of the germ cell population. In our previous study, the spermatocyte capacity to express meiosis-related genes was observed to be markedly reduced in spermatogenic failure, contributing to meiosis

impairment (Terribas *et al.*, 2010). Our present data demonstrate that the low spermatogenic efficiency in infertile men is accompanied not only by meiotic but also by premeiotic events in spermatogenesis, which contribute to spermatogenic blockade. Furthermore, the differences in expression during the initial stages of spermatogenesis in SpF-MA individuals suggest that this phenotype is already determined or arises in the premeiotic stages of the germ line.

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AUTHOR'S CONTRIBUTION

S.B. performed the RNA experiments and analysed the data. F.A. performed immunohistochemistry and histological interpretation of data. E.F. provided samples and clinical data. L.B. performed clinical assessment, provided samples, and critically reviewed the manuscript. S.L. conceived and designed the experiments, supervised the analysis of data and wrote the manuscript.

FIGURE LEGENDS

Supplemental Figure 1. Absolute expression levels of candidate reference genes (a.) in SCO, MA, HS and CS groups and (b.) in GCT and CS groups.

○, □, outlying values. * $p < 0.05$, (a.) Kruskal-Wallis test and (b.) Mann-Whitney test.

SCO: Sertoli cell only syndrome; MA: maturation arrest at spermatocyte stage; HS: hypospermatogenesis; CS: conserved spermatogenesis

Figure 1. Spermatogonia-preferentially expressed genes whose relative expression values were statistically altered in patient SpF group (MA and HS sub-phenotypes) compared with CS controls. Expression levels relative to *PGK1/PGM1* are shown.

* $p < 0.05$, Mann-Whitney test; ** $p < 0.002$, Mann-Whitney test and Bonferroni correction.

MA: maturation arrest at spermatocyte stage; HS: hypospermatogenesis; CS: conserved spermatogenesis.

Figure 2. Testicular somatic cell-preferentially expressed genes that showed differences in gene expression in infertile SCO, MA and HS patients relative to CS controls. Expression levels relative to *PGK1/PPIA* are shown..

* $p < 0.0083$, Mann-Whitney test and Bonferroni correction.

SCO: Sertoli cell only syndrome; MA: maturation arrest at spermatocyte stage; HS: hypospermatogenesis; CS: conserved spermatogenesis.

Figure 3.

Tissular expression profiling of *CCNE1* (a), *DAZL* (b), *RBM15* (c) and *STRA8* (d) by quantitative real-time qPCR in testis with conserved spermatogenesis (CS),

hypospermatogenesis (HS) and maturation arrest at the spermatocyte (MA) Expression levels relative to *PGK1* and *PGM1* are shown. Expression per cell profiling of *CCNE1*, *DAZL*, *RBM15* and *STRA8* displayed as expression ratio per spermatogonium (x100) (e, f, g, h) and expression per cell profiling of *CCNE1*, *DAZL*, *RBM15* and *STRA8* displayed as expression ratio per spermatogonium and spermatocyte (x100) (i, j, k, l). The horizontal bar indicates median value. Significant differences from the control are indicated: *p<0.05; **p<0.005.

Figure 4.

Immunohistochemical localization of selected proteins in sections of human testes with different phenotypes. From left to right, first column (A,E,I,M) shows sections of CS, second column (B,F,J,N) corresponds to SpF-HS, third column (C,G,K,O) represents SpF-MA, and the fourth column (D,H,L,P) displays SCO pattern. CCNE1 protein staining is shown in panels A-D, DAZL in E-H, CDKN1C in I-L, and DLK1 expression in M-P, were stained Leydig cells are indicated by arrows. See explanation of the cellular localization of different proteins in the text. Original magnification was X400 for panels A-L, and X200 for M-P. Scale bar in A and M = 100 µm.

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Table 1 Quantitative histological evaluation of the testicular samples included in the study

Histological pattern	Number of samples	Spermatogoniae	Spermatocytes I	Round spermatids	Elongated spermatids	Sertoli cells	JS
SCO	14	0	0	0	0	21.03 ± 7.00	1-2
SpF-MA	7	20.28 ± 4.38	23.30 ± 11.73	2.27 ± 3.10	0.52 ± 0.81	13.87 ± 3.01	4-6
SpF-HS	12	20.53 ± 6.68	29.77 ± 10.44	16.48 ± 8.07	5.70 ± 4.81	15.74 ± 5.61	7-8
CS	17	21.96 ± 4.27	32.30 ± 6.64	25.38 ± 12.04	21.14 ± 7.07	13.62 ± 3.11	9-10

The mean number ± SD of different type of cells/tubule are given for each Johnsen score classified group

Abbreviations: SCO: Sertoli cell only syndrome, SpF-MA: spermatogenic failure diagnosed with meiotic arrest, SpF-HS: spermatogenic failure diagnosed with hypospermatogenesis, CS: conserved spermatogenesis, JS: Johnsen score

Table 2. List of genes differentially expressed in GCT

Gene name	Relative expression values (PGK1/PPIA)		fold-change	*p* -value
	CS group	GCT group		
Spermatogonia target genes that are under-expressed				
ATM	1.05 ± 0.25	0.32 ± 0.18	-3.28	0.000076**
BARD1	1.02 ± 0.15	0.55 ± 0.25	-1.85	0.002202**
CCND1	0.73 ± 0.17	0.35 ± 0.36	-2.08	0.030987*
CCNE1	1.04 ± 0.25	0.16 ± 0.12	-6.50	0.000076**
CDKN1C	0.49 ± 0.13	0.19 ± 0.13	-2.58	0.000911**
DAZL	1.04 ± 0.20	0.06 ± 0.08	-17.33	0.000076**
DICER1	1.13 ± 0.22	0.21 ± 0.12	-4.90	0.000076**
E2F3	1.00 ± 0.14	0.58 ± 0.18	-1.72	0.000304**
FBXO32	0.82 ± 0.18	0.17 ± 0.18	-4.82	0.000076**
MRE11A	1.09 ± 0.25	0.29 ± 0.19	-3.75	0.000076**
POLD1	1.14 ± 0.36	0.59 ± 0.38	-1.93	0.030987*
RAD50	1.09 ± 0.26	0.16 ± 0.12	- 6.81	0.000076**
RBM15	0.99 ± 0.28	0.12 ± 0.06	-8.25	0.000076**
SIRT6	1.00 ± 0.21	0.36 ± 0.13	-2.77	0.000076**
STRA8	0.78 ± 0.31	0.13 ± 0.16	-6.00	0.000304**
TM9SF2	0.93 ± 0.14	0.24 ± 0.18	-3.87	0.000076**
XPA	1.06 ± 0.21	0.43 ±0.41	-2.46	0.006304*
Spermatogonia target genes that are over-expressed				
BAX	0.97 ± 0.14	1.19 ± 0.18	+1.22	0.019291*
Somatic cell target genes that are under-expressed				
AMHR2	1.01 ± 0.25	0.20 ± 0.26	-5.05	0.000076**
BCL3	0.93 ± 0.29	0.45 ± 0.15	-2.06	0.000076**
BMPR1A	0.97 ± 0.10	0.65 ± 0.28	-1.49	0.014962*
CDKN1A	0.97 ± 0.35	0.40 ± 0.38	-2.42	0.030987*
SCIN	1.15 ± 0.45	0.06 ± 0.05	-19.16	0.000076**
SMARCA1	1.02 ± 0.14	0.23 ± 0.29	-4.43	0.000076**
SOX9	0.95 ± 0.21	0.18 ± 0.23	-5.27	0.000076**
SPAG7	0.98 ± 0.12	0.38 ± 0.24	-2.58	0.000076**
VEGFA	0.85 ± 0.23	0.40 ± 0.19	-2.12	0.001443**
Somatic-cell target genes that are over-expressed				
FASLG	0.71 ± 0.84	3.89 ± 2.95	+5.48	0.002850*

Normalisers are described in brackets.

* p<0.05, **p<0.002, Mann-Whitney test.

Figure 1

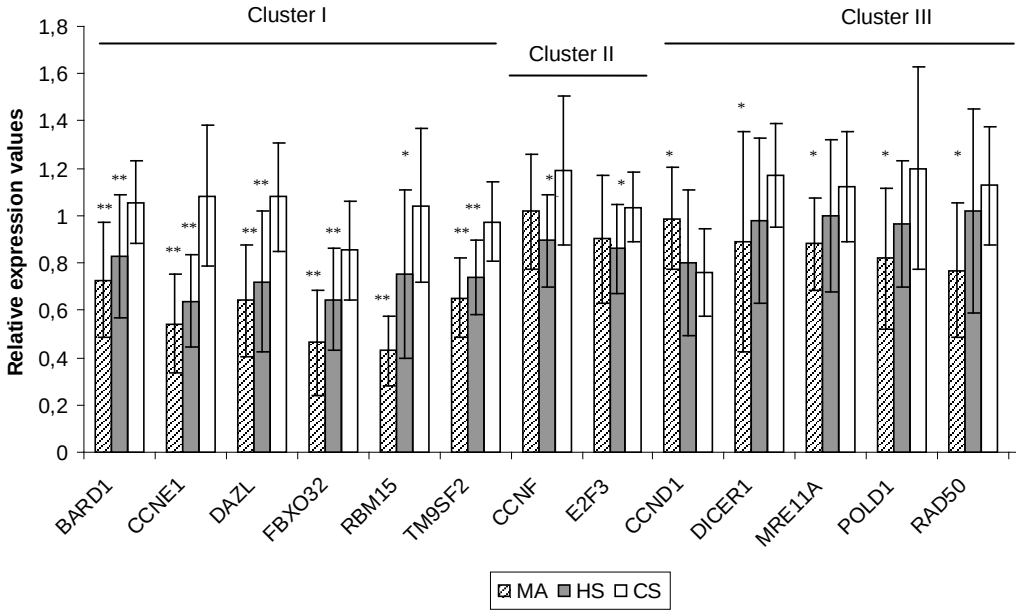


Figure 2

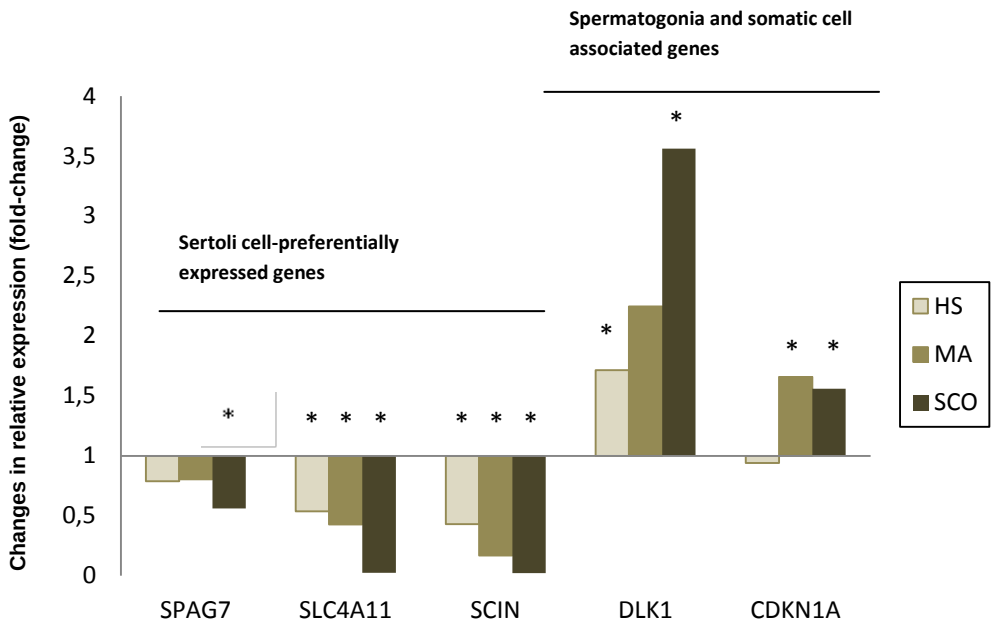
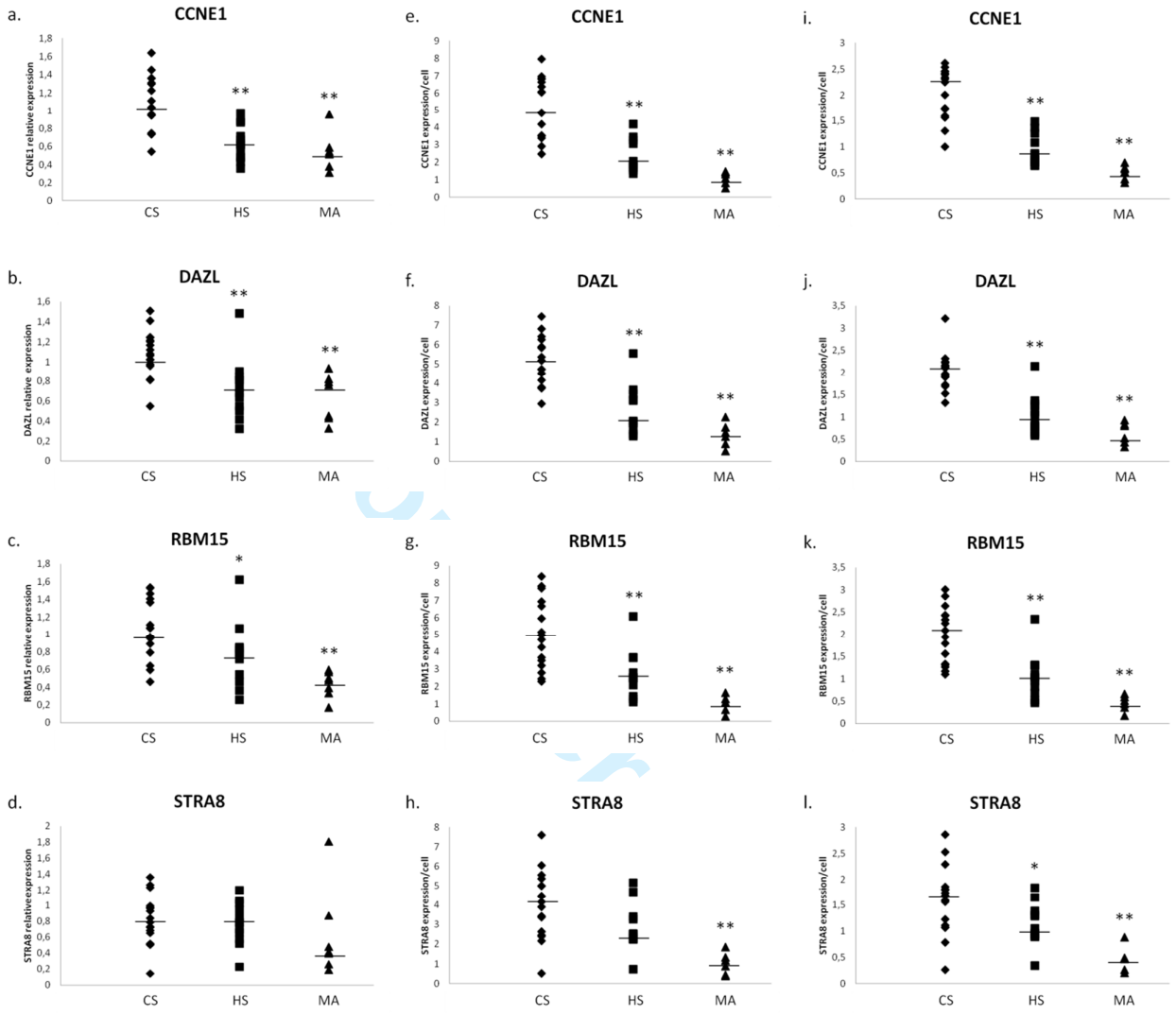
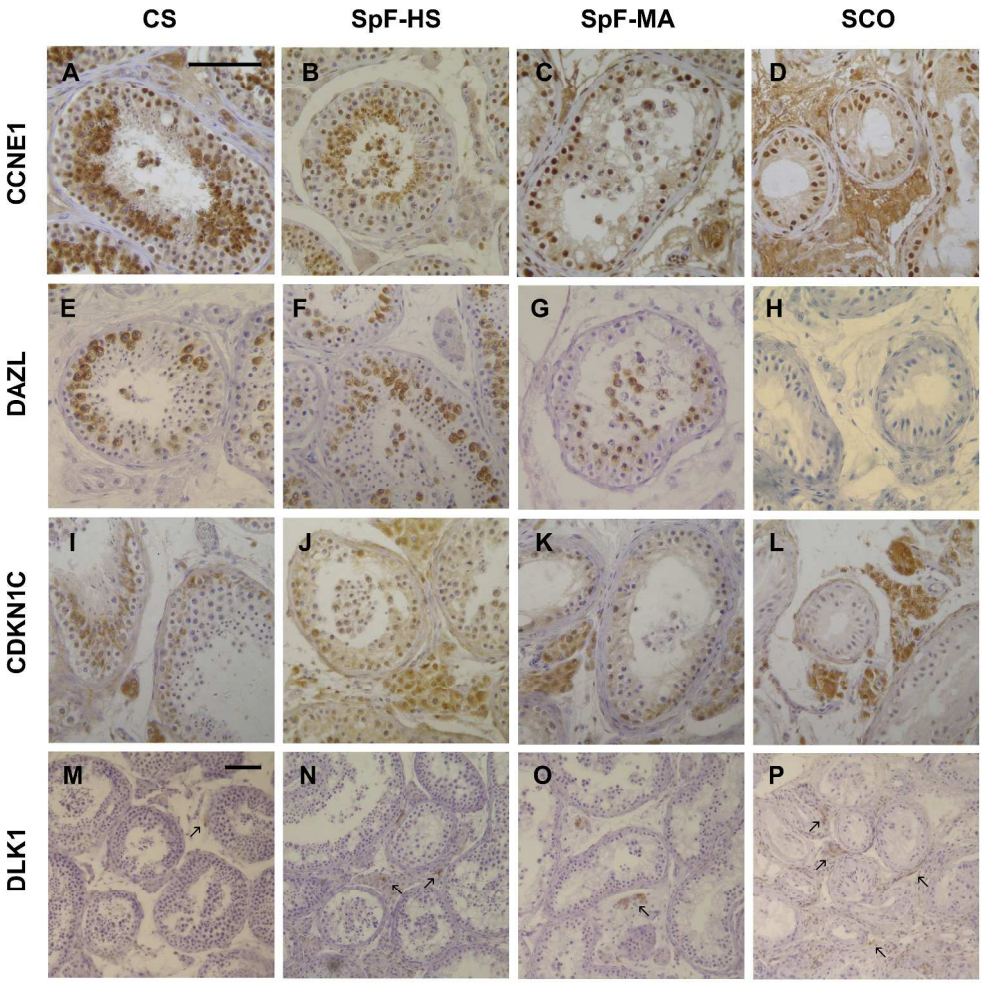


Figure 3


a.
CCNE1
CCNE1 relative expression
CS
HS
MA
b.
DAZL
DAZL relative expression
c.
RBM15
RBM15 relative expression
d.
STRA8
STRA8 relative expression
e.
CCNE1
CCNE1 expression/cell
f.
DAZL
DAZL expression/cell
g.
RBM15
RBM15 expression/cell
h.
STRA8
STRA8 expression/cell
i.
CCNE1
CCNE1 expression/cell
j.
DAZL
DAZL expression/cell
k.
RBM15
RBM15 expression/cell
l.
STRA8
STRA8 expression/cell


CS
SpF-HS
SpF-MA
SCO
CCNE1
DAZL
CDKN1C
DLK1
A
B
C
D
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G
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P

ALTERED GENE EXPRESSION SIGNATURE OF EARLY STAGES OF THE GERM LINE SUPPORTS THE PREMEIOTIC ORIGIN OF HUMAN SPERMATOGENIC FAILURE

Running title: Early germ line gene expression in impaired sperm production

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ABSTRACT (300 words)

The molecular basis of spermatogenic failure (SpF) is still largely unknown. Accumulating evidence suggests that a series of specific events such as meiosis, are determined at the early stage of spermatogenesis. The present study aims to ~~reveal~~ assess the expression profile of pre-meiotic genes ~~expression changes~~ of infertile testicular biopsies that might help to define the molecular phenotype associated with human deficiency of sperm production. An accurate quantification of testicular mRNA levels of genes expressed in spermatogonia was carried out by RT-qPCR in individuals showing SpF due to germ cell maturation defects, Sertoli cell-only syndrome (SCO) or conserved spermatogenesis (CS). Additionally, the gene expression profile of SpF was compared with that of testicular tumour (GCT), which is considered to be a severe developmental disease of germ cell differentiation. Protein expression from selected genes was evaluated by immunohistochemistry.

Our results indicate that SpF is accompanied by differences in expression of certain genes associated with spermatogonia in the absence of any apparent morphological and/or numerical change of this specific cell type. In SpF testicular samples, we observed down-regulation of genes involved in cell cycle *(CCNE1* and *POLD1)*, transcription and post-transcription regulation (*DAZL, RBM15* and *DICER1*), protein degradation (*FBXO32* and *TM9SF2*) and homologous recombination in meiosis (*MRE11A* and *RAD50*) which suggests that the expression of these genes is critical for a ~~successful~~ proper germ cell development. Interestingly, a decrease in the *CCNE1*, *DAZL*, *RBM15* and *STRA8* cellular transcript levels was also observed, suggesting that the gene expression capacity of spermatogonia is altered in SpF contributing to an unsuccessful sperm production. Altogether, these data point to the spermatogenic derangement being already determined at, or arising in, the initial stages of the germ

line~~, although these cells maintain their transcriptional activity related to mitosis and cell proliferation~~.

Key words: gene expression, testis, early stages of germ line, spermatogenic failure, male infertility

INTRODUCTION

Spermatogenesis is a highly orchestrated developmental process by which spermatogonia develop into mature spermatozoa. During the course of spermatogenesis the three major forms of cell cycle are represented: mitosis of primitive spermatogonia; two rounds of meiosis, from primary spermatocytes to haploid round spermatids; and differentiation including structural and nuclear changes to generate mature spermatids and spermatozoa. These processes are unique in male germ cell differentiation and depend on precise, developmental stage- and germ cell type- specific gene expression. However, the regulatory network that confers specific germ line gene expression in mammals is not properly understood, especially at the mitotic and meiotic stages. Understanding the regulatory step is essential for determining the molecular requirements for the progression of spermatogenesis, and thus for understanding male infertility which is often based on lack of replication of spermatogonia or meiotic blockade.

Accumulating evidence suggests that a series of specific events during spermatogenesis, such as meiosis and morphological changes, are determined at the early stage of spermatogenesis. Spermatogonia, and specifically the type B spermatogonia, should be an important preparation stage for meiosis. These data are supported by the description of activation or up-regulation of many genes during this specific germ cell stage (Guo *et al.*, 2004) and the generation of recombinant mouse models of spermatogonia-expressed genes exhibiting severe defects in meiosis (Wang *et al.*, 2001). Many of these genes codify germ cell specific proteins involved in transcriptional or post-transcriptional regulation of gene expression (Wang *et al.*, 2001).

Furthermore, cellular interactions between germ line and somatic components of the testicular seminiferous tubule, where spermatogenesis takes place, are essential to

achieve germ cell development, and thus for maintaining male fertility. The relevance of these cellular interactions is supported by physiological events (for review, see (Mclachlan *et al.*, 2007).

Some studies have used microarray technology to characterize the transcriptional profile in germ and somatic cells at different steps of testicular development (Chalmel *et al.*, 2007; Diederichs *et al.*, 2005; Namekawa *et al.*, 2006; Pang *et al.*, 2003; Schlecht *et al.*, 2004; Schultz *et al.*, 2003; Sha *et al.*, 2002; Shima *et al.*, 2004). We have used information from cDNA microarrays and mouse models to focus mainly on genes specifically or preferentially expressed in immature germ cells in mammals, to determine whether early gene expression changes are associated with subsequent spermatogenic disorders ~~that could potentially lead and/or contribute to defective or absent spermatogenesis~~ and thus, male infertility. Additionally, the gene expression profile of germ cell maturation failure was compared with that seen in testicular tumour, which is considered to be a developmental disease of germ cell differentiation, in order to give additional clues about the functional pathways involved in spermatogenic derangement.

MATERIALS AND METHODS

Subjects of study

Our study recruited nineteen patients (range 30-49 yr) infertile due to spermatogenic failure (SpF) at different germ cell stages, with a phenotype consistent with non-obstructive azoospermia or severe oligozoospermia (<5 million sperm per mL). In addition, fourteen patients with Sertoli cell-only syndrome (SCO) phenotype or germ cell aplasia, were studied as controls of somatic cells (negative controls), and seventeen infertile patients (range 32-50 yr), who were diagnosed with obstructive azoospermia (as a consequence of congenital absence of vas deferens or previous vasectomy) and showed conserved spermatogenesis (CS) were studied as gene expression controls of a complete spermatogenic process (positive controls). Patients were selected on the basis of the testicular histological pattern of >20 tubules from the same testicular section; only testicular samples with a homogeneous histological pattern were included in the study.

An extra group of individuals (n=5) diagnosed with germ cell tumour (GCT) (range 29-46 yr) were analyzed. Two GCT samples were histologically classified as carcinoma *in situ* (CIS) whereas the remaining three samples were classified as non-CIS or GCT of advanced stages: one as classic seminoma, one as embryonal carcinoma and one as mixed germ cell tumour (80% embryonal carcinoma; 20% classic seminoma).

Infertile individuals were selected from men referred for couple infertility to the Andrology Service of the Fundació Puigvert, whereas GCT samples were recruited from the Andrology Service of the Fundació Puigvert and the Urology Service of the Hospital Universitari de Bellvitge. The study was approved by the Institutional Review Board of both Centres, and all the participants signed a written informed consent.

The clinical procedures for infertile patients included medical history, physical examination, semen analyses [performed in accordance with World Health Organization guidelines (World Health Organization, 1999)] and hormonal study. The routine genetic study for all non-obstructive samples included karyotype and analysis of chromosome Y microdeletions, the latter performed according to the European guidelines (Simoni *et al.*, 1999; Simoni *et al.*, 2004). Men with a chromosomal aberration or a Y-chromosome microdeletion were not included in the study.

Testicular samples

Testicular biopsies from infertile men were obtained when necessary to confirm the clinical diagnosis and for sperm retrieval (TESE) and cryopreservation purposes. Each specimen was divided into three aliquots, one piece (≈10-20 mg) was fixed in Bouin's solution and reserved for histological analysis, a second aliquot (≈100-200 mg) processed for sperm extraction and the third (≈10 mg) was immediately transferred to liquid nitrogen and stored at –80ºC until analysis for gene expression experiments.

Referring to GCT, testicular samples were obtained directly after orchidectomy and macroscopic pathological evaluation. For gene expression studies, one tissue fragment was taken from the tumour portion of the testis and was immediately frozen at –80ºC.

Histological analysis

An assessment of spermatogenic status and the severity of the alteration were performed after hematoxylin-eosin staining of paraffin samples from infertile patients (5-µm sections) by quantification of specific germ cells (spermatogonia, spermatocytes I, round spermatids and elongated spermatids) and Sertoli cells. The average number per tubule

was calculated after analysis of at least 15-20 cross-sectioned tubules per testis. A modified Johnsen score (JS) count (Schulze *et al.*, 1999) was calculated on the basis of the number of different cell types per tubule and infertile samples were classified as CS, SpF-HS (hypospermatogenesis), SpF-MA (meiotic arrest) and SCO (Table 1).

RNA extraction and cDNA synthesis

Total RNA was obtained from the testicular biopsy using Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA), following the manufacturer's instructions. The quality of RNA [28S/18S ratio and RNA Integrity Number (RIN)] was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Testicular RNA from the five groups of study (SCO, SpF-MA, SpF-HS, GCT and CS) showed similar quality values: both 28S/18S ratio and RIN presented no significant differences among them ($p = 0.056$ and $p = 0.072$, respectively). Mean 28S/18S ratio value between all samples (mean±SD) was 1.17±0.18 and mean RIN value was 7.77±0.62.

Single-stranded cDNA was obtained by reverse transcription (RT) of 500 ng of RNA, using random primers and the High Capacity cDNA Reverse Transcription Kit (AB, Foster City, CA). The resulting cDNA solution was aliquoted and stored at –20ºC until use.

Gene expression quantification and statistical evaluation

Quantitative real-time PCR (qPCR) assays were performed by means of the application of the PCR arrays on micro fluidic cards (MFC), using 384-well TaqMan® Low Density Arrays (TLDAs) on an Applied Biosystems 7900HT Fast Real-Time PCR System (AB, Foster City, CA). The 48-gene format MFC (47 experimental assays and 1 TLDA amplification control, *18S*) allowed simultaneous measurement of 34 target

genes that were selected based on a preferential expression in spermatogonia among germ cells (n=26) and/or in Sertoli cells (n=8) -information obtained from cDNA microarrays and mouse models bibliography data- (Supplemental Table 1), three marker genes of the presence and/or function of spermatid (*PRM1*), Leydig (*INSL3*) and myoid cells (*S100A6*) and 10 potential reference genes. Genes and the corresponding assays on demand used for the setup of the TLDA are listed in Supplemental Table 2. Selected target genes are involved in different functional pathways (Supplemental Table 1 and Supplemental Table 2).

Patient and control samples were always analysed as paired samples in the same analytical run in order to exclude between-run variations. Additionally, a calibrator sample was included in all the plates to compare the change in expression of a nucleic acid sequence against the expression in all samples in the same study. Real-time PCR data (Ct values) were pre-processed and stored in SDS 2.2 software (AB, Foster City, CA).

Expression stability of the candidate reference gene/s was calculated with the GeNorm software (Vandesompele *et al.*, 2002), in order to select the most stable reference genes and improve normalization of target genes. GeNorm software calculates the gene expression stability value M of multiple candidate genes as the average pair-wise variation of a particular gene compared with all other candidate reference genes. Lower M values indicate genes with less expression variation among samples. Therefore, target gene expression was calculated relative to the expression of *PGK1* and *PGM1* reference genes for SpF and control samples, whereas *PGK1* and *PPIA* combination was selected as the most appropriate for GCT and controls. They showed no statistical differences in absolute expression levels between groups (Kruskal-Wallis

test) (Supplemental Figure 1) and low M-value (GeNorm software ~~(Vandesompele *et al.*, 2002)~~ indicating stable expression among samples.

Thus, raw data (Ct values) were normalised to the two reference genes and relative quantification (RQ) values were calculated using the qBase program (Hellemans *et al.*, 2007). and the $2^{-\Delta\Delta Ct}$ strategy. The Mann-Whitney U test was used to evaluate differences in relative expression of target genes in each patient group or subgroup compared with controls. Multiple test adjustment was applied by using Bonferroni correction.

Pearson product-moment correlation coefficients were calculated to determine the correlation between the expression ratios of the target genes and the various histological parameters in patient groups and controls.

All statistical analyses were performed using the SPSS software version 12 (Lead Technologies, Chicago, USA)

Immunohistochemistry

Tissue sections were prepared from Bouin-fixed, paraffin-embedded fragments of testicular biopsies. For this study, the following commercially available polyclonal rabbit antibodies were used: DAZL, HPA019777, Sigma-Aldrich, Inc; CCNE1 (C-19): sc-198, Santa Cruz Biotechnology, Inc; CDKN1C (C-20):sc-1040, Santa Cruz Biotechnology, Inc; DLK1 (H-118): sc-25437, Santa Cruz Biotechnology, Inc.

Immunohistochemistry was performed using the Dako EnVision+ kit (DAKO, Hamburg, Germany) in conjunction with the Dako autostainer, according to the instructions provided by the manufacturer.

Endogenous peroxidase was quenched by incubation in 0.5% hydrogen peroxide. Dilutions of primary antibodies were adjusted at 1:300 to 1:100 to optimize

the results. Secondary goat anti-rabbit antiserum was coupled to a labelled polymer-HRP, and staining was done with DAB and haematoxylin-eosin. Incubation with non immune serum was used as a negative control (data not shown). Stained sections were evaluated in bright-field microscopy (Axioskop 40, Zeiss, Göttingen, Germany) and images captured with a Nikon Coolpix 5400 digital camera. The immunoexpression of proteins was determined on tissue samples from at least six different individuals showing each of the spermatogenic phenotypes.

RESULTS

Quantitative determination of spermatogenic status in defective spermatogenesis

Paraffin-embedded testicular specimens from infertile patients and controls were available for histological quantification. Considering the heterogeneity of human testicular pathologies, we needed a detailed definition of the spermatogenic status of each sample in order to acquire high quality data related to germ cell specific transcriptional changes. To this end, the average number of Sertoli cells and specific germ cell types per tubule were determined in haematoxylin and eosin stained testicular sections and additionally the JS value was calculated (Table 1).

Using this strategy we confirmed the diagnosis of SCO (JS score 1-2) and CS (JS score 9-10) phenotypes. With respect to SpF patients, seven of them presented maturation arrest at primary spermatocyte level (SpF-MA) (JS score 4-6) and twelve presented hypospermatogenesis (SpF-HS) (JS score 7-8) (Table 1).

Round and elongated spermatids were absent in 4 out of 7 SpF-MA samples whereas the other three had very low values: round spermatids ranged between 2-4 per tubule and elongated spermatids ranged between 1-2 per tubule suggesting a 80-90% of meiotic arrest. This result confirms that only histological phenotypes with a defined and highly homogeneous pattern of individual tubules were included in the study.

The number of Sertoli cells showed a near two-fold increase in the group SCO (21.03 ± 7.00) compared with the CS samples (13.69 ± 3.12). Interestingly, the number of spermatogonia and Sertoli cells showed no significant differences (p = 0.318 and p =0.447 respectively) among SpF-MA, SpF-HS and CS groups (Table 1).

Gene expression related to impaired spermatogenesis

PRM1 expression confirms the histological phenotype

In order to confirm the histological quantification of samples, we first analyzed the expression of *PRM1,* the marker gene for the presence of spermatids. As expected, negligible transcript level values were found in SCO and 100% MA samples; very low values were determined in incomplete MA samples and decreased levels in HS samples when compared with CS controls, showing a fold decrease in expression of $2.24x10^4$ (SCO), 42.59 (SpF-MA) and 4.45 (SpF-HS) ~~fold decrease in expression compared with controls~~ ($p<0.001$). The absence of quantifiable *PRM1* expression values in GCT samples confirmed the absence of mature germ cells.

Our *PRM1* gene expression results consistently agreed with the histological diagnosis of all samples included in the study.

Expression behaviour of Leydig and myoid cells in spermatogenic disorders

Referring to the expression of the marker genes for the presence and/or function of Leydig (*INSL3*) and myoid cells (*S100A6*), no statistically significant differences were found between SpF-HS and CS groups (p=0.245 and p=0.059 respectively) and between SpF-MA and CS samples (p=0.065 and p=0.075 respectively), however these genes were significantly over-expressed in SCO group when compared with CS samples (p=0.001 and p=0.000 respectively), probably attributable to the absence of germ cells; since total testis samples are analysed, the loss of germ cells ~~changes the~~enrich the relative contribution of the remaining somatic cells in SCO phenotype.

GCT samples also showed no statistically significant differences in *INSL3* and *S100A6* expression when compared with the CS control group (p=0.401 and p=0.542 respectively)

Most of the spermatogonia-transcriptionally associated genes analyzed show a preferential germ line expression

Spermatogonia-associated genes included in the study have been previously described as having a preferential gene expression in the early germ line stages among germ cells (Supplemental Table 1). The assessment of expression of these genes in the SCO phenotype would provide important new information about whether these genes ~~are~~ can be expressed also in somatic cells of the testis.

Selective or preferential germ cell expression was confirmed for 21 out of the 26 spermatogonia-related genes of our study. First, the negligible transcript level values found for *CCNE1*, *DAZL, RBM15* and *STRA8* in complete SCO samples supported their selective germ cell lineage gene expression. Furthermore, statistically significant reduced transcript values in SCO compared with CS suggested a preferential germ cell expression: *ATM*, *BARD1*, *CCND1*, *CCNF*, *DICER1*, *E2F3*, *FBXO32*, *c-KIT*, *MRE11A*, *POLA1*, *POLD1*, *RAD50*, *SIRT6* and *TM9SF2* presented a very significant fold-change decrease in SCO ranging from 1.46 to 6.10 ($p<10^{-4}$ – 10^{-6}) whereas *BAX*, *CDKN1C* and *XPA* showed less marked decrease, with a fold-change ranging from 1.22 to 1.55 ($p<0.05$) (Supplemental Table 3).

However, for the five remaining spermatogonia-associated genes studied, a preferential expression in the germ cell lineage could not be demonstrated. *CDKN1A* and *DLK1* were found significantly over-expressed in SCO samples when compared with CS controls ($p=0.003$ and $p<0.002$ respectively), showing a fold-change increase of 1.77 and 3.99 respectively. Also, *BMPR1A, SMAD3* and *VEGFA* were not differentially expressed between SCO and CS samples ($p>0.05$) (Supplemental Table 3). Taken together, these results indicate a substantial expression of these five genes in testicular somatic cells.

Differential gene expression in defective and conserved spermatogenesis

We then looked for differences in target gene expression between SpF samples and CS controls.

For the 21 spermatogonia-associated genes preferentially expressed in the germ line, we identified 13 differentially expressed genes and grouped them into three gene clusters by their expression behaviour (Figure 1). In cluster I, both SpF-MA and SpF-HS phenotypes were associated with notable significant decreases in transcript levels of *BARD1, CCNE1*, *DAZL*, *FBXO32*, *RBM15* and *TM9SF2* genes (p<0.002), the reduction of expression being more pronounced in the SpF-MA phenotype. Cluster II contained genes significantly decreased in the SpF-HS (p<0.05) but not in the SpF-MA phenotype (*CCNF* and *E2F3).* Cluster III included all those genes differentially expressed in SpF-MA but not in SpF-HS phenotype: *DICER1*, *MRE11A, POLD1* and *RAD50* expression levels were decreased whereas *CCND1* was increased in the SpF-MA (p<0.05) when compared with controls. *CDKN1C* presented a similar pattern of gene expression deregulation to *CCND1,* although the difference in expression was not statistically significant (CS: 0.51±0.15~~—0.75±0.18~~; HS: 0.61±0.54 ~~0.80±0.31~~p=0.394; MA: 1.04±0.73 ~~0.98±0.21~~p=0.172) (Supplemental Table 3).

Interestingly, significant positive correlation coefficients (Pearson's correlation r≥0.6; p<0.0001) were found between the number of elongated spermatids and the transcription levels of genes from cluster I (Supplemental Table 4.A), suggesting that these changes in gene expression could be of physiological relevance.

In regard to the spermatogonia and somatic cell-expressed genes, *CDKN1A* was significantly increased in the SpF-MA and SCO samples (p<0.05). *DLK1* was found highly over-expressed in SpF samples, although the differences were only statistically

significant for the SpF-HS and SCO (p=0.001) and not the SpF-MA phenotype (probably due to a high standard deviation value) when compared with controls (Figure 2; Supplemental Table 3).

When considering the Sertoli cell-preferentially expressed genes, *SCIN* and *SLC4A11* were found very significantly decreased in MA and HS samples when compared with CS. SCO samples, although presenting an increased number of Sertoli cells, showed reduced levels of *SPAG7, SCIN* and *SLC4A11* compared with CS controls (p<0.006) (Figure 2; Supplemental Table 3). The expression of *FASLG* mRNA in SpF samples was increased compared with CS, although no statistically significant changes in the average of *FASLG* expression were observed among groups due to high standard deviation values in the infertile groups (CS control: 1.10±0.71; HS: 1.79±1.19; MA: 3.44±2.87).

Reduced cellular expression levels of germ cell–specific genes in SpF

We additionally analyzed the transcript levels per cell of spermatogonia-associated genes with a selective germ cell expression, in SpF subgroups compared to CS controls in order to exclude the differences in gene expression due to changes in testicular cellularity and to determine whether transcript level per cell is also altered in SpF. Selective germ cell expression of *CCNE1*, *DAZL*, *RBM15* and *STRA8* was previously confirmed as negligible transcript level values were found in SCO samples as previously described, furthermore, *DAZL* and *STRA8* were previously described to be expressed in spermatogonia but not in somatic tissues (Wang *et al.*, 2001). Values of transcript amount per cell, in arbitrary units, were obtained for each testicular sample by dividing the *CCNE1*, *DAZL*, *RBM15* and *STRA8* expression values by either the proportion of the spermatogonia (Figure 3e, f, g and h), ~~being~~as it is the germ cell stage

that predominantly expresses *CCNE1*, *DAZL*, *RBM15* and *STRA8* or by the proportion of the spermatogonia plus spermatocytes (Figure 3i, j, k and l), ~~being the latter~~as the meiotic germ cells ~~stage that~~ potentially expresses these genes although at much lower levels (GermSAGE, http://germsage.nichd.nih.gov; GermOnline http://www.germonline.org), present in a seminiferous tubule of the sample. Significant differences in cellular transcript levels were additionally found for *CCNE1*, *DAZL*, *RBM15* genes between SpF-HS patients and controls and between SpF-MA patients and controls, when considering either the proportion of spermatogonia or the proportion of spermatogonia and spermatocytes in the tubule ($p \leq 0.002$). Interestingly, cellular transcript levels for *STRA8* were found statistically decreased in SpF-MA when compared with CS when considering either the proportion of spermatogonia or the proportion of spermatogonia and spermatocytes in the tubule ($p \leq 0.002$). These results suggest that the decreased tissular expression levels in SpF are not attributable to a decreased number of spermatogonia or spermatocytes in the tubule but to a reduced ~~expression capability of the~~number of transcripts in immature germ cells. Furthermore, the decreased cellular *STRA8* expression levels observed in SpF-MA suggest that the number of genes whose expression is altered in immature germ cells might be higher than that initially observed in the whole tissue with meiotic arrest.

Strikingly, the decrease cellular expression observed in patients was accompanied by a higher severity in spermatogenic impairment, and cellular expression levels of *CCNE1*, *DAZL*, *RBM15* and *STRA8* genes were highly significantly and positively correlated with the number of elongated spermatids in the tubule (Pearson's correlation range; r=0.68-0.87; $p<0.0001$) (Supplemental Table 4.B.)

Gene expression pattern in Germ cell tumours

Relative expression values of fourteen spermatogonia preferentially- expressed genes were found to have extremely significant differences in expression between GCT and CS samples: *ATM*, *BARD1, CCNE1*, *CDKN1C*, *DAZL*, *DICER1*, *E2F3, FBXO32*, *MRE11A*, *RAD50*, *RBM15, SIRT6, STRA8* and *TM9SF2* (p<0.002). Less consistent statistical differences were found for four additional genes *BAX, CCND1, POLD1* and *XPA* (p<0.05). All these differentially expressed genes, with the exception of *BAX*, were found to be under-represented in GCT samples compared with controls (Table 2; Supplemental Table 3).

Regarding the somatically expressed genes; ten genes were found to have statistical differences in expression: *AMHR2*, *BCL3, SCIN*, *SMARCA1*, *SOX9, SPAG7, VEGFA* (p<0.002) *BMPR1A, CDKN1A* and *FASLG* (p<0.05). *FASLG* was found to be over-expressed, whereas the other nine genes were under-expressed, in GCT samples compared with controls (Table 2; Supplemental Table 3).

Some differences in expression behaviour were found when GCT samples were divided into CIS and non-CIS samples (Supplemental Table 3): over-expression of the *KIT* gene (an established marker for early-stage GCT) in CIS samples consistently agreed with the histological diagnosis of samples. No difference in expression was found for *BARD1*, *BMPR1A*, *CCND1*, *CDKN1A*, *CDKN1C*, *SMAD3* and *XPA* in CIS samples compared with CS controls, whereas their transcript values were found highly decreased in non-CIS samples (p<0.002). *DLK1* was significantly over-expressed in CIS samples (p=0.012), contrary to the marked under-expression found in non-CIS samples (p<0.002). Thus, this set of genes is, somehow, associated with different stages in tumour progression.

Expression signature by functional categories

In order to obtain some clues about the functional pathways that are affected in testis with SpF, genes were grouped into functional clusters according to the process they are involved in: cell proliferation, apoptosis/cell cycle, meiosis, DNA repair, transcription regulation, post-transcriptional regulation and degradation. The functional expression signature was compared with that obtained from the GCT samples (Supplemental Table 3).

Some of the spermatogonia-preferential transcripts differentially represented between the SpF subgroups and controls encode proteins involved in the regulation of the mitotic and meiotic cell cycle (cyclins, cyclin –dependent-kinase-inhibitors and DNA polymerases). *CCNE1* and *POLD1* were significantly under-expressed, this being particularly noticeable for *CCNE1*, whereas *CCND1* was over-expressed in the MA samples. In GCT only the *CCNE1* transcript value of this group of genes was significantly decreased compared with controls. Furthermore, we observed decreased expression levels of several genes that encode for putative RNA-binding proteins (such as the germ cell specific *DAZL* and *RBM15*), other proteins essential for production of miRNAs (such as *DICER1)*, proteins involved in the protein degradation pathway (*FBXO32* and *TM9SF2*) and also proteins implicated in the homologous recombination in meiosis (*MRE11A* and *RAD50)* in both SpF and GCT groups.

There were no differences in expression of genes such as *BMPR1A, c-KIT* and *VEGFA* (cell population proliferation), *BAX* gene (apoptosis), *ATM*, *SIRT6* and *XPA* (DNA repair genes) observed between SpF and CS groups, in contrast to the observed expression alteration of these genes in GCT.

The expression of all the Sertoli-specific expressed genes, with the exception of *SLC4A11* was significantly affected in the GCT samples, whereas only those genes

involved in cell cycle/apoptosis, *SLC4A11* and *SCIN,* were significantly decreased in the SpF meiotic altered samples.

Protein expression

We sought to determine whether the changes in transcript levels would correlate with modifications at the protein level. At the same time we aimed to determine whether the suggested alterations in gene expression affected expression levels of encoded proteins in the germ line. We focused first on robust differences in expression levels which might be discerned by immunohistochemistry and chose *CCNE1* and *DAZL* as examples of spermatogonia-associated genes specifically expressed in the germ line. Secondly, we selected proteins whose coding genes presented an upregulated expression in SpF samples such as DLK1 and CDKN1C (Figure 4).

CCNE1 immunostaining (A-D; Figure 4) in CS samples was observed mainly in both the nucleus and the cytoplasm of postmeiotic secondary spermatocytes/round spermatids, and in the in nucleus of spermatogonia. Sertoli cells showed less intense CCNE1 expression in the nucleus; some staining of the cytoplasms of interstitial Leydig cells and Sertoli cells was also seen, which tended to increase in samples with MA and SCO; in the germ line of SpF samples CCNE1 expression was exclusively detected in the spermatogonia and not detected in primary spermatocytes and elongated spermatids, when present. The DAZL protein (E-H, Figure 4) was almost exclusively present in the cytoplasm of primary pachytene spermatocytes in CS samples and therefore expression was negative in testis with SCO. Overall, immunoexpression in testis sections of both CCNE1 and DAZL protein decreased within seminiferous tubules, in the germ line, as spermatogenic damage progressed, showing good correlation with RNA expression.

CDKN1C (I-L; Figure 4) in CS samples was seen in the cytoplasm of Sertoli cells and Leydig cells; the immunostaining for Leydig cells was more intense in MA and SCO samples; peritubular cells were positive in SCO; however in SpF samples some spermatogonia and primary spermatocytes also exhibited moderate expression of CDKN1C, leading to a global increase in CDKN1C expression in biopsies with SpF. The immunoreactivity of DLK1 (M-P; Figure 4) in CS samples seemed to be restricted to the cytoplasm of a few Leydig cells, which were more frequently stained in MA, and more so in SCO suggesting a relevant contribution of Leydig cells to DLK1 expression. Overall, immunoexpression of both CDKN1C and DLK1 protein in testis sections, preferentially in somatic cells for DLK1, increased as spermatogenic damage progressed, showing good correlation with RNA expression.

DISCUSSION

The aim of this study was to ~~reveal~~ assess early testicular transcriptional changes that ~~are~~ could be involved in human severe deficiency of sperm production. We focused our attention mainly on the expression profile of premeiotic germ cells as this is a key step in male germ cell maturation. The accurate quantification of testicular mRNA levels in SpF by RT-qPCR experiments led to the identification of differences in expression of certain genes associated with spermatogonia in the absence of any apparent morphological and/or numerical change of this specific cell type.

The gene expression profile in SpF~~shown by the altered genes~~ can be used as a basis for identification of candidate genes ~~involved in the aetiology of~~that contribute to spermatogenic impairment. SpF and GCT expression signature comparison could additionally give some clues about the molecular mechanisms underlying the origin of these alterations. Our data indicate that, in the SpF patients, a large proportion of spermatogonia-preferentially expressed genes exhibited reduced testicular expression levels when compared with CS individuals. As expected, the number of genes whose expression was altered as well as the magnitude of increase or decrease of gene expression in GCT was even higher, possibly related to the fact that the germ line in testicular tumour has undergone a dedifferentiation process representing an extreme situation of gene expression deregulation of spermatogenic impairment. Interestingly, gene expression signatures of both phenotypes, SpF and GCT, share some aberrant patterns of gene expression supporting the idea that the participation of these genes is essential for physiological germ cell development. In contrast, other genes are differentially affected in both pathological groups suggesting that they contribute to the phenotype and could be used as potential molecular markers.

The cellular complexity of the testis is an inherent problem which should be taken into account when studying gene expression profiles in this organ. As the pathological seminiferous tubules lack germ cells to varying degrees, changes in gene expression at the tissue level can reflect changes in the capability for transcribing the mRNA in a specific cell type as well as changes in the cell type composition in pathological testis. The absence of significant differences in the spermatogonia and Sertoli cell number among the SpF-MA, SpF-HS and CS groups in our study indicates that transcript levels cannot be attributed to the presence or absence of these specific cell types. Additionally, we are aware of the fact that the levels of different mRNAs could change as the proportion of immature germ cells is different in tubules in conserved spermatogenesis, in which all stages of germ cell are present, and in those in maturation arrest, where only some stages are present. In this condition, we assume that there would be less mRNA from most spermatogonia-expressed genes in CS testis than in SpF testis. Our study shows that most of the differentially expressed genes showed higher expression in testis with conserved spermatogenesis suggesting that the reduction of target genes could not be attributable to either the spermatogonia cell number or to different proportion of this germ cell stage in the tubule, but to real differences in the expression capability of the cell. Furthermore, we describe reduced cellular expression levels of four germ line specific genes in SpF samples supporting this premise.

The spermatogonia-related genes whose transcripts were differentially represented between SpF subgroups and controls included genes involved in specific functional pathways. A first group of genes encodes proteins involved in the regulation of the mitotic and meiotic cell cycle such as cyclins, cyclin-dependent-kinase-inhibitors and DNA polymerases indicating that the regulation of this specific functional gene cluster in the initial stages of spermatogenesis is critical for further differentiation and

meiosis of germ cells. Additionally, decreased expression levels of several genes encoding putative RNA-binding proteins (such as the germ cell specific *DAZL* and *RBM15*), other proteins essential for production of miRNAs such as *DICER1*, and also proteins involved in the protein degradation pathway (*FBXO32* and *TM9SF2*) in both SpF and GCT groups, underlie the complexity of post-transcriptional control in proliferation and differentiation of germ cells. *MRE11A* and *RAD50,* involved in homologous recombination in meiosis, also showed altered expression in meiotic blockade. Unexpectedly, we observed no difference in testicular expression for *STRA8*, a well known gene involved in meiotic cell cycle, participating in chromosome pairing and in the process that leads to stable commitment to the meiotic cycle (Mark *et al.*, 2008), in SpF group nor in SpF-MA subtype samples when compared with controls. ~~The reduced transcript levels of other genes expressed in spermatogonia as the ones involved in piRNA processing machinery such as *PIWIL2* and *TDRD1* in SpF, as we recently described (Heyn *et al.*, 2012) further supports the role of a proper gene expression in early germ line stages for a successful sperm production.~~ Interestingly, an additional statistically significant reduction in the expression levels of germ cell–specific genes per spermatogonia was observed in MA and HS when compared to CS samples, demonstrated for *CCNE1*, *DAZL* and *RBM15* genes, being more pronounced in the MA phenotype suggesting that the expression capacity in immature germ cells correlates with the severity of testicular damage. More intriguingly, a decreased cellular expression level was even observed for *STRA8* in MA pattern, suggesting that in MA phenotype, premeiotic cellular expression could be affected ~~in a lesser extend~~ for a larger number of genes. The remarkable correlation coefficient between the *CCNE1*, *DAZL, RBM15* and *STRA8* transcript levels per cell and the number of elongated spermatids in the testicular tubule additionally underlines the determinant role of

premeiotic *CCNE1*, *DAZL, RBM15* and *STRA8* expression in the progression of the spermatogenic process.

In previous studies, the reduction of gene expression in SpF patients has been mainly attributed to the decreased number of germ cells that specifically express the gene of interest (i.e. significantly lower *DAZL* mRNA concentrations were previously found in testes of non-obstructive azoospermic men (Lin *et al.*, 2001)), although germ cell quantification was not properly performed. Here, we demonstrate that the changes in expression observed among groups could be not exclusively explained by the immature germ cell number but the contribution of the reduced cellular expression of i.e. *DAZL* mRNA in spermatogenic impairment should be also taken into account.

Protein data on nonobstructive testicular tissue corroborate our mRNA expression results: CCNE1 and DAZL protein decreased within seminiferous tubules, in the germ line, as spermatogenic damage progressed. The reduced transcript levels of other genes expressed in spermatogonia as the ones involved in piRNA processing machinery such as *PIWIL2* and *TDRD1* in SpF, as we recently described (Heyn *et al.*, 2012) further supports the role of a proper gene expression in early germ line stages for a successful sperm production.

The expression levels of genes participating in cell population proliferation, mitochondria-mediated apoptosis and DNA repair (assuring the maintenance of genome integrity) are in general maintained in SpF, contrary than in GCT, supporting the idea that they are similarly ~~normally~~ processed in meiotic derangement and in conserved spermatogenesis. However, there is one exception possibly due to other regulatory pathways: the levels of spermatogonia-specific full-length *BARD1* transcript (Feki *et al.*, 2004; Irminger-Finger *et al.*, 2001), involved in germ cell apoptotic events, could be

repressed in SpF by the high levels of FSH in spermatogenic failure samples (Feki *et al.*, 2004).

Although the transcription profile of spermatogonia-associated genes in SpF is different from that in GCT, some functional clusters are affected in both phenotypes: genes with functions in cell cycle, transcription and post-transcriptional regulation and protein degradation. Meanwhile, other spermatogonia-expressed genes encoding proteins involved in cell proliferation, apoptosis and DNA repair pathways are not affected in SpF, suggesting that in spermatogenic failure, although the abnormal initiation of the meiotic process is already determined in these immature germ cells, these cells maintain their activity related to mitosis and cell proliferation.

Regarding the Sertoli-specific expressed genes, the absence of differences in gene expression of six out of eight genes studied in the SpF-MA and SpF-HS phenotypes suggest that, in spermatogenic derangement, the functions of Sertoli cells are in general maintained, but not those involved in cell cycle/apoptosis (*SLC4A11* and *SCIN*) related to germ cell support. Previous studies have shown that, in mouse, chemically-induced germ cell depletion can alter expression of several Sertoli cell genes (Jonsson *et al.*, 1999; Maguire *et al.*, 1993; O'shaughnessy *et al.*, 2008) demonstrating that germ cells regulate Sertoli cell activity by means of the regulation of Sertoli cell gene expression. Here we observed a similar pattern in a pathological naturally-occurring phenotype. We observed a gradual decrease of *SLC4A11* and *SCIN* transcript levels attributable to the progressive depletion of germ cell stages (SCO>MA>HS>CS). It is noteworthy that even in the presence of a two-fold increase of the number of Sertoli cells as a compensatory phenomenon in SCO, the decrease in expression of certain Sertoli cell genes becomes much more noticeable in the total absence of germ cells.

Some recent microarray studies have assessed global gene expression analysis in testicular biopsies from infertile men in order to identify the genes critical for spermatogenesis (Chalmel *et al.*, 2012; Von Kopylow *et al.*, 2010; Ellis *et al.*, 2007; Feig *et al.*, 2007; Rockett *et al.*, 2004; Fox *et al.*, 2003). In these studies specific germ cell transcription patterns are inferred from infertile testicular phenotypes in men and a pattern of significantly decreased regulated genes has been attributed to the degree of spermatogenic failure and the loss of specific stages of germ cells. We provide data suggesting that the molecular basis for severe spermatogenic impairment is more complex than initially proposed. In SpF the immature germ cells present an altered and decreased transcriptional pattern of certain genes, and thus the number of genes associated with these cells could be underestimated from microarray studies of infertile samples. Furthermore, our results should be helpful to better interpret microarray or future NGS transcriptome studies. Elucidation of a more extensive transcriptional profile with the detailed analysis of testicular cellular composition could be important in understanding the molecular mechanisms that underlie male infertility.

It is conspicuous that most of the altered spermatogonia-related genes are involved in essential processes during spermatogenesis and aberrant expression is often associated with spermatogenic defects. Whether the observed differential expression profiles represent the cause or consequence of maturation arrest remains to be elucidated. Considering the heterogeneous aetiologies and highly individual molecular causes which may underlie spermatogenic failure in humans, the molecular changes described here may represent common symptoms, but may also reflect early dysfunction events affecting germ cells which may causally contribute to the pathology. This data should be useful in delineating the patterns of gene expression involved in

male germ cell maturation deficiency, which may contribute to understanding male infertility.

In summary, our study provides evidence that the premeiotic stage of germ cell differentiation, exhibits associated patterns of gene expression deregulation in spermatogenic impairment, which is more severe in meiotic arrest. This altered gene expression pattern is observed despite there being no apparent morphological and/or numerical change observed in this early stage of the germ cell population. In our previous study, the spermatocyte capacity to express meiosis-related genes was observed to be markedly reduced in spermatogenic failure, contributing to meiosis impairment (Terribas *et al.*, 2010). Our present data demonstrate that the low spermatogenic efficiency in infertile men is accompanied not only by meiotic but also by premeiotic events in spermatogenesis, which contribute to spermatogenic blockade. Furthermore, the differences in expression during the initial stages of spermatogenesis in SpF-MA individuals suggest that this phenotype is already determined or arises in the premeiotic stages of the germ line.

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AUTHOR'S CONTRIBUTION

S.B. performed the RNA experiments and analysed the data. F.A. performed immunohistochemistry and histological interpretation of data. E.F. provided samples and clinical data. L.B. performed clinical assessment, provided samples, and critically reviewed the manuscript. S.L. conceived and designed the experiments, supervised the analysis of data and wrote the manuscript.

FIGURE LEGENDS

Supplemental Figure 1. Absolute expression levels of candidate reference genes (a.) in SCO, MA, HS and CS groups and (b.) in GCT and CS groups.

○, □, outlying values. * $p < 0.05$, (a.) Kruskal-Wallis test and (b.) Mann-Whitney test.

SCO: Sertoli cell only syndrome; MA: maturation arrest at spermatocyte stage; HS: hypospermatogenesis; CS: conserved spermatogenesis

Figure 1. Spermatogonia-preferentially expressed genes whose relative expression values were statistically altered in patient SpF group (MA and HS sub-phenotypes) compared with CS controls. Expression levels relative to *PGK1/PGM1* are shown.

* $p < 0.05$, Mann-Whitney test; ** $p < 0.002$, Mann-Whitney test and Bonferroni correction.

MA: maturation arrest at spermatocyte stage; HS: hypospermatogenesis; CS: conserved spermatogenesis.

Figure 2. Testicular somatic cell-preferentially expressed genes that showed differences in gene expression in infertile SCO, MA and HS patients relative to CS controls. Expression levels relative to *PGK1/PPIA* are shown..

* $p < 0.0083$, Mann-Whitney test and Bonferroni correction.

SCO: Sertoli cell only syndrome; MA: maturation arrest at spermatocyte stage; HS: hypospermatogenesis; CS: conserved spermatogenesis.

Figure 3.

Tissular expression profiling of *CCNE1* (a), *DAZL* (b), *RBM15* (c) and *STRA8* (d) by quantitative real-time qPCR in testis with conserved spermatogenesis (CS),

hypospermatogenesis (HS) and maturation arrest at the spermatocyte (MA) Expression levels relative to *PGK1* and *PGM1* are shown. Expression per cell profiling of *CCNE1*, *DAZL*, *RBM15* and *STRA8* displayed as expression ratio per spermatogonium (x100) (e, f, g, h) and expression per cell profiling of *CCNE1*, *DAZL*, *RBM15* and *STRA8* displayed as expression ratio per spermatogonium and spermatocyte (x100) (i, j, k, l). The horizontal bar indicates median value. Significant differences from the control are indicated: *p<0.05; **p<0.005.

Figure 4.

Immunohistochemical localization of selected proteins in sections of human testes with different phenotypes. From left to right, first column (A,E,I,M) shows sections of CS, second column (B,F,J,N) corresponds to SpF-HS, third column (C,G,K,O) represents SpF-MA, and the fourth column (D,H,L,P) displays SCO pattern. CCNE1 protein staining is shown in panels A-D, DAZL in E-H, CDKN1C in I-L, and DLK1 expression in M-P, were stained Leydig cells are indicated by arrows. See explanation of the cellular localization of different proteins in the text. Original magnification was X400 for panels A-L, and X200 for M-P. Scale bar in A and M = 100 µm.

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