The interaction of modified histones with the bromodomain testis-specific (*BRDT*) gene and its mRNA level in sperm of fertile donors and subfertile men

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Abstract

As histone modifications have been suggested to be involved in the regulation of gene expression after fertilisation, the present study aimed to analyze the interaction between the bromodomain testis-specific (*BRDT*) gene and differentially modified histones in human spermatozoa. The *BRDT* transcript level was studied to identify possible correlations between epigenetic changes, mRNA level and subfertility associated with impaired sperm chromatin condensation. Chromatin immunoprecipitation (ChIP) was performed with ejaculates from fertile and subfertile men using antibodies against specifically acetylated and methylated histone H3. Immunoprecipitated DNA was analysed by real-time quantitative PCR with primer pairs for *BRDT*. The *BRDT* mRNA level was screened by real-time RT-PCR. ChIP assay revealed co-localisation of acetylated and methylated histones within promoter and exon regions of the *BRDT* gene in fertile men. Interestingly, reduced binding of investigated modified histone modifications was observed in the *BRDT* promoter of subfertile patients. Different mRNA levels of *BRDT* have been detected in a group of infertile patients, as well as in fertile men. Enrichment of methylated histones within the *BRDT* promoter of fertile sperm suggests that this epigenetic mark may cause repression of *BRDT* after fertilisation, and may be changed in infertile patients. Our data suggest that reduced histone methylation in the promoter of *BRDT* may be associated with increased transcript levels in subfertile patients.

Reproduction (2010) 140 435–443

Introduction

During human spermiogenesis, ~85% of histones are replaced by protamines (Tanphaichitr *et al.* 1978, Gatewood *et al.* 1987, Steger *et al.* 1998), while the remaining histones exhibit acetylated and methylated lysine residues within their N-terminal tails (Allfrey *et al.* 1964). It has been proposed that modified histones are involved in the regulation of gene expression after fertilisation (Bernstein *et al.* 2007). Genes associated with modified histones, therefore, might be essential for early embryo development, as it has been demonstrated that genome regions expressed during early embryogenesis are histone-enriched (Gardiner-Garden *et al.* 1998, Wykes & Krawetz 2003, Rousseaux *et al.* 2008). In addition, Hammoud *et al.* (2009) reported that nucleosomes and especially histone modifications are enriched at genomic regions of developmental importance, such as imprinted gene clusters, microRNA clusters, *HOX* gene clusters and promoters of stand-alone developmental transcription and signalling factors. Arpanahi *et al.* (2009) demonstrated that acetylated histone 4 at

lysine 12 (H4K12ac) is associated with gene regulatory regions, including promoter regions and sequences recognised by the CCCTC-binding factor.

In addition to epigenetic marks transmitted by modified histones from the spermatozoon to the oocyte, certain mRNAs are synthesised prior to transcriptional arrest during spermatogenesis and then stored in mature spermatozoa which have been postulated to make a paternal contribution to gene expression in the early embryo (Ostermeier *et al.* 2004, Lalancette *et al.* 2008). Although most of the spermatozoal transcripts disappear at the four-cell stage of embryo differentiation, studies derived from mouse implantation embryos confirmed that some RNAs are translated after fertilisation. For example, paternal PLC ζ (*PLCZ1*) RNA has been suggested to activate the oocyte through calcium oscillation after fertilisation (Swann *et al.* 2006). Another transcript, *Kit*, may epigenetically alter the phenotype of murine offspring (Rassoulzadegan *et al.* 2006).

The *BRDT* gene and its transcripts (bromodomain testis-specific gene, also referred to as *FSRG3* or *BRD6*)

represent potential candidates for studying the role of epigenetic changes for fertility and paternal contribution to the embryo, as they play an important role in the establishment of proper germ cell differentiation (Jones *et al.* 1997, Pivot-Pajot *et al.* 2003, Shang *et al.* 2004). The first bromodomain of the BRDT protein recognises the acetylation marks of histone 4 in order to start chromatin remodelling (Morinière *et al.* 2009). In addition, male mice carrying a mutation within the allele of the first bromodomain are subfertile (Shang *et al.* 2007). *BRDT* transcripts have been demonstrated to be specifically expressed in human and murine testis. In the latter, *Brdt* mRNA has been observed from pachytene spermatocytes to spermatids (Haynes *et al.* 1992, Zheng *et al.* 2005, Shang *et al.* 2007). In man, a splicing variant of the *BRDT* gene (*BRDT*-NY) was found in embryonic and adult human testis and spermatozoa from fertile men (Zheng *et al.* 2005). By contrast, transcripts of this splicing variant could not be detected in testis of some azoospermic men including arrest of spermatogenesis at the level of round spermatids (Zheng *et al.* 2005). As spermatozoa are known to contain a variety of transcripts (Zao *et al.* 2006) and mRNAs could be delivered from sperm to oocyte (Ostermeier *et al.* 2002), it might be possible that *BRDT* mRNA influences chromatin compaction during spermiogenesis and/or expression of *BRDT* during early embryogenesis (Miller *et al.* 2005, Zheng *et al.* 2005).

The aim of the present study was to analyse whether, in human spermatozoa, the *BRDT* gene is associated with modified histones. Based on our preliminary experiments testing specificity of several antibodies against modified histones H3 and H4 by immunohistochemistry (Hecht *et al.* 2009) and chromatin immunoprecipitation (ChIP) assay, we selected following histone modification for our study with fertile and subfertile patients: histone H3 acetylated at lysine 9 (H3K9ac), histone H3 dimethylated at lysine 9 (H3K9dm), histone H3 trimethylated at lysine 9 (H3K9tm) and histone H3 trimethylated at lysine 27 (H3K27tm).

Binding loci for histones in the *BRDT* gene were investigated as shown in Fig. 1. In addition, we analysed whether there are differences in *BRDT*-histone interactions between fertile and infertile men. Our recent results obtained from ChIP using antibodies

against H4K12ac in combination with a promoter array (ChIP-on-chip) provided additional information on the binding of H4K12ac to the promoter region of *BRDT* in fertile (Arpanahi *et al.* 2009) and infertile patients (data not published). Finally, we analysed the *BRDT* mRNA level in sperm of both fertile and subfertile men, as the population of RNA in mature ejaculated spermatozoa may reflect transcriptional activity during normal or impaired spermatogenesis.

Results

ChIP in combination with real-time quantitative PCR (qPCR) indicated an enrichment of H3K9ac, H3K9dm, H3K9tm and H3K27tm associated with the promoter region and exon of the *BRDT* gene in fertile men. We used unmodified histone H3 as positive control, as it is present in all nucleosomes. H3 exhibited the lowest enrichment in promoter region 1 (10%) and the highest enrichment in the exon (35%). Non-specific IgG served as a negative control, which showed low enrichment values of non-specific binding to the genomic regions of the *BRDT* gene (Fig. 2).

In spermatozoa from fertile men, H3K9ac exhibited a relative enrichment to the input in all regions of the *BRDT* gene. The highest enrichment was found in promoter region 2 (9%) and the lowest enrichment in promoter region 1 (2%; Fig. 2). Furthermore, histones H3K9dm, H3K9tm and H3K27tm showed enrichment at the *BRDT*-binding site (2–21%) with the lowest values in the exon (Fig. 2).

In spermatozoa of subfertile patients, ChIP in combination with qPCR was performed using two different pooled groups of patients ($n=5$ and $n=6$). The negative control IgG revealed nearly no enrichment (0.04–0.6%). The non-specific binding was lower than in fertile men (Fig. 2). The positive control H3 revealed an enrichment which was by trend higher than in fertile men (9–98%; Fig. 2). Interestingly, enrichment values of acetylated H3K9 were lower in the promoter regions (1–4%), but four times higher in the exon when compared with fertile men (Fig. 2). Methylated histones exhibited a lower enrichment than in fertile men (0–5%), except for H3K27tm (3–24%; Fig. 2). Within the exon, H3K27tm displayed enrichment 12 times higher than in fertile men.

Regarding the s.d., all values were lower in infertile patients than in fertile men, except for IgG at promoter 1 and exon region, H3 at promoter 3, H3K9dm at promoter 1 and H3K27tm at the exon.

Data obtained from ChIP-on-chip promoter microarray revealed that histone H4 acetylated at lysine 12 (H4K12ac) was not associated with the *BRDT* promoter, neither in sperm from fertile men, nor from subfertile patients (Fig. 3A and B). Using material from three fertile donors, the validation of microarray data confirmed no binding sites. However, interaction with another member of the BRD family, namely *BRD9*, was identified

Figure 1 Part of the genomic region of the *BRDT* gene (chromosome 1, p 22.1). Primer pairs are located within the promoter (Pr1–3) and the first exon (Ex). TSS, transcription start site.

Figure 2 Results from ChIP in combination with real-time PCR. Immunoprecipitated DNA of fertile (A) and subfertile men (B) and different modified histones (H3K9ac, H3K9dm, H3K9tm and H3K27tm) were used. H3 served as positive control, and IgG served as negative control. Three different primer pairs were applied to analyse binding sites within the promoter region, while one primer pair was specific for a binding site within the first exon. Lane 1 on each gel represents a 1000-bp marker (Bio-Rad). (C) Enrichment pattern of H3K9ac, H3K9dm, H3K9tm and H3K27tm in the promoter region (1–3) and the first exon of the *BRDT* gene in a group of fertile and subfertile men. Range of s.d.s of modified histones in different gene regions: fertile men: IgG: 0.3–3.3, H3: 4.1–30.6, H3K9ac: 1.9–9.4, H3K9dm: 1.0–9.5, H3K9tm: 1.5–15.4, H3K27tm: 1.2–15.1; subfertile men: IgG: 0.0–0.4, H3: 1.3–9.1; H3K9ac: 0.0–4.8, H3K9dm: 0.3–2.9, H3K9tm: 0.1–0.4, H3K27tm: 0.6–2.1.

by binding peaks in DNA of fertile donors (false discovery rate score, FDR=0.140; Fig. 3C). In subfertile patients, the peak corresponds to the *BRD9*-binding site with FDR=0.367 (Fig. 3D). We did not detect a binding position for H4K12ac in the promoter, suggesting that this histone modification represents no potential interaction partner with the testis-specific *BRDT* gene. *BRDT* mRNA was identified in sperm from fertile and subfertile men (Fig. 4A), and a difference was found between both groups (*t*-test: $P=0.017$). A range of

one- to six-fold changes was observed in ejaculates of fertile men. By contrast, in ejaculates of subfertile patients, there was a range of 1- to 11-fold changes. Means of the fold changes from fertile versus subfertile men were calculated, as shown in Fig. 4B.

Statistical analysis based on a calculation of correlation coefficients between *BRDT* mRNA level and diagnostic parameters revealed no significant correlation with any of the investigated variables (Fig. 5). There was no significant relation of *BRDT* mRNA level to pregnancy. However, a tentative relationship could be observed when *BRDT* mRNA level was correlated with sperm morphology (Spearman correlation coefficient $r=-0.261$, $P=0.073$) and aniline blue staining (2+3; %, $r=0.260$, $P=0.084$). In addition, patients exhibiting aniline blue staining of spermatozoa lower than 25% (2+3) also displayed a lower *BRDT* mRNA level ($<25\%$ aniline blue (2+3)—mean *BRDT* mRNA level = 5.77 ± 2.49 vs $>25\%$ aniline blue (2+3)—mean *BRDT* mRNA level 7.11 ± 2.0). As normal sperm morphology and efficient chromatin condensation are required for successful fertilisation, the correspondence between *BRDT* mRNA level with sperm morphology and chromatin condensation is not surprising.

Discussion

In the present study, we analysed the interaction of histones H3K9ac, H3K9dm, H3K9tm and H3K27tm with the *BRDT* gene, as well as the *BRDT* mRNA level, in sperm of fertile and subfertile men. ChIP analysis provided evidence that the promoter region of the *BRDT* gene may interact with more than one histone modification. These findings are in agreement with data from Bernstein *et al.* (2005, 2006, 2007) and Azuara *et al.* (2006), who described overlapping regions of two different modified histones as 'bivalent domains'. In addition, identical binding patterns of several modified histones have been reported by Wang *et al.* (2008). The combinatorial patterns of modified histones at these domains might, therefore, conserve the epigenetic information for chromatin remodelling (Wade *et al.* 1997, Strahl & Allis 2000, Turner 2000, Schreiber & Bernstein 2002). However, when pluripotent cells start to differentiate, only one of two marks is preserved (Bernstein *et al.* 2005, Azuara *et al.* 2006) being involved in either expression or repression of a gene.

Concerning enrichment of H3K9ac, H3K9dm, H3K9tm and H3K27tm at the *BRDT* gene in spermatozoa, we observed only small differences between fertile and subfertile men. In subfertile men, only a few values were not within the range of the s.d. of fertile men. The association of methylated histones with *BRDT* suggests its transcriptional repression during late spermatogenesis, as the association and repressive function of the three states of H3K9 methylation (in combination with H3K9ac) were described for the

Figure 3 ChIP-on-chip analysis with H4K12ac peak visualisation within *BRDT* and *BRD9* promoters by SignalMap Genome Browser (NimbleGen). Log₂ ratios were calculated from the input signals for the experimental (10% genomic DNA) and test samples (H4K12ac) that were co-hybridised to the array. No significant peaks within *BRDT* promoters on chromosome 1 could be identified in fertile (A) and subfertile (B) men. The *BRD9* peak with FDR score=0.140 (orange bar) indicated high confidence binding of H4K12ac in fertile men (C). In subfertile patients (D), low probability of the peak in *BRD9* was observed, as indicated by grey bar. FDR=0.367. The lower the FDR score, the more likely the peak corresponds to a histone-binding site on the DNA.

stromal membrane-associated GTPase-activating protein (*SMAP*) gene in human CD4⁺T cells (Wang *et al.* 2008).

In general, it has been reported that lysine acetylation correlates with transcriptional activity, while methylation can have both effects: transcriptional activation (H3K4, H3K36; Santos-Rosa *et al.* 2002), or repression (H3K9, H3K27 and H4K20; Bernstein *et al.* 2007, Li *et al.* 2007). However, the function of modified histones in transcriptional regulation is not fully clarified. Furthermore, it has been proposed that every modification has the potential to activate or repress by different conditions. In our study, dimethylated H3K9 could have a repressive function in the promoter and an activating function in the exon, as Vakoc *et al.* (2005) proposed for this modification in mammalian chromatin.

Using microarray analysis, Dobson *et al.* (2004) detected *BRDT* mRNA expression in primary and secondary oocytes, and that a two- to three-fold

downregulation of *BRDT* on days 1–2 post-fertilisation suggests a paternal contribution to the regulation of *BRDT* expression in the embryo. These data support our hypothesis that an increase in histone methylation in the promoter of spermatozoal DNA may arrest the expression of *BRDT* until day 3 after fertilisation which is then followed by rearrangement of paternal epigenetic marks with maternal histones.

According to ChIP-seq results (Hammoud *et al.* 2009), the *BRDT* promoter was found to be occupied by H3K4me3. Furthermore, authors showed that trimethylated H3K27 (H3K27tm) is significantly enriched at developmental promoters that are repressed in early embryos, including many bivalent (H3K4tm/H3K27tm) promoters. Concerning *BRDT*, we could identify high enrichment of H3K27tm to promoter 1 and promoter 2 regions in fertile donors (Fig. 2) giving rise to the idea of bivalent promoter occupancy in *BRDT* gene. In contrast,

Figure 4 Analysis of *BRDT* transcripts. (A) *BRDT* mRNA transcripts in spermatozoa of fertile ($n=6$, white bars) and subfertile ($n=49$, grey bars) men. The lowest expression value was reset to 0 as reference; therefore, for subfertile men, only 48 grey bars are shown instead of 49. The range of one- to six-fold changes was observed in ejaculates of fertile men, while higher individual variation of 1- to 11-fold changes has been recognised in the group of subfertile patients. (B) Distribution of *BRDT* fold change by group of fertile versus subfertile patients. The *BRDT* level was significantly higher in the group of subfertile men (mean $6.53 \pm \text{s.d. } 2.27$) when compared with the group of healthy controls (mean $4.12 \pm \text{s.d. } 2.12$; $P_{t\text{-test}}=0.017$).

Figure 5 Scatter plot matrix showing correlation between BRDT level and following diagnostic parameters from infertile patients ($n=49$): sperm density/ml (Spearman correlation coefficients $r=-0.120$, $P=0.413$), sperm morphology (%) ($r=-0.261$, $P=0.073$), aniline blue staining (2+3) (%) ($r=0.260$, $P=0.084$), number of inseminated oocytes ($r=-0.108$, $P=0.460$), number of embryos in 2nd pronucleus stage ($r=-0.071$, $P=0.629$), correctly fertilised oocytes ($r=0.054$, $P=0.714$).

we found 33 gene promoters that were associated with acetylated histone H4K12ac (known as activating mark) and were expressed in four-cell embryos (Zhang *et al.* 2009). Among them were the following genes: *MTMR3*, *PAX9*, *MAGEA1*, *CAMSAP1* and *SLC41A2*.

In our ChIP experiment, unmodified histone H3 exhibited a high enrichment in fertile and subfertile men. This may be due to higher amounts of unmodified H3 than modified H3. Other possibilities could be a better H3 antibody affinity and a poor input DNA preparation.

Finally, we were able to demonstrate *BRDT* mRNAs in sperm of both fertile and subfertile men. A significant difference could be detected between fertile and subfertile men. This is in contrast with data from Wawrzik *et al.* (2009), who showed similar results for germ cells of oligozoospermic and normozoospermic men. The presence of sperm mRNAs could be explained by the fact that these transcripts are stored for further function in the zygote, as proposed by Miller *et al.* (2005). However, differential *BRDT* mRNA levels were observed within the group of fertile donors and subfertile patients suggesting individual variations. Nevertheless, comparing mean values of mRNA level from subfertile and fertile group, we could demonstrate a clear difference between both groups. Owing to high level of variability observed in the *BRDT* mRNA of donors, we suggest to use larger cohorts of control ejaculates for further experiments in order to establish the normal, physiological range of mRNA levels in spermatozoa.

The fact that spermatozoa contain high levels of *BRDT* mRNA, and both the promoter and the coding region of the *BRDT* gene are associated with modified histones representing epigenetic marks that may be transmitted to the oocyte suggests a possible role for *BRDT* during early embryo development.

Materials and Methods

Ejaculate samples of fertile donors and subfertile patients

Ejaculate samples of fertile donors ($n=5$ for ChIP and $n=6$ for mRNA experiments) were collected at the Department of Urology, Pediatric Urology and Andrology, University of Giessen, Germany. All samples were classified according to WHO criteria (World Health Organization 2010).

Ejaculate samples of subfertile donors ($n=11$ for ChIP and $n=49$ for mRNA experiments) were obtained from patients who underwent ICSI procedure at the IVF Center of Middle Hessa, Germany. Subfertile patients were selected due to teratozoospermia and asthenozoospermia. In addition, aniline blue staining has been performed (Henkel *et al.* 1994) representing an indicator for defective chromatin condensation during spermiogenesis, as it stains lysine-rich histones, but not arginine or cysteine-rich protamines. In total, 64.3% of patients and 37.5% of fertile donors displayed defects in chromatin compaction. All samples for this study were obtained after patients signed written informed consent. Ejaculate and ICSI parameters of fertile and subfertile men are summarised in Tables 1 and 2.

Chromatin immunoprecipitation

ChIP assay requires at least 10^6 sperm cells per immunoprecipitation. Owing to low sperm concentration, ejaculate samples from infertile patients were pooled. The first pool consisted of five patients, and the second pool contained sperm samples from six individuals. Formaldehyde cross-linking and ChIP assays of ejaculated sperm were performed, according to the manufacturer's instructions (Chromatin Immunoprecipitation Assay Kit, Millipore, Temecula, CA, USA). DNA was recovered by phenol/chloroform extraction and ethanol precipitation. The size of sonicated DNA was monitored by gel electrophoresis. Sonicated cells were then diluted tenfold in

Table 1 Ejaculate parameters of fertile donors.

	<i>n</i>	%
Ejaculate volume (ml)		
<2	0	0
≥2	8	100
Sperm density (million/ml)		
<20	0	0
≥20	8	100
Morphology (%)		
<15	3	37.5
≥15	5	62.5
Motility (%)		
<50	1	12.5
≥50	7	87.5
WHO a <20	1	12.5
WHO a ≥20	7	87.5
WHO b <20	6	75
WHO b ≥20	2	25
WHO c <10	4	50
WHO c ≥10	4	50
WHO d <50	1	12.5
WHO d ≥50	7	87.5
Aniline blue staining (2+3) (%)		
<25	5	62.5
≥25	3	37.5

ChIP dilution buffer with protease inhibitors and processed for ChIP. For each condition, 10% of immunoprecipitation without antibody was saved as total input chromatin. Immunoprecipitations were performed using 2–8 µl of polyclonal rabbit anti-IgG (negative control; Abcam, Cambridge, UK), polyclonal rabbit anti-H3 (positive control; Abcam), polyclonal rabbit anti-H3K9ac (Abcam), monoclonal mouse anti-H3K9dm (Abcam), polyclonal anti-H3K9tm (Abcam) and polyclonal rabbit anti-H3K27tm (Millipore, Schwalbach, Germany) antibodies. After the final step, each immunoprecipitation sample was resuspended in 20 µl of aqua bidest. For chip array with H4K12ac (Abcam ab1761), purified IP-probe (Qiaquick Purification Kit, Qiagen) and 10 ng of input material (total chromatin) were prepared by adapting the protocol for whole genome amplification using the Sigma GenomePlex WGA kit (Sigma–Aldrich) as described in O'Green *et al.* (2006). The required amount of DNA for microarray analysis (4 µg/sample) was generated using WGA Reamplifikation Kit (Sigma). Amplicons were applied to the HG18 human 5 kb promoter array from NimbleGen System. Labelling and hybridisation of the probes for ChIP-on-chip analysis was performed by NimbleGen Service ImaGenes GmbH (Berlin, Germany), for details see Arpanahi *et al.* (2009). Microarray data are available for download at GEO <http://www.ncbi.nlm.nih.gov/geo/>, accession numbers GSM 327833 and GSM327832. Data from subfertile patients are not yet published.

Real-time PCR

For evaluation of ChIP assays, amplification of 105–176 bp products from the genomic *BRDT* promoter region and exon was applied (Fig. 1). PCR products did not overlap. Primers (MWG, Ebersberg, Germany) are listed in Table 3. We chose

three primer pairs for the promoter region, as they represent the most likely binding sites for histone modifications (Arpanahi *et al.* 2009, Hammoud *et al.* 2009). Although binding of histones to exon regions seems to have no impact on the transcriptional activity in the embryo, histone binding to the *BRDT* exon could not completely be excluded. Therefore, we applied one primer pair that specifically amplifies a sequence within the first exon of the *BRDT* gene in order to investigate, whether there is a possible disorder due to impaired chromatin condensation in the coding region of infertile patients. PCRs contained 2 µl of immunoprecipitation sample, 10 pmol/µl of each primer, 12.5 µl iQ SYBR Green Supermix (Bio-Rad) in 23 µl of total volume. PCR conditions were an initial step of 3 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 58–62 °C and 1 min at 72 °C. After amplification, 10 µl of the PCR product was electrophoresed on an 1.5% agarose gel, and DNA was visualised under u.v. light. Each PCR generated only the expected specific amplicon, as shown by melting temperature profiles of final products and gel electrophoresis. In addition, PCR products were checked by sequencing (Scientific Research and Development GmbH, Oberursel, Germany).

Table 2 Ejaculate and post-fertilisation parameters of patients with subfertility treated by ICSI.

	<i>n</i>	%
Ejaculate volume (ml)		
<2	11	18.3
≥2	49	81.7
Sperm density (million/ml)		
<20	19	31.7
≥20	41	68.3
Morphology (%)		
<15	53	88.3
≥15	7	11.7
Motility (%)		
<50	43	71.7
≥50	17	28.3
WHO a <20	57	95.0
WHO a ≥20	3	5.0
WHO b <20	47	78.3
WHO b ≥20	13	21.7
WHO c <10	36	60.0
WHO c ≥10	24	40.0
WHO d <50	11	18.3
WHO d ≥50	49	81.7
Aniline blue staining (2+3) (%)		
<25	20	35.7
≥25	36	64.3
No. of inseminated oocytes		
<10	39	65.0
≥10	21	35.0
No. of embryos in 1st pronucleus stage		
<10	49	81.7
≥10	11	18.3
No. of embryos in 2nd pronucleus stage		
<10	49	81.7
≥10	11	18.3
Correctly fertilised oocytes (%)		
<60	16	26.7
60–90	31	51.7
100	13	21.7
Pregnancy		
Yes	33	55.0
No	27	45.0

Table 3 Primer pairs used in real-time PCR and standard PCR experiments.

Name	Accession number	Primer sequence 5'-3'	Product size (bp)
BRDT-pr1-F	NM_001726	GAG GAA TCA GTG TGG GAT CG	119
BRDT-pr1-R	NM_001726	TCA GTA GTG CCC CCA TCT TC	119
BRDT-pr2-F	NM_001726	GAA GAT GGG GGC ACT ACT GA	176
BRDT-pr2-R	NM_001726	GCG AGA CTG CAT CTC AAA CA	176
BRDT-pr3-F	NM_001726	GTG CGT TGC TTT CTT GTG AA	105
BRDT-pr3-R	NM_001726	TTT CCT GAA GCG GAA GTC TG	105
BRDT-ex-F	Jones <i>et al.</i> (1997)	AAT GTC TCT GCC AAG TCG ACA A	175
BRDT-ex-R	Jones <i>et al.</i> (1997)	AGC ATC CAC AGG ACG TTG AAA G	175
ACTB-F	NM_001101	TTC CTT CCT GGG CAT GGA GT	90
ACTB-R	NM_001101	TAC AGG TCT TTG CGG ATG TC	90
HSP90AB1-F	NM_007355	AAG AGA GCA AGG CAA AGT TTG AG	120
HSP90AB1-R	NM_007355	TGG TCA CAA TGC AGC AAG GT	120
GAPDH-F	NM_002046	TCT CCT CTG ACT TCA ACA GCG AC	126
GAPDH-R	NM_002046	CCC TGT TGC TGT AGC CAA ATT C	126
ATP5B-F	NM_001686	TCA CCC AGG CTG GTT CAG A	80
ATP5B-R	NM_001686	AGT GGC CAG GGT AGG CTG AT	80
CD45-F	Lambard <i>et al.</i> (2004)	TGC AGA TGC CTA CCT TAA TGC	844
CD45-R	Lambard <i>et al.</i> (2004)	CAC ATT GCA GCA CTT CCA TT	844
KIT-F	Lambard <i>et al.</i> (2004)	AGT ACA TGG ACA TGA AAC CTG G	780
KIT-R	Lambard <i>et al.</i> (2004)	GAT TCT GCT CAG ACA TCG TCG	780

Calculations for graphs were processed as follows: C_t = cycle number at which each PCR reaches a predetermined fluorescence threshold. It is set within the loglinear range of all reactions. Enrichment of the immunoprecipitated sample compared with input material was calculated as follows: $\Delta C_t = C_t(\text{input}) - C_t(\text{immunoprecipitated sample})$ and % total = $2^{\Delta C_t} \times 10$ (according to 10% input chromatin of total immunoprecipitated chromatin) (Frank *et al.* 2001). For fertile men, PCRs with material of different donors ($n=6$) were performed. The mean of the C_t values of different donors was calculated. In subfertile men, the ejaculate of five and six donors was pooled to perform ChIP. In this case, the mean of different PCRs with the same material was calculated.

RNA isolation and first-strand cDNA synthesis

RNA extraction from ejaculates was performed using the RNA extraction kit RNeasy MINI (Qiagen) including a DNase treatment. The purity of extracted RNA was assessed by measuring the optical density at wavelengths of 230, 260 and 280 nm with Nanodrop Photometer (Eppendorf, Hamburg, Germany). In addition, RNA integrity was analysed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) using the RNA 6000 Nano Assay according to the manufacturer's instructions and the Agilent 2100 Bioanalyzer Software (Fig. 6). The RNA 6000 ladder was purchased from Ambion (Austin, TX, USA).

First-strand cDNA synthesis was performed using Sensiscript (Qiagen). For cDNA synthesis, 2 μ l 10 $\times$ RT buffer, 2 μ l dNTP mix (5 mM each dNTP), 2 μ l Oligo-dT primer (10 μ M), 1 μ l (10 IU) RNase inhibitor and 1 μ l Sensiscript reverse transcriptase were added. Samples were incubated at 37 °C for 60 min.

Furthermore, contaminations with somatic cells or immature germ cells were checked using primer pairs for leukocytes (*CD45*) and testicular germ cells (*KIT* (*C-KIT*); Lambard *et al.* 2004). We did not detect any contamination in our samples (Fig. 7).

Real-time RT-PCR

Real-time RT-PCR was performed using iQ SYBR Green Supermix and iCycler (Bio-Rad). Per sample, 25 ng total cDNA of fertile ($n=6$) and subfertile ($n=49$) men were used for amplification of heat shock protein (*HSP90AB1*; previously known as *HSPCB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β -actin (*ACTB*) and ATP-synthase subunit 5B (*ATP5B*) as reference genes and *BRDT* as target gene. For the detection of the *BRDT* gene, the primer pair for the first exon was used. The PCR efficiency for each single primer pair was determined using serial tenfold dilutions of cDNA transcripts of each gene. All PCR assays yielded specific products of the expected sequence, and the efficiency of all assays was between 95 and 99%. The linear correlation coefficient (R^2), an indicator of fit for the standard curve (equation: PCR efficiency = $(10^{(-1/\text{slope})} - 1) \times 100$), plotted to the standard data points of all genes ranged from 0.988 to 1.000. Cycling conditions were 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. Primer

Figure 6 Quality control of sperm RNA using agarose gel electrophoresis (3 = RNA) and Agilent 2100 Bioanalyzer (1, RNA 6000 ladder, 2, RNA, 4, electropherogram). High amount of RNA (>4000 nucleotides) is indicative for intact RNA. 18S RNA was absent and 28S RNA was reduced in sperm RNA. This was expected as sperm contain no or a reduced amount of rRNA (Ostermeier *et al.* 2002).

Figure 7 Contamination check of sperm mRNA using *CD45* and *KIT* as markers. Agarose gel showing PCR products of *CD45* and *KIT* in fertile donors (A) and subfertile patients (B). Lanes 1–3 indicate samples free of contamination isolated from ejaculated sperm. Lane 4 shows the PCR product when blood cDNA (*CD45*) and accordingly testis cDNA (*KIT*) were used as positive controls. Lane 5 contains Rnase-free water.

pairs are summarised in Table 3. Values recorded for quantification were the fractional cycle numbers (C_f) where the background-corrected amplification curves crossed a threshold value. Subsequently, candidate reference genes have been selected using GeNorm software (GenEx pro 4.3.7, MultiD Analyses AB, Göteborg, Sweden), as previously described by Vandesompele *et al.* (2002) and Normfinder software (GenEx pro 4.3.7; Andersen *et al.* 2004).

Applying GeNorm software, *ACTB* and *GAPDH* were determined as best combination of two reference genes (M value = 1.3). Normfinder software determined *ACTB* and *HSP90AB1* as the most stable reference genes with lowest variability examined (variability of the genes: 0.6). For further real-time RT-PCR data analysis, we combined *GAPDH* and *ACTB* as reference genes. Relative expression levels were calculated using the GenEx pro 4.3.7 software.

Statistical analysis

The statistical analysis was performed using SAS V9.2 (SAS Institute, Cary, NC, USA). As normal distribution could be assumed for the parameter *BRDT* fold change, comparing the two groups healthy donors ($n=6$) and subfertile patients ($n=49$), t -test could be applied. However, normal distribution could not be assumed for other parameters of interest (ejaculate parameters from subfertile patients and healthy donors). Therefore, Spearman correlation coefficients were calculated for the evaluation of possible associations between *BRDT* level and diagnostic parameters. These analyses were done in explorative sense, which means the computed P values are only a measure for the reproducibility of the observed results, under the null hypothesis there is no association between *BRDT* fold change and the diagnostic parameter of interest.

Declaration of interest

The authors declare that they have no direct or indirect commercial or financial incentive associated with publishing this manuscript.

Funding

This work was supported by the Bayer Vital GmbH and the German Society of Andrology (grant: Bonn – Bad Godesberg, 01.12.07).

Acknowledgements

We thank Barbara Fröhlich, Kerstin Wilhelm and Angela Erkel, Department of Urology, Pediatric Urology and Andrology, Justus Liebig University of Giessen, for technical assistance, as well as Christine Scheibelhut, Institute for Medical Informatics, Justus Liebig University of Giessen, for discussion regarding statistical analysis.

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Received 15 March 2010

First decision 12 April 2010

Revised manuscript received 25 May 2010

Accepted 10 June 2010