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Expression of Somatic DNA Repair Genes in Human Testes

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Abstract Meiosis is the key process for recombination and reduction of the diploid chromosome set to a haploid one. Many genes that have been found in yeast or mouse models to play a role in meiosis are also important for the repair of DNA damage in somatic cells. To study the DNA repair gene transcriptome during male germ cell development, we have developed a specialized cDNA microarray with 181 human genes which are involved in different somatic DNA repair pathways and/or cell cycle control and 45 control house-keeping genes. This DNA repair gene chip was used to quantify the mRNA expression levels in three human testes samples versus a fibroblast RNA pool. Two hundred twenty genes on the chip (including house-keeping genes) showed detectable expression levels in adult testes. Sixty-four DNA repair- and cell cycle-associated genes showed higher expression levels in testicular cells than in mitotically dividing fibroblasts and, therefore, are likely to be implicated in meiosis. The microarray results of 17 genes with increased expression levels were validated with reverse Northern blots or real-time quantitative RT PCR. Systematic analyses of the meiotic DNA repair gene transcriptome may provide new insights into the genetics of male (in)fertility. J. Cell. Biochem. 100: 1232–1239, 2007.

Key words: cDNA microarray; DNA repair genes; meiosis; spermatogenesis; testes

Male germ cell development is a complex process that involves stem-cell renewal, meiosis, and dramatic reorganization of the resulting haploid genome. Because human spermatogenesis continues throughout adult life, it requires mitotic growth of spermatogonial stem cells before they enter the meiotic differentiation pathway. Separation of homologous chromosomes during the first (reductional) meiotic division and separation of sister chromatids during the second (equational, as in mitosis) division occurs without an intervening S phase. The major difference between meiosis and mitosis is formation of the synaptonemal complex and recombination between the aligned sister chromatids of homologous chromosomes in meiotic prophase I. Many DNA repair proteins that are required for the detection and processing of damaged DNA in somatic cells are also functional during meiotic recombination [for review, see Marcon and Moens, 2005]. Some somatic DNA repair proteins (i.e., RAD51) also have meiosis-specific homologs (DMC1).

Each meiotic recombination event is initiated by the formation of a double-strand break (DSB) in one sister chromatid during early prophase I [Keeney et al., 1997]. However, unlike DNA breaks in somatic cells that result from DNA damage or replication machinery slippage, the approximately 300 meiotic DSBs in mouse spermatocytes are induced by expression of specific genes, in particular *SPO11*. The serine/threonin protein kinases ATM and ATR may function in detection and signaling of SPO11-induced DSBs [Moens et al., 1999]. The homologous recombination proteins RAD51 and DMC1 associate with these breaks and promote strand invasion and homology search [Shinohara and Shinohara, 2004]. The resulting RAD51/DMC1 nodules also bind replication protein A and later on the mismatch repair protein MSH4 and the DNA-helicase BLM

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[Moens et al., 2002]. The majority (>90%) of the approximately 300 nodules are resolved without cross-overs. Only approximately 25 recombination nodules in mouse mid-pachytene spermatocytes contain the mismatch repair proteins MLH1 and MLH3, which are thought to be active in reciprocal recombination [Kolas et al., 2005]. The breast cancer susceptibility gene products BRCA1 and BRCA2, which are involved in DNA damage detection and repair in somatic cells, bind to the unpaired chromosome cores in meiotic cells [Chen et al., 1998]. Biallelic inactivation of BRCA2 causes an autosomal recessive disorder, Fanconi anemia. In addition to BRCA2/FANCD1, several other FANC proteins, that is, FANCA, FANCC, and FANCD2 are active during meiosis [Nadler and Braun, 2000; Wong et al., 2003; Sharan et al., 2004].

Accumulating evidence suggests that meiotic recombination evolved from ancestral somatic DNA repair mechanisms. Already existing DNA repair proteins were adapted to meiosis where they perform related but not necessarily identical functions as in somatic cells. In order to systematically identify somatic DNA repair proteins which are involved in meiotic differentiation, we have compared the expression patterns of known DNA repair genes between testicular cells and exponentially growing fibroblasts. To this end, we have constructed a specialized DNA repair gene chip. We assumed that genes showing equivalent expression levels in testicular cells and fibroblasts are involved primarily in somatic cell cycle control and mitotic division. Higher expression of a gene in testicular cells was considered a good indicator for its involvement in meiosis-specific functions. In addition to DNA repair genes that were already known to be present and functional during meiosis, we identified several dozen genes whose function during meiosis remains to be elucidated.

MATERIALS AND METHODS

Cell Substrates

Human testes samples were obtained from three patients of proven fertility, by open incision biopsy, in association with an orchitectomy. Tissues samples were either shock-frozen in liquid nitrogen and stored at −80°C or used immediately for RNA preparation. Primary skin fibroblast cultures from four unrelated individuals were grown in minimal essential medium with Earle's salts (Gibco BRL) supplemented with amino acids, vitamins, antibiotics, and 15% fetal bovine serum.

cDNA Microarrays

Two independent cDNA clones each of 181 DNA repair and cell cycle genes and 45 control genes were obtained from the resource center of the German Human Genome Project (http://www.rzpd.de). Clone inserts were amplified with M13 forward and reverse primers. Amount and size of the generated PCR products were checked on agarose gels. Purified PCR products were resuspended in 10 μl each of 3 × SSC, 1.5 M Betain (Sigma), and printed with a robotic spotting device (OmniGrid 100, Genemachines) and stealth print head (SPH32, Telechem/arrayIT) on glass slides (Corning GAPS II).

Total RNAs of testicular and fibroblast cells were isolated using Trizol reagent (Invitrogen). The cDNA targets were directly labeled by incorporation of fluorescent nucleotides during reverse transcription. Total RNA (30 μg) was used in a random priming reaction containing 100 μM Cy3- or Cy5-dUTP (Amersham), 200 μM dTTP, and 500 μM each of dATP, dCTP, and dGTP (Roche). The labeled probe was incubated with 1 μl RNase H (BioLabs) for 20 min at 37°C and then purified with the NucleoSpin Extract kit (Macherey-Nagel).

Differentially labeled cDNA targets were resuspended in 50% formamide, 6 × SSC, 0.5% SDS, 5× Denhardt's solution. Then 1.5 μl tRNA (10 μg/μl) and 1 μl human Cot-1 DNA (20 μg/μl) (Invitrogen) were added. The resulting 220 μl of hybridization mixture were denatured at 95°C for 5 min and then cooled on ice. cDNA microarrays were hybridized in a Lucidea SlidePro Hybridizer (Amersham) for 15 h. Each hybridization experiment was done at least twice, swapping the dyes for testicular and fibroblast RNA labeling.

Images were acquired with an Affymetrix Array Scanner 428. Cy3- and Cy5-fluorescence intensities were measured separately at 532 and 635 nm, respectively. The resulting 16-bit data files were imported into Microarray Imagene 4.2 software (BioDiscovery). Local background of raw spot intensities was subtracted with the Gene Sight Light software. Empty spots were excluded from further analysis. Expression data from different hybridization experiments were

normalized using the Lucidea Score Card (GM Health Care), which was printed on each slide. This normalization tool is particularly suitable for low- and middle-density chips, where normalization across the fluorescence intensity of all spotted genes does not produce reliable results. Means of normalized log-products of all (>6) microarray hybridization experiments were used for further analysis.

Reverse Northern Blots

Gene arrays for reverse Northern blot experiments were fabricated by spotting 2 μl (200–400 ng) aliquots of purified PCR products of 12 DNA repair-associated genes (*CDKN1A*, *DMAP1*, *DNMT3A*, *FANCF*, *HK1*, *ILF2*, *MBD4*, *MSH6*, *POLQ*, *RFC2*, *TRF4*, and *XRCC3*) in duplicates onto nylon filters. In addition, 2 μl (200–400 ng) aliquots of two house-keeping genes (A*CTIN* and *GAPDH*) and 2 μl (6–8 ng) aliquots of a 1:100 dilution of the unlabeled cDNA sample (corresponding to the hybridization probe) were spotted as controls.

Reverse transcription of approximately 30 μg target RNA resulted in 9–12 μg cDNA. Approximately one half (4–6 μg) of this cDNA sample was labeled with the digoxigenin DNA labeling kit (Roche) according to the manufacturer's instructions. Filters (macroarrays) were hybridized overnight at 42°C with digoxygenated cDNA targets dissolved in 5 ml hybridization mixture (0.5 M NaH_2PO_4, 1 mM EDTA, pH 7.2, 7% SDS, and 1% BSA). Then they were washed 2× for 15 min at 50°C in 2 × SSC, 1% SDS, and 2× for 15 min at 50°C in 0.5 × SSC, 1% SDS. The digoxigenin luminescent detection kit (Roche) was used for signal detection.

Auto-exposure of all filters was done with an AIDA chemiluminescence imager. The resulting TIFF files were imported into the AIDA image analysis program 3.40 (Raytest). The low spot density allowed us to discriminate background hybridization intensity (indicated by black squares in Fig. 1) versus spot intensity for each spot (indicated by black circles in Fig. 1). The background intensity surrounding each spot was subtracted from the signal (spot) intensity. Negative intensity values were called "no signal." Hybridization intensities of all spots on a particular filter were normalized to the amount of cDNA. cDNA spot intensity after hybridization of the same cDNA sample correlated well with the amount of cDNA used in this experiment. The intensities of the two or four spots for each gene or cDNA sample on the blot were averaged and the standard deviation was calculated. To compare the hybridization intensities between two different filters, the average cDNA spot intensity on one filter was divided by that on the other filter. The resulting ratio was used as normalization factor for all genes on the same filters.

Real-Time Quantitative RT PCR

Quantitative RT PCR analysis of six DNA repair-associated genes (*LIG3*, *POLQ*, *RAD51L3*, *RECQL*, *RFC5*, and *TOP3A*) was performed with predesigned and optimized Qiagen QuantiTect Primer Assays (QT00017155, QT00040838, QT00021168, QT00201740,

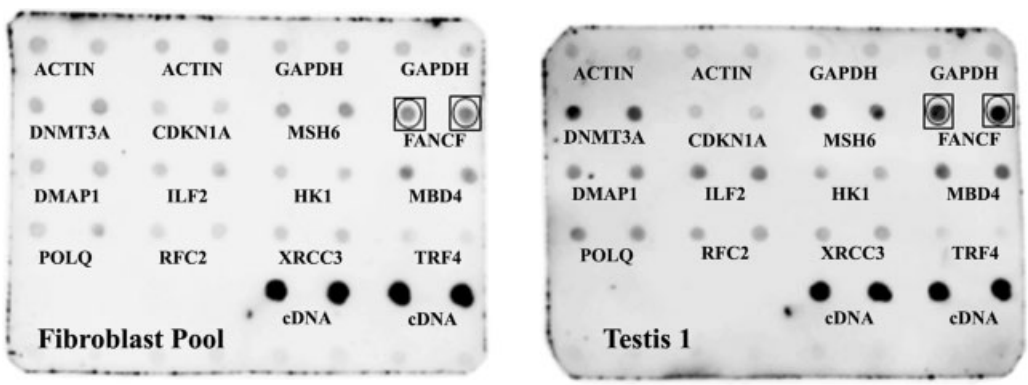

Fig. 1. Northern blot analysis of 12 genes that showed expression differences between testicular cells and fibroblasts in microarray experiments (Table I). Two house-keeping control genes, *ACTIN* and *GAPDH*, as well as the diluted cDNA sample were spotted in quadruplicates, the 12 test genes in duplicates. The first blot was hybridized with pooled fibroblast RNA and the second blot with testicular RNA. As an example only, *FANCF* (indicated by black circles) displays a four- to fivefold higher expression in testicular cells than in fibroblasts.

QT00071288, QT00051814) on an Applied Biosystems 7500 Fast Real-Time PCR system. Each 25 μl reaction volume contained 100 ng cDNA template, 3 μl 10× QuantiTect Primer Assay, 15 μl 2× QuantiTect SYBR Green I PCR Master Mix, and RNase-free PCR graded water. PCR was performed with one cycle of 95°C for 15 min (first stage) and 40 cycles of 94°C for 15 s, 57°C for 30 s, and 72°C for 40 s (second stage). Relative quantification was carried out with the delta-delta-CT method (Applied Biosystems 7500 Fast System SDS Software version 1.3), using 18S rRNA (QT00199367) as endogenous control.

RESULTS

We used a customized cDNA microarray to quantify the expression levels of 181 genes that are known to be involved in DNA repair [Wood et al., 2005] and/or cell cycle control in three human testicular samples versus pooled fibroblast RNA. In addition, the microarray contained 45 human house-keeping genes and 30 plant genes as positive and negative hybridization controls, respectively, as well as the Lucidea Score Card for normalization of hybridization intensities. At least two hybridization experiments with dye swapping were performed with each testicular RNA sample. Thus, relative expression levels of our study genes in testicular versus fibroblast cells were calculated from at least six independent hybridization experiments. Our working hypothesis was that genes that are mainly important for somatic DNA repair and mitotic cell cycle control are transcribed at approximately equivalent levels in exponentially growing fibroblasts and testes. We assumed that genes with meiosis-specific functions are transcriptionally upregulated in testicular cells, containing a high percentage of meiotic cells.

One hundred seventy five (of the 181) DNA repair- and cell cycle-associated genes on our chip and all 45 house-keeping genes showed detectable expression levels in testicular cells. This implies that the vast majority (97%) of somatic DNA repair proteins are also active during meiosis. Forty-eight DNA repair- and cell cycle-associated genes showed at least twofold increased expression levels in testicular cells (Table I). Sixteen additional genes had 1.5- to twofold increased expression levels (Table II). Previous studies in rodents and/or humans demonstrated that 15 of the 48 genes with at least twofold increased expression, for example, *ATM* [Hamer et al., 2004], *BRCA1* [Xu et al., 2003], *FANCC* [Nadler and Braun, 2000], and *MSH2* [Richardson et al., 2000], and 8 of the 16 genes with 1.5- to twofold increased expression, for example *BRCA2* [Sharan et al., 2004] and *MLH1* [Kolas et al., 2005], are essential for meiosis. *DNMT3A* and *MBD4* may be involved in epigenetic gene silencing during germ cell differentiation [Galetzka et al., 2006]. The remaining 41 transcriptionally upregulated genes have not been linked to meiosis so far. Interestingly, only one gene, *CDKNA1*, showed a lower expression by microarray analysis in testicular cells than in fibroblasts.

Two independent experimental techniques for quantification of single gene expression were employed to judge the quality of our microarray data. First, 12 differentially expressed genes were analyzed by reverse Northern blots (Fig. 1). Two control house-keeping genes, *ACTIN* and *GAPDH*, as well as diluted cDNA were spotted in quadruplicates, the 12 test genes in duplicates. Northern blots were hybridized with pooled fibroblast RNA and with total RNAs from each of the three testicular samples. Because we and others [Dheda et al., 2004; Neuvians et al., 2005] found *ACTIN* and *GAPDH* expression to vary between cell substrates, the hybridization intensities of all spots on a particular filter were normalized to the amount of cDNA. All 11 test genes that showed at least twofold higher expression levels in testicular cells by microarray analysis were validated with reverse Northern blot experiments (Table III). The fact that expression levels varied considerably (by a factor of 2 to 3) among the three testicular samples may be explained by interindividual differences, that is, in the percentages of meiotic cell types versus contaminating tubular and stromal cells, or technical differences, that is, time from biopsy to shock-freezing. Testis 2 consistently showed the highest expression levels. *CDKNA1*, the only gene with decreased expression in our microarray experiments displayed a 1.5- to twofold higher expression level in testicular cells by reverse Northern blots. Because reverse Northern blots are generally more sensitive than microarrays and *CDKNA1* has been reported to be involved in the regulation of meiosis [West and Lahdetie, 1997], the microarray data for this gene are likely to be false. Conflicting results of microarray and reverse

TABLE I. Genes Showing at Least Twofold Higher Expression Levels in Human Testicular Cells than in Fibroblasts

Gene	Increased expression	UniGene cluster	Accession number	Meiotic function reported
ATM	4×	Hs.435561	BC043617.1	Hamer et al. [2004]
BRCA1	3×	Hs.194143	ENST00000272146	Xu et al. [2003]
CCNH	4×	Hs.146607	BT006764.1	
DMAP1	8×	Hs.8008	BC072418.1	
DNMT3A	8×	Hs.515840	U12685.1	Galetzka et al. [2006]
ERCC3	2×	Hs.469872	AF265228.1	
ERCC4	4×	Hs.460019	M31899.1	
FANCC	3×	Hs.494529	U64315.1	Nadler and Braun [2000]
FANCF	3×	Hs.523543	BC015748.1	
GTF2H2	4×	Hs.191356	BC064557.1	
HK1	6×	Hs.370365	AK128226	Mori et al. [1993]
ILF2	6×	Hs.75117	BG121872	Lopez-Fernandez et al. [2002]
ILF3	5×	Hs.465885	NM012218	
LIG3	4×	Hs.100299	BX640996.1	
MBD4	4×	Hs.369849	AF072250	Galetzka et al. [2006]
MDM2	5×	Hs.156519	M92424	
MSH2	6×	Hs.597656	AK223284	Richardson et al. [2000]
MSH6	6×	Hs.445052	BC071594	
NTHL1	4×	Hs.66196	NM000179.1	
PARP1	4×	Hs.177766	NM002528.4	Gotoh et al. [1999]
PMS1	6×	Hs.111749	NM007266.1	Lipkin et al. [2002]
PMS2L4	4×	Hs.278468	CR749432	
PMS2L5	2×	Hs.397073	BC029419.1	
POLQ	5×	Hs.241517	AB017004	
POLR1C	3×	Hs.520146	CR936627	
POLS	5×	Hs.481542	AF076838	
RAD17	2×	Hs.16184	BX647297	Freire et al. [1998]
RAD51L3	3×	Hs.125244	NM004584.2	
RAD9	4×	Hs.240457	NM002884.2	
RAP1	3×	Hs.190334	BC020496	
RECQL4	3×	Hs.31442	AF078695	
REV3L	4×	Hs.232021	NM006341.2	
REV7/MAD2L2	3×	Hs.19400	NM181471	
RFC2	4×	Hs.139226	NM181578	
RFC5	7×	Hs.506989	NP061907.2	
RIF	4×	Hs.223617	NM004875.2	
SIRT5	4×	Hs.282331	BC035196	
SIRT6	4×	Hs.423756	AF233396	
SPO11	4×	Hs.159737	AF169385	Romanienko and Camerini-Otero [2000]
TOP2A	4×	Hs.156346	NM001067	Cobb et al. [1999]
TOP3A	10×	Hs.435124	NM004618	
TOP3B	5×	Hs.436401	AL833505	
TP53AP1	6×	Hs.274329	NP009164.1	
UBE2B	4×	Hs.385986	NM006999.3	Baarends et al. [2003]
UBE2N	5×	Hs.524630	NM003337	van der Laan et al. [2004]
UBE2V1	5×	Hs.420529	NM003348	
XAB1	5×	Hs.18259	NM199203	
XRCC3	6×	Hs.549075	AK126706	

Total RNAs from three testicular samples were compared to a human fibroblast pool, using a specialized cDNA microarray.

Northern blot analyses for a few genes are not unexpected, because these two methods rely on different normalization tools. Each normalization strategy can reveal and unmask expression differences.

Increased expression levels of six additional genes of interests were validated by real-time quantitative RT PCR. Because we found comparable 18S rRNA levels in our fibroblast pool and testicular samples, relative gene expression was normalized to the amount of 18S rRNA. By real-time quantitative RT PCR, all six test genes showed an at least twofold increased average expression level in testicular cells and considerable variation in expression between testicular samples (Fig. 2).

Validation of 17 of 18 differentially expressed genes tested (95%) by either reverse Northern blot or real-time RT PCR demonstrates that overall our microarray results are reliable. Collectively, our expression profiling data support the hypothesis that most somatic DNA repair genes are active during meiosis and that many of them are transcriptionally upregulated in meiotic versus mitotic cells.

DISCUSSION

The aim of our study was to compare the expression levels of a large number of DNA repair- and cell cycle-associated genes in meiotically and mitotically dividing cells. The

TABLE II. Genes Showing 1.5- to Twofold Higher Expression Levels in Human Testicular Cells than in Fibroblasts

Gene	Increased expression	UniGene cluster	Accession number	Meiotic function reported
ADPRT	1.7×	Hs.177766	M18112.1	Meyer-Ficca et al. [2005]
BRCA2	1.6×	Hs.34012	U43746.1	Sharan et al. [2004]
CDK7	1.5×	Hs.184298	BC000834.2	Kim et al. [2001]
DDB1	1.9×	Hs.290758	BC051764.2	Holmberg et al. [2005]
GTF2H3	1.6×	Hs.355348	BC065250.1	
HCNP	1.7×	Hs.9822	AF226051.1	
IKBKB	1.7×	Hs.413513	AF031416.1	
MLH1	1.7×	Hs195364	BC006850.1	Kolas et al. [2005]
NTPBP	1.6×	Hs.18259	AB044661.1	
RAD50	1.6×	Hs.242635	AF057299.1	Bannister and Schimenti [2004]
RBBP5	1.5×	Hs.519230	BC075060.2	
SIRT1	1.8×	Hs.369779	AF083106.2	
TERF1	1.8×	Hs.584810	U40705.1	Scherthan et al. [2000]
TINF2	1.6×	Hs.496191	AF195512.1	
WRN	1.7×	Hs.567358	AF091214.1	
XRCC1	1.9×	Hs.98493	BC023593.2	Walter et al. [1996]

Total RNAs from three testicular samples were compared to a human fibroblast pool, using a specialized cDNA microarray.

observation that >35% of the identified genes with increased mRNA levels in testicular cells (Tables I and II) are already known to play a role in meiosis supports our assumption that genes that are more highly expressed in testicular cells than in fibroblasts are fundamental to meiotic differentiation. However, because testicular tissue consists of many different cell types, we cannot totally exclude the possibility that the increased expression levels of some identified genes are not related to meiosis but reflect their functions in haploid (postmeiotic) cells, Sertoli cells, and/or contaminating tubular and stromal cells.

In conceptually related expression profiling studies (http://germonline.org/), transcript levels were compared between total human testis, chondrocytes, and vascular smooth muscle as well as between spermatocytes and spermatids. Fifteen of 48 genes with at least twofold increased expression levels in testicular cells in our study, *BRCA1*, *CCNH*, *ERCC3*, *GTF2H2*, *MSH6*, *PMS2L5*, *RAD17*, *RAD9*, *RECQL4*, *RFC5*, *POLR1C*, *POLS*, *SPO11*, *TOP2A*, and *TOP3A*, also showed higher expression in testicular cells versus chondrocytes and smooth muscle cells and in spermatocytes versus spermatids. Eighteen genes, *ATM*, *DNMT3A*, *FANCC*, *HK1*, *LIG3*, *MDM2*, *PMS1*, *PMS2L4*, *POLQ*, *RAP1*, *REV3L*, *SIRT5*, *SIRT6*, *TOP3B*, *TP53AP1*, *UBE2B*, *UBE2V1*, and *XRCC3*, only showed higher expression in testicular cells versus mitotically dividing fibroblasts (this study). Five of these genes, *ATM* [Hamer et al., 2004], *FANCC* [Nadler and Braun, 2000], *HK1* [Mori et al., 1993], *PMS1* [Lipkin et al., 2002], and *UBE2B* [Baarends et al., 2003] are known to perform important

TABLE III. Relative Expression Levels of 12 Genes in Three Testicular Cell Samples Compared to Human Fibroblasts, as Determined by Reverse Northern Blot Analysis

		Relative expression in microarrays	Relative expression in reverse Northern blots		
Gene	IMAGE clone	Pooled testes 1–3	Testes 1	Testes 2	Testes 3
CDKN1A	p956 B0758	−3	2	2	1.5
DMAP1	p956 C1370	8	3	6	2
DNMT3A	p956 I23112	8	5	8	4
FANCF	p956 B1567	3	4	5	4
HK1	p998 H213644	6	3	4	2
ILF2	p956 H1042	6	5	8	4
MBD4	p956 I1453	4	2	6	2
MSH6	p956 D0464	6	3	5	3
POLQ	p956 D2053	4	6	10	4
RFC2	p956 L19177Q2	4	6	12	4
TRF4	p956 C141177Q	6	2	4	1.5
XRCC3	p956 M1588	6	3	6	3

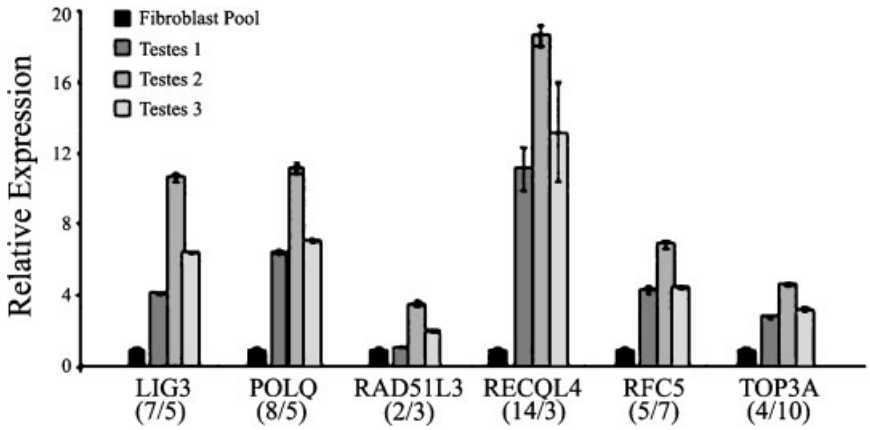

Fig. 2. Validation of six differentially expressed genes by real-time quantitative RT PCR. Relative expression of a given gene in three independent testicular samples (gray bars) is compared to the mRNA level in the fibroblast pool (black bar), which was chosen as a reference. The first number in parentheses below a given gene name represents the average of increased fold expression in testes, as measured by RT PCR, the second number represents the increased fold number from microarray experiments.

meiotic functions. This clearly indicates that our experimental approach is capable of identifying meiosis-relevant genes and provides novel insights into the meiotic transcriptome.

DNA repair and cell cycle genes are fundamental for maintaining genome integrity in somatic cells. Unrepaired somatic DNA damage may result in cell death or tumorigenic transformation. During meiosis, many somatic DNA repair genes may perform modified functions that are essential for meiotic recombination and germ cell development [Marcon and Moens, 2005]. It is tempting to speculate that mutations or interindividual variations in the DNA repair gene inventory contribute to the high rate of fertility problems in humans, which affect 10–15% of couples [De Kretser and Baker, 1999]. Infertility can be caused by many environmental, behavioral, and genetic factors. However, the genetic component (mainly autosomal-recessive factors) may be responsible for up to 60% of cases of male infertility [Liford et al., 1994]. In this light, the 64 genes that we found to be highly expressed in human testicular cells (Tables I and II) including 41 genes which have not been associated with meiosis so far, may provide excellent candidate genes for human male (in)fertility. As an example only, *RFC2* which encodes the 40 kDa subunit of the heteropentameric replication factor C lies within the microdeletion region for Williams Beuren syndrome [Peoples et al., 1996]. Because to the extent of present knowledge patients with Williams Beuren syndrome are infertile, *RFC2* haploinsufficiency may be associated with fertility problems.

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