ORIGINAL ARTICLE

Screening of genes involved in chromosome segregation during meiosis I: toward the identification of genes responsible for infertility in humans

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Prophase I of male meiosis during early spermatogenesis involves dynamic chromosome segregation processes, including synapsis, meiotic recombination and cohesion. Genetic defects in the genes that participate in these processes consistently cause reproduction failure in mice. To identify candidate genes responsible for infertility in humans, we performed gene expression profiling of mouse spermatogenic cells undergoing meiotic prophase I. Cell fractions enriched in spermatogonia, leptotene/zygotene spermatocytes or pachytene spermatocytes from developing mouse testis were separately isolated by density gradient sedimentation and subjected to microarray analysis. A total of 726 genes were identified that were upregulated in leptotene/zygotene spermatocytes. To evaluate the screening efficiency for meiosis-specific genes, we randomly selected 12 genes from this gene set and characterized each gene product using reverse transcription (RT)-PCR of RNA from gonadal tissues, *in situ* hybridization on testicular tissue sections and subcellular localization analysis of the encoded protein. Four of the 12 genes were confirmed as genes expressed in meiotic stage and 2 of these 4 genes were novel, previously uncharacterized genes. Among the three confirmation methods that were used, RT-PCR appeared to be the most efficient method for further screening. These 726 candidates for human infertility genes might serve as a useful resource for next-generation sequencing combined with exon capture by microarray.

INTRODUCTION

Infertility is a commonly observed clinical problem in humans with a similar incidence as diseases associated with lifestyle, such as diabetes or hypertension. Approximately 10% of couples experience some form of infertility.[1] In most cases, the etiology is distributed fairly equally among male factors, ovarian dysfunction and tubal factors. A small percentage of cases are attributed to other female factors such as endometriosis, uterine factors or cervical factors. Although genetic factors are thought to underlie the etiology of some cases of infertility, there is little direct evidence showing the involvement of gene mutations or polymorphisms in this condition.[2]

Meiosis is a key developmental process that is essential for sexual reproduction. The most characteristic feature of meiosis is dynamic chromosomal behavior during prophase I, and in particular a physical connection between homologous chromosomes (synapsis) that is required for correct chromosomal segregation in the meiotic I stage. Experimental analyses using gene-modified mouse models have demonstrated that a genetic defect in early meiotic genes consistently causes reproduction failure, suggesting the possibility that infertility in humans may be caused by defects in meiotic regulatory genes.[3] However, only a limited number of candidate genes have been surveyed for causative mutations. Indeed, although some infertility studies have identified disease-causing mutations,[4–6] others have failed to identify any causal mutations, possibly due to the heterogeneous etiology of infertility.[7–14] Even when samples are appropriately collected from patients with a meiotic defect, this complicated biological process in prophase I is mediated by numerous meiotic genes responsible for homologous recombination, synapsis or sister chromatid cohesion. Indeed, even in yeast it is estimated that hundreds of genes may be involved in the meiotic process.[15,16] In addition, it is likely that many meiotic regulatory genes remain to be identified in mammals, partly due to the small degree of homology with yeast meiotic genes. Thus, an effective strategy for screening a large number of uncharacterized genes functionally important for meiosis is required to facilitate investigation of the etiology for infertility in humans and mammalian meiotic mechanisms.

Steady progress has been made toward the identification of novel genes expressed in meiotic stage by means of gene expression analyses using testicular tissues. Recently, global expression profiling in developing testis from mice has been documented using cDNA microarrays or serial analysis of gene expression (SAGE).[17–19] A considerable number of whole genome expression profiles using oligonucleotide microarrays have also been reported.[20,21] Such analyses have allowed investigators to rapidly identify a number of new meiosis-specific genes.[22,23] However, these gene data sets often include many non-meiotic genes, preventing individual characterization of each identified gene. Thus, development of a strategy for further screening candidate genes from a microarray data set would be indispensable.

In this study, we report a novel strategy for the identification of the candidate genes responsible for infertility in humans. Using meiotic cells that were fractionated and enriched by gravity sedimentation from mouse developing testis, we first investigated the expression profiles of different enriched meiotic cells. Candidate genes functionally important or indispensable for meiosis were further screened using several different approaches, and comparisons of the efficiency of each screening methodology were carried out.

MATERIALS AND METHODS

Isolation of meiotic cells from mouse testis
A modified discontinuous Percoll density gradient was used to fractionate mouse meiotic cells from testicular cells undergoing first round of spermatogenesis.[24] Briefly, male C57BL6 mice were killed and their testes were removed for further analysis. We used 20 testes each from 8-day-old mice for spermatogonia, 12-day-old mice for leptotene/zygotene spermatocytes and 15-day-old mice for pachytene spermatocytes. All washing steps and enzymatic digestions were performed in Eagle's minimum essential medium (Sigma, St Louis, MO, USA). Testes were decapsulated, the tubules were teased apart with forceps, and the separated tubules were incubated with $1 \, mg \, ml^{-1}$ hyaluronidase, $1 \, mg \, ml^{-1}$ trypsin, and $1 \, mg \, ml^{-1}$ collagenase for 10 min. After the tubular fragments were repeatedly pipetted and washed by sedimentation, a second enzymatic incubation was performed for 10 min. After washing, the isolated cells were collected by filtration through a $70 \, \mu m$ screen. A discontinuous density gradient was prepared in a polypropylene tube with 1 ml of each Percoll density suspension prepared by diluting an iso-osmotic Percoll suspension. Each percentage of Percoll and its respective density is listed in Supplementary Figure 1. The cell suspensions were layered on top of the gradient, which was then centrifuged at $800 \times g$ for 30 min at $25 \, ^\circ C$. Cells that sedimented at the interface between the different density suspensions were collected as fractions 1–10. Each cell fractions was characterized by morphological analysis using phase contrast microscopy and Nomarski interference contrast microscopy. The expression of cell lineage- and spermatogenesis stage-specific genes was then examined using reverse transcription (RT)-PCR (see Supplementary Table 1 and Results section).

Microarray analysis
Microarray experiments were performed using the Affymetrix GeneChip system (Affymetrix, Santa Clara, CA, USA). Total RNA was isolated from gradient cell fractions using the RNeasy kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). The quality of each RNA sample was determined by electrophoresis through denaturing agarose gels and subsequent staining with ethidium bromide. The RNA was also quantified and evaluated for purity by ultraviolet spectrophotometry. Approximately $1 \, \mu g$ of each total RNA sample was labeled with biotin using the MessageAmp aRNA Kit (Ambion, Austin, TX, USA). After fragmentation into $\sim 200 \, bp$, synthesized cRNAs were hybridized to Affymetrix Mouse Expression Sets 430, MOE430A and MOE430B. Signal intensities were amplified using a second staining with a biotin-labeled anti-streptavidin antibody followed by phycoerythrin-streptavidin staining. Array fluorescent images were obtained using the GeneChip Scanner 3000 (Affymetrix). Microarray experiment data analyses were performed using Microsoft Excel (Microsoft, Redmond, WA, USA) and Genespring

software, Versions 6–11 (Silicon Genetics, Redwood City, CA, USA). All data were subjected to per chip and per gene normalization, and then used for further analysis. To avoid 'false-positive' signals, we excluded certain genes from the analysis for which the signal intensity was under 50, as the expression levels for genes with low absolute signal intensity are not reliable. Microarray data were deposited in the GEO database with the detailed hybridization information according to the MIAME guidelines and assigned the accession number GSE19532.[25]

RT-PCR
Total RNA was extracted from isolated spermatogenic cells, fetal mouse gonads at 15.5 days postcoitum, and adult mouse gonads at 10 weeks postpartum. First-strand cDNAs were synthesized using Superscript II reverse transcriptase according to the manufacturer's instructions (Invitrogen, Grand Island, NY, USA). Semiquantitative PCR was performed using standard methods and the primer sets listed in Supplementary Table 1.

In situ hybridization
The cDNA templates for in situ hybridization probes were amplified by RT-PCR using primer sets containing T7 and T3 promoter sequences. Digoxigenin-labeled sense and antisense RNA probes were generated using T7 or T3 RNA polymerase and digoxigenin RNA labeling mixture (Roche, Basel, Switzerland). In situ hybridization was performed on paraffin sections of adult mouse testis according to standard protocols. Hybridization signals were detected using an alkaline phosphatase-labeled anti-digoxigenin antibody (Roche), followed by color development with nitroblue tetrazolium and 5-bromo-4-chlor-3-indolyl phosphate.

Expression of epitope-tagged proteins in cell lines
The entire coding region of each selected candidate gene was obtained by RT-PCR and individually cloned into the vector pcDNA3.1/myc-His (Invitrogen). Constructs were individually transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h, cells were fixed with neutral-buffered 3% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 5 min, and treated with 3% bovine serum albumin before immunostaining. Transfected myc-tagged proteins were detected using an anti-myc monoclonal antibody, 9E10, and a fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin-G antibody.

RESULTS

Expression profiling to yield candidate genes functionally important or indispensable for meiosis
To analyze gene expression during meiosis, male germ cells were isolated from the testis of developing mice. Spermatogonia were isolated from fraction 2 of 8-day-old testis, leptotene/zygotene spermatocytes were obtained from fraction 5 of 12-day-old testis and pachytene spermatocytes from fraction 8 of 15-day-old testis (Supplementary Figure 1 and Results section). The gene expression profiles of the fractionated spermatogenic cells were analyzed using an oligonucleotide microarray. Scatter plot analysis of spermatogonia versus leptotene/zygotene spermatocytes (G vs L/Z) with all genes demonstrated that most of the data points occurred along a 45° angle, confirming the accuracy of this technique (Figure 1a, left). In contrast, the scatter plot between leptotene/zygotene versus pachytene spermatocytes (L/Z vs P) showed a significantly reduced correlation for gene expression (Figure 1a, right). The correlation coefficient of G vs L/Z was 0.9673, whereas those for G vs P and L/Z vs P were 0.6390 and 0.6944, respectively. These results indicate that gene expression was remarkably altered during the transition from zygotene to pachytene spermatocytes. To assess the purity of each cell fraction, we evaluated the expression levels of known lineage- or stage-specific genes. Spermatogonia-specific genes were upregulated in G, whereas genes known to be related to meiotic function were upregulated in L/Z or P. In contrast, expression levels of genes specific for Sertoli cells remained
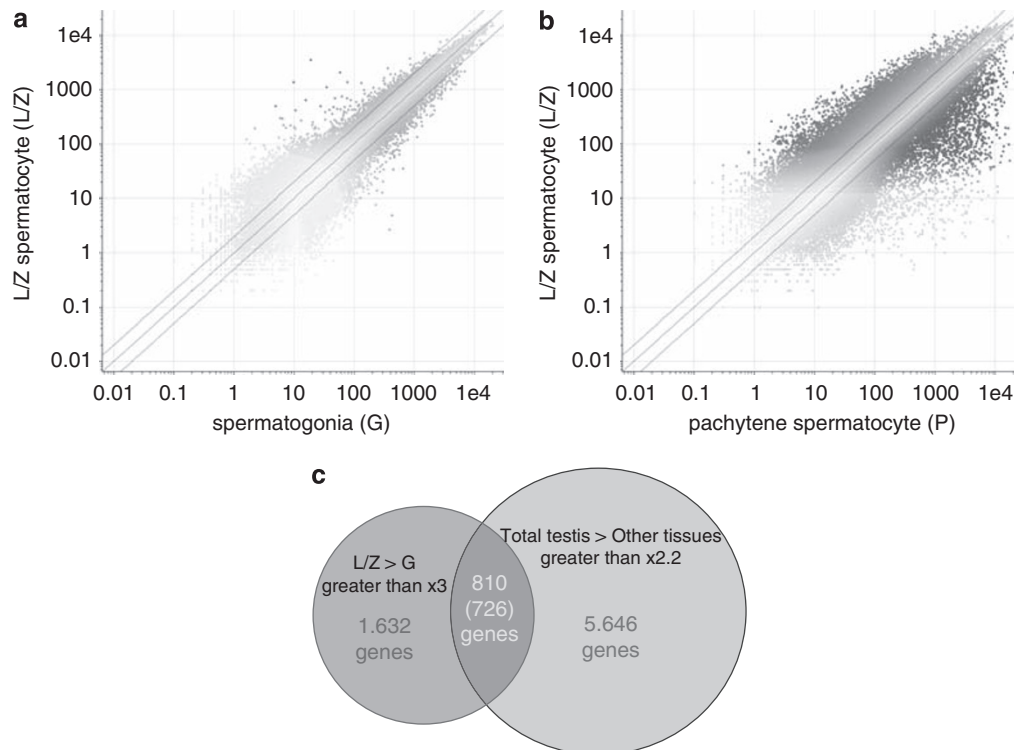
Figure 1 Gene expression profiling for the identification of novel meiotic genes. (**a**) Scatter-plot of all genes spotted on the microarray. Axes show the fluorescence intensity associated with each gene (log10). The normalized signal for leptotene/zygotene (L/Z) spermatocytes (*y* axis) and spermatogonia (*x* axis) is indicated by a color code. Red spots signify genes that are upregulated and green spots signify genes that are downregulated. The lines on the scatter plots indicate the twofold and 0.5-fold boundaries, respectively. Linear regression was performed and the resultant correlation coefficients are presented in the text. (**b**) Scatter-plot of the comparison between the gene expression patterns in spermatogonia and pachytene spermatocytes. (**c**) Venn diagram indicating the number of selected genes using two criteria: genes with an expression level in leptotene/zygotene (L/Z) spermatocytes higher than that in spermatogonia (G: left) and genes with an expression level in testis higher than that in other tissues (right). A full color version of this figure is available at the *Journal of Human Genetics* journal online.

unchanged in each fraction, indicating that effect of somatic cell contamination to the germcell fractions appears to be minimal (Supplementary Figure 2).

As a screen for early meiosis-related genes, we attempted to set a criterion that would enable most known meiotic genes to be included in the selected gene set. These genes are known to be involved in double-strand break formation, meiotic recombination or synapsis. As a first criterion, we selected genes showing more than threefold upregulation from spermatogonia to L/Z spermatocytes. Among the approximately 40 000 genes examined, this parameter selected 1632 transcripts, which was still excessive due to the inclusion of ubiquitously expressed genes. To further reduce the number of genes, we used tissue specificity as a further filter, as many meiosis-specific genes are known to show preferential gonadal expression.[26] We compared the gene expression levels in mouse testis with those in a mixture of 11 selected somatic organs (spleen, intestine, stomach, skin, brain, colon, heart, kidney, liver, muscle and lung) from 15-day-old mice. We used a 2.2-fold increase as the cut-off value for testis-preferred expression to reduce the number of selected genes to less than 1000 genes, which would be right for an exon capture by microarray for subsequent next-generation sequencing. Using this cut-off value, a total of 5646 genes were selected. The combination of the two gene sets yielded a new gene set that consisted of 810 genes (Figure 1b), which is listed in full in Supplementary Table 2. After removing redundancy, 726 non-redundant genes are referred to as MLZ-001-726 due to their expression in male leptotene and zygotene spermatocytes.

Table 1 Classification of 726 candidate meiotic genes

Genes	Characteristics	Number	Subtotal	Total
Known	Meiosis-preferred expression	35		
	Testis-preferred expression	101		
	Others	442	578	
Unknown	Testis-preferred expression	51		
	Others	97	148	726

The composition of the selected genes is summarized in Table 1. Among these 726 candidates, 578 are named genes, with the remaining 148 previously uncharacterized. Among the named genes, 35 genes (~6%) are known to fulfill vital functions during meiosis. Most of the established genes associated with chromosome behavior in meiosis were included in the MLZ gene set as shown in Table 2. Our data thus suggest that our selection criteria efficiently selected most of the genes functionally important or indispensable for meiosis. However, this list also contained genes reported to be involved in late spermatogenesis, such as *Calmegin* (MLZ-095), *Daz1* (MLZ-114), *Piwi2* (MLZ-356) and *Zpbp* (MLZ-591). Thus, additional methods were required to further screen for genes expressed in meiotic stage.

RT-PCR analysis of selected genes
To further screen for genes expressed specifically in meiosis, we performed RT-PCR analysis of 12 genes selected randomly from our

Table 2 Microarray data for 12 known meiotic genes

Rank in 726	Expression ratio	Symbols	Description	Function	Expression
70	19.26	Msh4	mutS homolog 4	Meiotic recombination	Meiotic
123	10.32	Sycp1	Synaptonemal complex protein 1	Synapsis (central element)	Meiotic
158	8.02	Dmc1	Disrupted meiotic cDNA 1 homolog	Meiotic recombination	Meiotic
172	7.37	Rad51c	Rad51 homolog c	Meiotic recombination	Ubiquitous
188	6.88	Brca1	Breast cancer 1	Meiotic recombination	Ubiquitous
210	6.24	Smc1b	Structural maintenance of chromosome 1b	Sister chromatid cohesion	Meiotic
312	4.77	Sycp3	Synaptonemal complex protein 3	Synapsis (lateral element)	Meiotic
347	4.40	Stag3	Stromal antigen 3	Sister chromatid cohesion	Meiotic
395	4.13	Atr	Ataxia telangiectasia and rad3 related	Meiotic recombination	Ubiquitous
424	3.98	Spo11	Sporulation protein 11	Meiotic recombination	Meiotic
516	3.51	Fkbp6	FK506 binding protein 6	Synapsis in male	Meiotic
526	3.48	Pms2	Post-meiotic segregation increased 2	Synapsis in male	Ubiquitous

Table 3 Validation results for the 12 candidate genes

Gene ID	GenBank	Function	Description	RT-PCR	In situ hybridization	Subcellular localization
MLZ-136	AK006085	Unknown	Dipeptidase 3	Meiotic	Meiotic	Cytoplasm
MLZ-220	BG075885	Unknown	Heat-shock transcription factor family member 5	Testis	ND	ND
MLZ-264	NM_025281	Known	Ly1 antibody reactive clone	Ubiquitous	ND	ND
MLZ-278	BF021951	Known	Meiosis expressed gene 1	Testis	ND	ND
MLZ-312	NM_025719	Unknown	NF-κB-activating protein-like	TESTIS	ND	ND
MLZ-393	AK005802	Unknown	RNA-binding motif protein 26	Ubiquitous	ND	ND
MLZ-453	AV257384	Known	Structural maintenance of chromosome 5	Meiotic	Meiotic	Nuclear
MLZ-465	AK016064	Known	Speedy homolog A	Meiotic	Meiotic	Nuclear
MLZ-501	AK014855	Known	Tyrosyl-DNA phosphodiesterase 1	Ubiquitous	Ubiquitous	ND
MLZ-622	NM_025953	Unknown	RIKEN cDNA 1700019N12 gene	Testis	Spermatid	ND
MLZ-636	AA266367	Unknown	RIKEN cDNA 1700097N02 gene	Testis	ND	ND
MLZ-675	AK015924	Unknown	RIKEN cDNA 4930528F23 gene	Meiotic	Meiotic	Cytoplasm

Abbreviation: ND, not determined.

MLZ gene set (Table 3) and of three well-established meiotic genes (*Spo11*, *Sycp1*, *Rec8*). Among the 12 selected genes, 5 were reported genes and 7 were of unknown function. As the first round of meiosis is known to commence in the mouse testis at 11 days postpartum, meiosis-specific genes are expressed in adult testis but not in the fetal testis. In contrast, meiosis begins at 11–12 days postcoitum in the fetal ovary and arrests at the dictyate stage of meiotic prophase until adulthood. To examine whether the test genes were expressed only during spermatogenesis or during meiosis of both sexes, RT-PCR analysis was performed using fetal mouse male and female gonads at 15.5 days postcoitum, and adult mouse male and female gonads at 10 weeks postpartum.

All three known meiosis-specific genes exhibited a similar expression pattern with the intense expression in fetal ovary and adult testis, weak expression in fetal testis and negligible expression in adult ovary (Figure 2). Among the 12 genes selected for analyses, spermatogenesis-associated expression was confirmed in 9 genes. The exceptions were MLZ-264, -393 and -501. Among the nine confirmed genes, four (MLZ-220, 312, 622 and 636) were testis-specific and not expressed in the fetal ovary. MLZ-278 also showed a testis-preferred expression pattern. The remaining four genes (MLZ-136, -453, -465 and -675) exhibited an expression pattern that was almost identical to the known meiotic genes, suggesting that about one-third (4/12) of our selected genes appear to be involved in early meiosis (Table 3).

Developmental expression during meiosis

To confirm their meiosis-specific expression properties, the tissue expression of the candidate meiotic genes was evaluated using *in situ* hybridization. Seven genes including genes with a meiosis-related expression pattern by RT-PCR (MLZ-136, -453, -465, -675 and *Spo11*) and an additional two genes (MLZ-501 and -622) were examined (Figure 3). MLZ-501, which was found to be a ubiquitously expressed gene by RT-PCR, was detected in all testicular tissue cells. MLZ-622, which represented spermatogenesis-specific expression, was expressed in spermatids, suggesting a late-spermatogenesis function. Among the candidate meiosis-specific genes, three other genes as well as *Spo11* were found to be expressed specifically in meiotic spermatocytes. These *in situ* hybridization data were also in clear agreement with the findings obtained by RT-PCR (Table 3). Interestingly, although MLZ-136 and -453 were found to be expressed in a wide range of meiotic cells, the cells expressing MLZ-675 were observed in a more confined manner in the seminiferous tubules, similar to that associated with *Spo11*. These observations suggest that MLZ-675 functions in an initial step of meiotic recombination during early prophase of meiosis I.

Subcellular localization of the encoded proteins

Our final goal in this study was to identify meiotic genes that have a role in chromosome segregation during meiosis I. To screen our
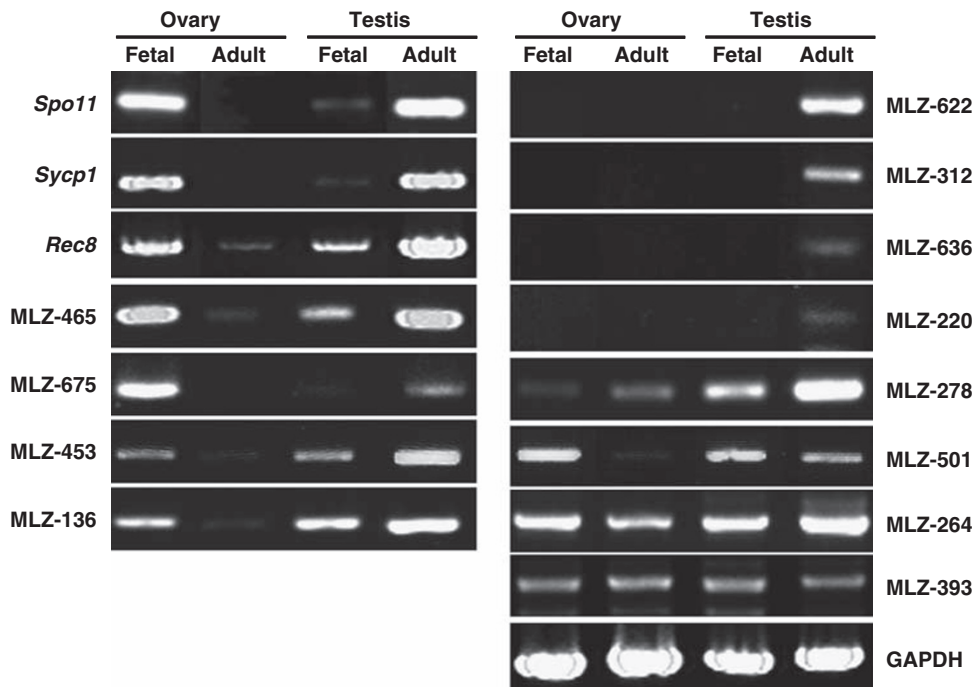
Figure 2 Semiquantitative RT-PCR analysis of selected candidate genes using RNA from fetal mouse gonads obtained from male and female at 15.5 days postcoitum and adult gonads obtained from male and female mouse at 10 weeks postpartum. The samples are indicated at the top and the individual gene names are listed on either side of the gel images. The genes shown in the left panel appeared to be expressed specifically in meiosis.
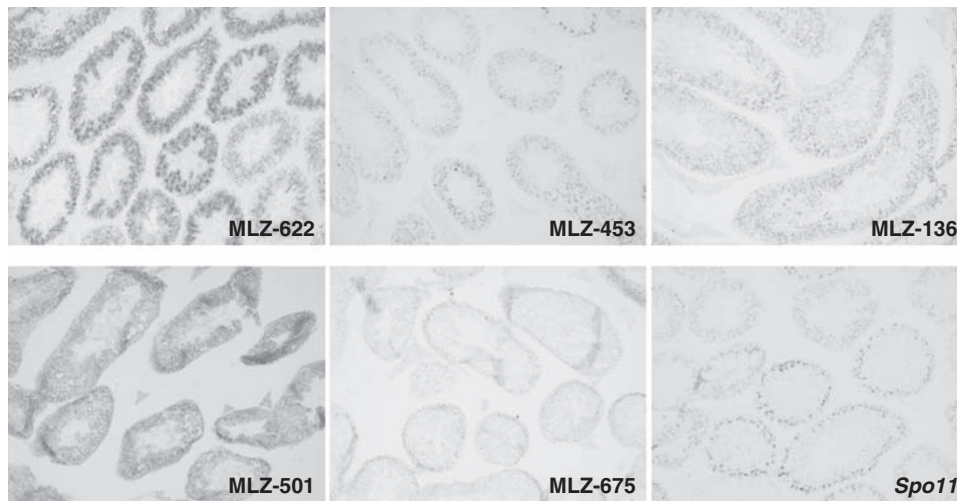
Figure 3 *In situ* hybridization analysis of tissue sections of adult mouse testis. Representative results (MLZ-136, -453, -501, -622, -675 and *Spo11*) are shown. The signals produced by MLZ-136, -453, -675 and *Spo11*, but not other candidates, demonstrate a meiotic cell pattern.

meiosis-specific candidate genes for a possible function in chromosome segregation, we examined the subcellular distribution of the corresponding myc-tagged gene products when overexpressed in 293 cells and looked for a nuclear localization pattern. Four candidate genes were examined for possible associations with meiotic chromosome function (MLZ-136, -453, -465, -675 and *Spo11*). Proteins encoded by MLZ-453 and -465 were detected in the 293 cell nucleus, whereas MLZ-136 and -675 were detected in the cell cytoplasm (Figure 4a).

Interestingly, *Spo11*, which is a known meiosis-specific gene that catalyzes the formation of DNA double-strand breaks, was unexpec-

tedly detected in the cell cytoplasm (Figure 4b). Although this method provided us some information regarding gene function, we could not select genes that were related to the segregation of meiotic chromosome solely based upon the nuclear localization of overexpressed proteins in this heterologous system.

DISCUSSION

In this study, we performed gene expression profiling of developing meiotic cells in the mouse. Several earlier reports have described methods for isolating such cells for use in microarray analysis.
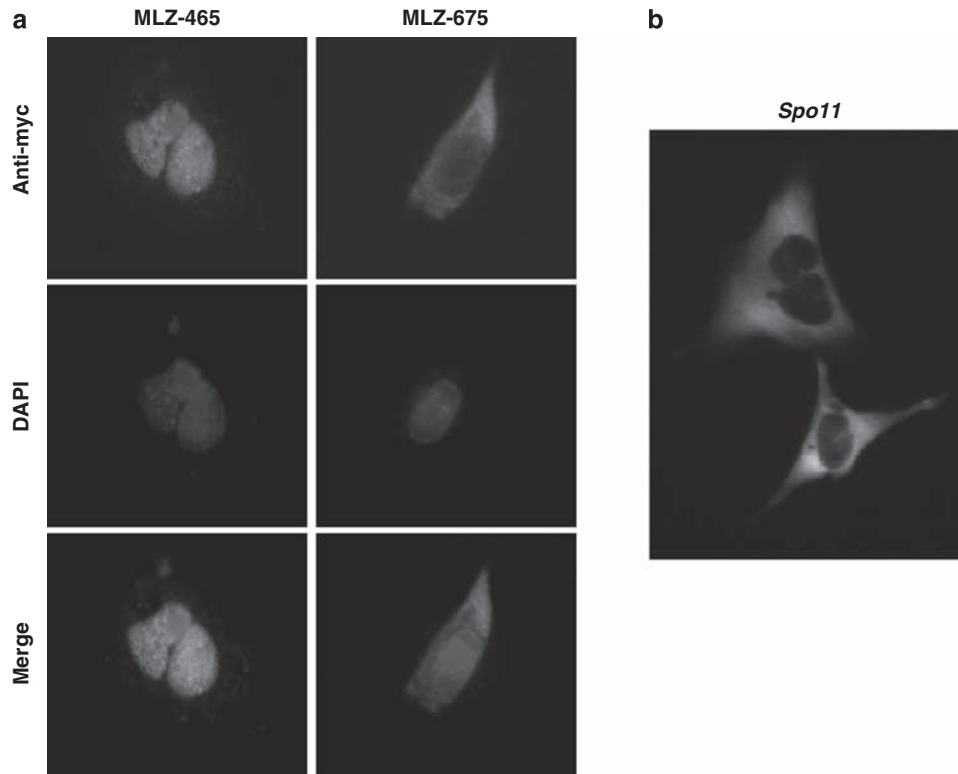
Figure 4 Subcellular localization of candidate meiotic gene products. (**a**) Results for candidate genes related to chromosomal behavior in meiosis. Overexpressed proteins were localized using immunostaining with anti-myc antibodies (green) and 4,6-diamidino-2-phenylindole (DAPI) (blue) as a nuclear counter stain. Representative staining results (MLZ-465 and -675) are shown. The left panel indicates nuclear localization and the right panel shows cytoplasmic localization of the expressed protein. (**b**) Results for *Spo11*, which functions during the initiation of meiotic recombination within the nucleus of meiotic cells. The green signals indicate cytoplasmic localization of the expressed protein. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Sedimentation or elutriation using a bovine serum albumin gradient is an already described standard procedure for this purpose.[27,28] Alternatively, stage-specific meiotic cells can be obtained by microdissection and can also be used as a source of RNA.[29,30] However, both of these techniques are technically demanding and time consuming. To simplify the strategy, total testis samples obtained during progression of the first round of spermatogenesis can also be applied for this purpose.[20,31] In addition, the Percoll gradient procedure was originally used for the isolation of rat spermatogenic cells.[24] In this study, we fractionated mouse testicular cells undergoing the first round of spermatogenesis using the Percoll gradient procedure after a few modifications, and successfully obtained gradient fractions of spermatogonia, leptotene/zygotene and pachytene cells.

In our analyses, a set of genes upregulated in the leptotene/zygotene fraction relative to spermatogonia was found to include most of the known genes functionally important or indispensable for meiosis. In addition, a large number of genes were found to be differentially expressed between spermatogonia and pachytene spermatocytes, which is consistent with previous reports.[19,21,22] RNA synthesis tapers during the leptotene/zygotene stage and then restarts at the pachytene stage when synapsis of all homologous chromosomes completes, with the exception of the sex chromosomes.[32] It is thus possible that the expression of genome-wide genes needs to change dramatically in order to complete a checkpoint for unsynapsed autosomes during the transition from zygotene to pachytene spermatocytes. Thus, the genes expressed in the leptotene/zygotene fraction are more likely to reflect the gene expression pattern of meiotic prophase.

We evaluated the efficiency of our selection for meiotic genes using RT-PCR and *in situ* hybridization. Among the 12 genes we randomly selected out of our entire set of 726 genes, one-third were found to be genes expressed in meiotic stage. Among the four meiotic genes in our selected group, two were of known functions (*Spdya*, *Smc5*), and the remaining two were as yet uncharacterized. One gene (MLZ-136) contains a renal-dipeptidase domain, whereas the other (MLZ-675) encodes a fork-head domain and shares weak homology with *Rpa*. Thus, our strategy for gene identification appears to be a useful and effective method to identify novel meiosis-specific genes. On the basis of efficiency estimated by the 12 genes we selected randomly, we predict that our 726 candidate gene cohort might contain 200–300 meiosis-specific genes.

In contrast, these 726 genes still included too many non-meiotic genes to realistically permit analysis of the function of each gene in any detail. Additional screenings will thus be required using other methods to select the genes specifically expressed in prophase I. In our present analysis, semiquantitative RT-PCR was found to clearly distinguish meiosis-specific genes from ubiquitously expressed genes or genes expressed specifically in spermatogenesis. This result indicates that the high expression both in female gonads at 15.5 days postcoitum and male gonads at 10 weeks postpartum is a good criterion for selecting genes involved in prophase I. Although our *in situ* hybridization experiments did successfully identify genes expressed in meiotic stage, RT-PCR may be a better tool for rapidly and accurately screening for meiosis-specific genes. Moreover, additional microarray experiments using the RNA samples from the four organs, fetal and adult gonads

obtained both from male and female mice, may be a more efficient way to further screen for novel genes involved in meiosis.

Our final aim was the identification of candidate genes for disease-causing mutations in patients with infertility. The genetic analysis of infertility is generally challenging due to the intrinsic nature of the disease and the resulting lack of familial cases. It is possible that most mutations leading to infertility arise *de novo*, suggesting that a positional cloning strategy, such as a genome-wide association study, cannot be used. Sequencing candidate genes may thus be the only way to identify mutations responsible for human infertility. Expression profiling using microarrays is a useful procedure for identifying sets of functional candidate genes. Other investigators have attempted to identify candidate genes using expression profiling of testicular tissue obtained from patients with azoospermia.[33,34] Indeed, one candidate polymorphism responsible for non-obstructive azoospermia has now been identified using this strategy.[35]

From our current experiments, we obtained 726 candidate meiosis-specific genes that may constitute a useful resource for identifying genes associated with non-obstructive azoospermia with meiotic arrest in humans. Human orthologues of these 726 candidate genes could be subjected to high-throughput mutational analysis using exon capture by custom-made microarray, followed by massive sequencing with next-generation sequencers.[36] Furthermore, we recently identified two women with recurrent pregnancy loss who carry mutations in a gene functionally indispensable for meiosis.[37] These 726 genes are also candidates for genes responsible for recurrent pregnancy loss due to susceptibility for aneuploidy and deserve further investigation.

ACKNOWLEDGEMENTS

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