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Novel Development-Related Alternative Splices in Human Testis Identified by cDNA Microarrays

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ABSTRACT: Alternative splicing of premessenger RNA is an important regulatory mechanism that increases the diversity of proteins transcribed from a single gene. This is particularly important in the testis because germ cell expansion and differentiation require many cellular changes and regulatory steps. To investigate novel development-related alternative splicings in the human testis, complementary DNA microarray studies were conducted with the use of probes from human fetal testes, adult testes, and human spermatozoa. Of a total of 386 Unigene clusters found to be related to the development of the testis, 67 clusters showed a total of 74 novel alternative spliceoforms. Developmental stage–dependent expression was also performed for a novel Unigene, *NYD-SP20* (Hs.351068), which had 4 possible novel spliceoforms and another Unigene, *CRISP2* (cysteine-rich secretory protein 2, Hs.2042), which had 3 possible novel spliceoforms. These results indicate that alternative splicing plays an important role in the complicated processes of testis development and spermatogenesis.

Key words: Alternative splicing, spermatogenesis, regulatory mechanism, protein diversity, spliceoforms.

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Alternative splicing is an important molecular mechanism for regulating eukaryotic gene expression and enabling protein diversity. Through alternative splicing, multiple forms of mitochondrial RNA (mRNA) and protein products can be produced from a single pre-mRNA molecule (Maniatis and Tasic, 2002; Boue et al, 2003). A recent genome-wide analysis of alternative splicing indicated that 30% to 60% of human genes have alternatively spliced forms, suggesting that alternative splicing is one of the most significant components of the functional complexity of the human genome (Xu et al, 2002).

The testis is a rich source for identifying gene regulation mechanisms because germ cell expansion and differentiation requires many cellular changes and regulatory steps. In developing germ cells, the lengths of mRNA transcripts often vary as the cells mature, reflecting ongoing regulatory changes (Eddy, 2002); alternative splicing is thought to be an ideal mechanism for controlling gene expression at transcription level in these tissues (Walker et al, 1999). Recently, a genome-wide analysis of expressed sequence tags (ESTs) showed that the testis has the greatest enrichment of tissue-specific splicing (Xu et al, 2002), which could be involved in various functions, including spermatogenesis and steroidogenesis (Carreau et al, 2001; Hammes et al, 2001; Venables, 2002). Although recent studies have collected alternative splicing information from annotated resources such as the alternative splicing annotation project (ASAP; http://www.bioinformatics.ucla.edu/ASAP/) and EST databases (Gelfand et al, 1999; Hu et al, 2001; Lee et al, 2003), there is a paucity of large-scale information on development-related alternatively spliced mRNAs in the human testis.

Here, we used a human testis complementary DNA (cDNA) microarray hybridized with ^{33}P-labeled human adult testis, fetal testis, and adult spermatozoa cDNA probes to identify novel testicular development-related alternatively spliced mRNAs. Sixty-seven Unigene clusters showed a total of 74 novel alternative spliceoforms, allowing us to investigate the relationship between alternative splicing and developmental state. Together, our results illustrate the significance of alternative splicing in human testis development and spermatogenesis.

Materials and Methods

Samples

Informed consent was received from either the participants or their kin, and the ethics committee of Nanjing Medical University granted research approval prior to sample collection. Testicular tissue samples were obtained from adult males and elderly males (Body Donor Center, Nanjing Medical University, China). Fetal testes were from accidentally aborted 6-month-old fetuses (Clinical Reproductive Center, Nanjing Medical University, China). Human ejaculates were obtained from healthy volunteers

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with proven fertility and normal semen quality according to WHO (1999) criteria (ie, greater than 20×10^6 spermatozoa/mL, with greater than 50% active sperm; greater than 25% of sperm moving forcefully in one direction [rapid and linearly progressive]; and less than 1 lymphocyte per high-power field [40×]). Samples and preparation of RNA probes was as described in previous papers (Sha et al, 2002; Wang et al, 2004). Macroscopic and histologic examinations showed every level of spermatogenic cells and Sertoli cells around seminiferous tubules in adult human testis and elderly testis; however, fewer spermatids and spermatozoa were seen in elderly testis than in adult testis. In fetal testis, only Sertoli cells and prospermatogonia converged at the mediastinum.

Screening of Genes Related to Testis Development

In previous studies, a human testis cDNA microarray was constructed in our laboratory (Sha et al, 2002; Wang et al, 2004). For identification of full-length cDNAs, we employed the Human Testis Large Insert λ Phage cDNA Library (HL5503U; Clontech, Mississauga, Ontario, Canada); this library has an average insert size of greater than 3.0 kilobases (kb) and an insert size range between 0.8 and 7.0 kb. Genes related to testis development and spermatogenesis were identified by hybridization of adult testis, fetal testis, and adult spermatozoa probes onto cDNA microarrays containing 9216 human clones (2 dots for each clone were spotted). In brief, after subtraction of the background from an area in which no clone was spotted, clones with an intensity density greater than 10 were considered positive signals, whereas if the standard difference of the signal intensity of the paired dots was greater than 0.3, the result was not analyzed. The hybridization intensity of correspondent dots in adult and fetus were compared. If the difference in values of spot intensity in adult and fetus was more than twofold higher or lower, the correspondent genes were considered differentially expressed. Genes expressed at a higher level in adult spermatozoa were considered potentially related to spermatogenesis (Sha et al, 2002; Wang et al, 2004).

Identification and Characterization of Novel Alternative Splices in Human Testis

All differentially expressed cDNA plasmids were proliferated, extracted, and purified. MegaBACE 1000 (Amersham Pharmacia, Stockholm, Sweden) was used to sequence the full insert length. The National Center for Biotechnology Information (NCBI) BLAST-nr tool was used to search for sequence homologies, and 3 types of genes were identified: known genes, novel genes, and novel alternative spliceoforms. Here, we focus on the last type. The NCBI Human Genome database (http://www.ncbi.nlm.nih.gov/genome/guide/human/) was searched for exon/intron information for each novel spliceoform, and the NCBI LocusLink database (http://www.ncbi.nlm.nih.gov/LocusLink) was examined for splice site information. Finally, the SMART (http://smart.embl-heidelberg.de/) and protein family (PFAM; http://www.sanger.ac.uk/Software/Pfam/) databases of protein domain sequences were used to assess the effects of the observed alternative splices on the predicted protein products.

Tissue Distribution of Novel Spliceoforms

Multiple tissue cDNA panels (including brain, heart, kidney, liver, lung, pancreas, placenta, and skeletal muscle) were purchased from Clontech (K1420-1). Testis cDNA was amplified by reverse transcription (RT)–PCR from normal human adult testis with the use of specific primers (AY009109 sense: 5′-CAG GAA CAG TGA CAG TGT AGG ACG-3′ and antisense: 5′-TGT GGA GAC GCT CTT GCT G-3′; AF327561 sense: 5′-TGT TAA AGA ACA GAG AAG TGA TCC T-3′ and antisense: 5′-GTG TTT ATA TTG CTT CAG GGC TTG T-3′; AY014284 sense: 5′-TGA CAA TCT CCA GGA ACA GT-3′ and antisense: 5′-TCT AGA TTC TGC ATG GAG AT-3′; AF332009 sense: 5′-TAA ATT GTG GGA CTC AAA GGA GAC-3′ and antisense: 5′-GCC AAG GTT GAT TCT GAA GGC CA-3′) designed to span at least 1 intron to guard against genomic contamination. Amplification of the glucose-3-phosphate dehydrogenase (G3PDH) housekeeping gene was used as the positive control. The PCR mixtures (20 μL) contained 2 μL of cDNA template, 2 μL of 10× reaction buffer, 1.5 μL of $MgCl_2$ (25 mM), 1.5 μL of desoxynucleotide triphosphate (dNTP; 2 mM), 0.1 μL of *Taq* DNA polymerase (5 U/μL), 10.9 μL of distilled water, and 1 μL of each primer (5 pmol/μL). PCR was performed with a PTC100 thermocycler (MJ Research Inc, Watertown, Mass) with an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, with a final extension for 7 minutes at 72°C. The PCR products were separated by electrophoresis and analyzed.

NYD-SP20 and CRISP2: Developmental Stage–Dependent Expression of Novel Spliceoforms

Template cDNAs were RT-PCR amplified from human fetal, adult, and elderly testis tissues and spermatozoa. Gene *NYD-SP20* (Hs.351068), which had 4 spliceoforms (a, AF367472; b, AY032684; c, AY035867; d, AY035868), was amplified by primer pair P1, which amplified all 4 spliceoforms (sense: 5′-GCC TAA GTG AAA ATT CAG CTC G-3′ and antisense: 5′-CAT CTC TGT AGC TCC AAC CAC C-3′); primer pair P2, which amplified spliceoforms c and d (sense: 5′-AGT TGT GTG TCG TGT TG-3′ and antisense: 5′-CAC TGT TTT CAT TAC AGG AGC-3′); and primer pair P3, which amplified spliceoforms a and b (sense: 5′-ACG GGG CCG CCT GGA CGA-3′ and antisense: 5′-CAT CTC TGT AGC TCC AAC CAC C-3′). One set of primers was used to amplify gene *CRISP2* (cysteine-rich secretory protein 2, Hs.2042; sense: 5′-TCT GGT TAC TGT GCT GCT TCC-3′ and antisense: 5′-TCG TTG TTA CCT CTC TGC TCC-3′). PCR was performed as described previously.

Results

Identification of Novel Development-Related Spliceoforms in Human Testes

Among the cDNA clones that gave positive signals in the tested tissues, 1522 clones showed at least twofold differences in intensity between adult and fetal testes; these were considered differentially expressed and related to testis development. After bioinformatics analysis, the

Table 1. *Fourteen Unigene clusters were discovered that had greater than 2 forms of alternative splicing in the microarrays, including novel and known isoforms; asterisks indicate novel alternative gene splicing that were transported to GenBank by our laboratory*

Unigene ID	Unigene Name	Accession No.
Hs.528525	RNA-binding protein 2-like 1 short isoform	NM_006267 AY354203*
Hs.224355	Serine/threonine kinase 31	NM_031414 AF332194*
Hs.74335	Heat shock 90 kd protein 1, beta	NM_007355 AY359878*
Hs.368605	Retinoic acid–induced 14	AY317139* AY354204*
Hs.436349	Reticulon 4	AY123249 AF333336*
Hs.381219	Ribosomal protein L15	NM_002948 AY347528*
Hs.408096	Fragile X mental retardation, autosomal homolog 1	NM_005087 AY341428*
Hs.99881	Lactate dehydrogenase C, transcript variant 1	AY286300* NM_002301
Hs.234573	TL132 protein	NM_145809 AY363305*
Hs.416032	Testis specific, 10	AY014284* BC028366 NM_025244
Hs.178281	Bromodomain, testis specific	BC017582 AY338951* NM_001726
Hs.351068	Testis development-related *NYD-SP20*	AF367472* AY035867* AY035868* AY032684*
Hs.129055	Outer dense fiber of sperm tails 2 isoform 1	NM_153437 NM_002540 AY366499* AY319414*
Hs.2042	Cysteine-rich secretory protein 2	AY289796* AY292862* AY292863* NM_003296

1522 clones could be grouped into 386 Unigene clusters, of which 120 were highly expressed in the fetal testis and the others highly expressed in the adult testis. Of the 266 Unigene clusters highly expressed in the adult testis, 149 were positively identified in spermatozoa. We identified 67 Unigene clusters as having novel alternative spliceoforms, 14 of which had greater than 2 alternatively spliced forms (Table 1). Of 74 novel development-related alternative splices identified, 63 were highly expressed in adult testis and 49 were also highly expressed in spermatozoa (Table 2). One, *FXR1P* (fragile X mental retardation autosomal homolog 1, Hs.408096), had 2 alternatively spliced forms: *FXR1P*-a (NM_005087) and *FXR1P*-b (AY341428). *FXR1P*-a was highly expressed in adult testis and spermatozoa, whereas novel splice variant *FXR1P*-b was highly expressed in only the adult testis (Figure 1).

Types of Novel Spliceoforms and Their Corresponding Open Reading Frame Changes

There are several types of alternative RNA processing. According to the classification of Lou and Gagel (2001), 3 types of alternative splicing exist: alternative exon (including cassette exon and mutually exclusive exon), alternative intron, and alternative splice site (including a 5′ splice site and 3′ splice site). All novel alternative splicings were classified on the basis of the described method. Although alternative splicing can remove or retain intronic sequences, this form of splice was not observed in this work. Interestingly, we found that 74.63% of spliceoform employed both alternative transcription then alternative splicing because they used different promoters to initiate transcription. Among them, 70% used a downstream promoter (Table 2). We also separated the spliceoforms into 2 groups on the basis of whether or not they showed open reading frame (ORF) changes (Table 2) and found that 57 (83.58%) of the spliceoforms changed the predicted ORFs.

Tissue Distribution of Novel Alternative Spliceoforms

After bioinformatic analysis, several novel alternative spliceoforms were considered to have important functions because the ORFs were changed. Previous studies had identified tissue distribution of 10 spliceoforms of various genes via RT-PCR, 7 of which were uniquely expressed in human adult testis (Cheng et al, 2002; Xiao et al, 2002; Yin et al, 2002; Zhou et al, 2002; Zhu et al, 2002; Fang et al, 2004; Huang et al, 2004; Zhu et al, 2004; Hu et al, 2004; Zheng et al, 2004). Here, we randomly chose another 4 novel alternative spliceoforms (accession numbers AF332009, AY009109, AF327561, and AY014284) and examined their tissue expression profiles with 9 tissues. PCR followed by electrophoresis showed that all 4 were highly expressed in human adult testis and 1 (AF332009) was widely expressed, whereas 3 (AY009109, AF327561, and AY014284) were uniquely expressed in adult testis (Figure 2).

NYD-SP20 and TPX1: Developmental Stage–Dependent Expression of Novel Spliceoforms

As shown in Figure 3, *NYD-SP20* was found to have 4 novel alternatively spliced forms (a, b, c, and d), and *CRISP2* was found to have a known spliced form (a, NM_003296) and 3 novel alternatively spliced forms (b, AY289796; c, AY292862; d, AY292863). All *NYD-SP20* spliceoforms were expressed in human adult testis and human spermatozoa, whereas human fetal and elderly testis tissues expressed only spliceoforms b, c, and d (Figure 4A through D). All forms of *CRISP2* were highly expressed in human adult testis and spermatozoa but were

Table 2. *Classification of novel alternative splicings according to their splicing types, open reading frame (ORF) changes, and profile in the microarrays**

Unigene ID	Gene Symbol	Accession No.	Alternative Exon	Alternative Splice Site	Alternative Usage of Promoter*	ORF Change	Highly Expressed in Adult Testis†
Hs.113319	*KIF2*	AY317140		●	●d	●	●s
Hs.118281	*ZNF266*	AY376240	●			●	
Hs.444982	*ALS2CR8*	AY032876	●	●		●	●
Hs.15250	*PECI*	AY297537	●		●d	●	
Hs.436133	*USP32*	AF350251	●		●d	●	●s
Hs.178576	*CTNNBL1*	AF367471	●		●d	●	●s
Hs.323634	*STAG3*	AY313779	●		●d	●	●s
Hs.407922	*RFX4*	AF332192	●		●d	●	●
Hs.350939	*p44S10*	AF359879		●	●	●	
Hs.364615	*SLC30A9*	AY319413		●		●	
Hs.279763	*FLJ10504*	AY334564		●	●d	●	●s
Hs.372719	*PHF7*	AY334564	●		●d	●	●s
Hs.413074	*NFX1*	AF332009	●			●	
Hs.411098	*NPM1*	AY347529		●	●		
Hs.159087	*RAD23B*	AY313777		●	●d	●	●s
Hs.409311	*FGD4*	AY367054	●	●	●d	●	●s
Hs.50334	*C9orf24*	AF367474		●	●d	●	●
Hs.59838	*FLJ10808*	AF359880	●		●d	●	●s
Hs.436921	*KIAA0586*	AY359881	●			●	●
Hs.221688	*SRPK2*	AY354201		●	●	●	●s
Hs.22315	*CREB1*	AY347527	●	●		●	●s
Hs.156232	*C6orf204*	AY313778	●	●	●	●	●s
Hs.401274	*SLC2A14*	AY357941	●		●d	●	●s
Hs.441069	*PIAS2*	AF361054	●		●	●	●s
Hs.231975	*CREM*	AY292864	●	●	●	●	●s
Hs.371698	*RAE1*	AY349350	●		●d		●
Hs.22587	*SSX2IP*	AY367055	●	●	●d		●s
Hs.273104	*ANKRD28*	AY367056	●	●	●d	●	●s
Hs.284491	*PDXK*	AY303972		●	●d		●s
Hs.468207	*NRD1*	AY360265	●		●d	●	●s
Hs.412370	*RPL9*	AY376242		●			
Hs.439202	*DPP8*	AY354202	●		●	●	●
Hs.443292	*CPS1*	AY317138		●	●d	●	●
Hs.55481	*ZNF165*	AY366500		●	●d		●
Hs.370123	*AP2B1*	AY341427	●	●		●	●s
Hs.386939	*USP7*	AY376241		●	●d	●	●s
Hs.373980	*IQGAP2*	AY351902	●	●	●d	●	●s
Hs.446140	*SEC8L1*	AF380839		●	●		
Hs.289097	*DDX19*	AF353720	●		●	●	●s
Hs136885	*RNF6*	AY009109	●		●	●	●
Hs.440961	*CAST*	AF327443		●	●	●	●s
Hs.117920	*WDR10*	AF302154	●			●	●s
Hs.388392	*DNAJA1*	AY186741	●		●	●	●s
Hs.247302	*TWSG1*	AF294628		●	●d		●
Hs.69360	*KIF2C*	AY026505		●	●d	●	●s
Hs.108646	*GPD2*	AF311325	●		●d	●	●s
Hs.164267	*DYRK3*	AF327561	●	●		●	●
Hs.178121	*MFAP3L*	AF327560		●	●d	●	●s
Hs.337625	*FLJ10081*	AF311326	●		●d	●	●s
Hs.8109	*SMYD3*	AY186742	●		●d	●	●s
Hs.125139	*FLJ11004*	AY313780	●		●d	●	●
Hs.78946	*CUL3*	AY337761		●	●d	●	●s
Hs.99344	*BGR*	AY009107	●	●		●	●s
Hs.528525	*LOC388974*	AY354203	●		●	●	●s
Hs.224355	*STK31*	AF332194		●		●	●s
Hs.74335	*HSPCB*	AY359878		●			
Hs.436349	*RTN4*	AF333336		●	●d	●	●s
Hs.381219	*RPL15*	AY347528		●			
Hs.408096	*FXR1*	AY341428		●	●d	●	●

Table 2. *Continued*

Unigene ID	Gene Symbol	Accession No.	Alternative Exon	Alternative Splice Site	Alternative Usage of Promoter*	ORF Change	Highly Expressed in Adult Testis†
Hs.99881	*LDHC*	AY286300		●			●s
Hs.234573	*LOC220594*	AY363305		●	●d	●	●s
Hs.416032	*TSGA10*	AY014284		●	●d	●	●s
Hs.178281	*BRDT*	AY338951	●		●	●	●
Hs.368605	*RAI14*	AY317139	●	●	●d	●	●s
		AY354204					
Hs.129055	*ODF2*	AY366499	●	●	●	●	●s
		AY319414					●s
Hs.2042	*CRISP2*	AY289796	●	●		●	●s
		AY292862					●s
		AY292863					●s
Hs.351068	*NYD-SP20*	AF367472	●	●		●	●s
		AY035867					●s
		AY035868					●s
		AY032684					●s

* A ''d'' indicates that the novel gene used a downstream promoter and 5; proterminal 5′-terminal was shorter than their splicing counterparts.
† An ''s'' indicates the novel gene was highly expressed in both the adult testis and spermatozoa.

not detected in fetal and elderly testis tissues (Figure 4E and F).

Discussion

cDNA microarrays are widely used for gene expression profiling, genotyping, DNA sequencing (Aitman, 2001; Smith and Greenfield, 2003), gene discovery, disease diagnosis, drug discovery, toxicological research, and so forth (Lockhart and Winzeler, 2000; Copland et al, 2003). Here, a human testis cDNA microarray was constructed and used to investigate novel development-related alternative spliceoforms in the human testis. We identified 386 testes-associated Unigene clusters, 67 (17.36%) of which possessed novel development-related alternative spliceoforms. Because known spliceoforms were not included in our analysis, greater than 17.36% of genes are likely alternatively spliced forms in the human testis. This is consistent with a recent genome-wide analysis of alternative splicing, which indicated that 30% to 60% of human genes have alternatively spliced forms and that alternative splicing is enriched in the testis (Xu et al, 2002). Of 74 novel alternatively spliced forms identified in this work, 49 were highly expressed in both adult testis and spermatozoa. Tissue distribution was examined for 4 of the novel alternatively spliced mRNAs, and we found 3 were highly and uniquely expressed in human adult testis. Previously, 10 other novel spliceoforms had been identified by RT-PCR (Cheng et al, 2002; Xiao et al, 2002; Yin et al, 2002; Zhou et al, 2002; Zhu et al, 2002; Fang et al, 2004; Huang et al, 2004; Zhu et al, 2004; Hu et al, 2004; Zheng et al, 2004). Of the 14 spliceoforms investigated (4 in this work, 10 in previous reports), 10 were highly and uniquely expressed in the testis, whereas 4 were widely expressed, but found at higher levels in the testis.

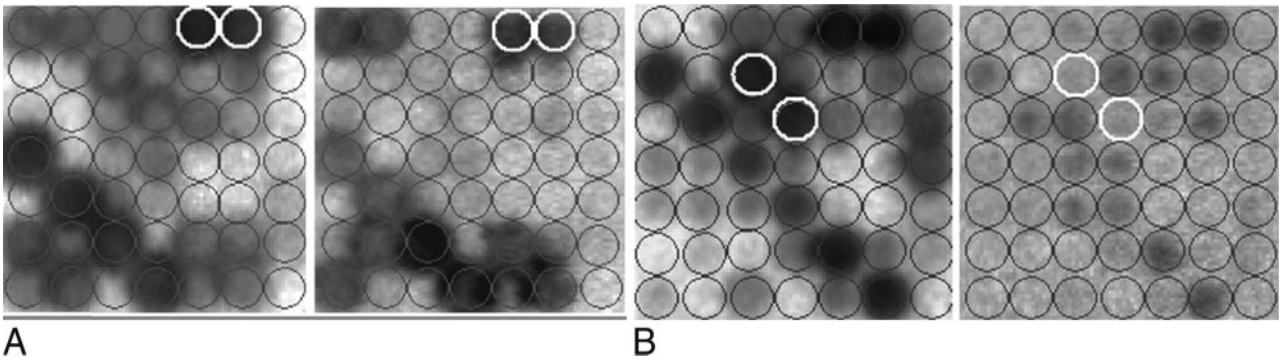

Figure 1. Hybridization signals of *FXR1P* (fragile X mental retardation autosomal homolog 1, Hs.408096) in human adult testis and spermatozoa. White rings indicate 2 alternatively spliced forms. **(A)** *FXR1P*-a (NM_005087) used to probe human adult testis (left) and spermatozoa (right) showed hybridization intensities of 189.99 and 91.12, respectively. **(B)** *FXR1P*-b (AY341428) used to probe human adult testis (left) and spermatozoa (right) showed hybridization intensities of 220.81 and 6.18, respectively.

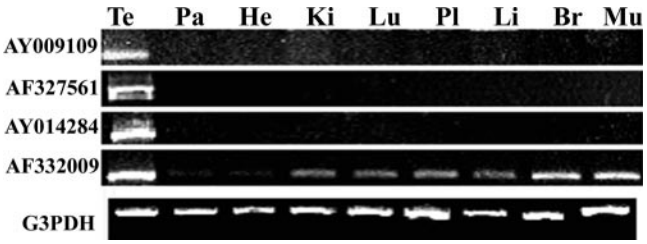

Figure 2. Tissue distribution of 4 novel alternative spliceoforms of various genes. Three alternative spliceoforms (accession numbers AY009109, AF327561, and AY014284) were all highly and uniquely expressed in the adult testis. One of the novel alternative spliceoforms (AF332009) was widely and highly expressed in the testis. Glucose-3-phosphate dehydrogenase (G3PDH) was used as the positive control. Te indicates testis; Pa, pancreas; He, heart; Ki, kidney; Lu, lung; Pl, placenta; Li, liver; Br, brain; and Mu, muscle.

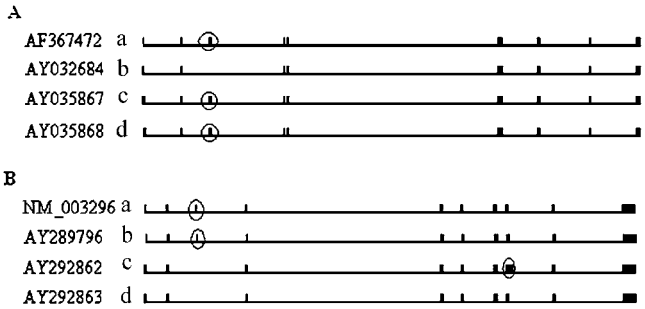

Figure 3. Alternative spliceoforms of the novel Unigene *NYD-SP20* and *CRISP2* (cysteine-rich secretory protein 2). Rings indicate the major differences among them. **(A)** Four alternatively spliced forms (a, b, c, and d) of *NYD-SP20.* All of them initiate translation from exon 2. Because spliceoform b lacks exon 3, it encodes a shorter predicted protein than the other 3 forms. **(B)** Four alternatively spliced forms of *CRISP2.* All of them initiate translation from exon 4. Because spliceoform c contains a longer form of exon 8, it encodes a longer predicted protein than the other 3 forms.

These preliminary results could be extrapolated to suggest that some, if not a majority, of the spliceoforms identified in this work are specifically or preferentially expressed in the testes and are likely to perform testis-specific functions.

Although spermatogenesis is the fundamental function of the testis (Eddy, 2002), it is not an autonomous process. It is subject to secondary regulation by endocrine cues transmitted indirectly through surrounding somatic cells. Both germ cells and testis somatic cells, such as Leydig cells and Sertoli cells, are important to testis development (Jegou and Sharpe, 1993; Griswold, 1998). We found that Unigene cluster Hs.408096 (*FXR1P*) had 2 alternatively spliced forms: known variant *FXR1P*-a and novel variant *FXR1P*-b (Table 1). Both of them were highly expressed in human adult testis, but *FXR1P*-a was highly expressed in human spermatozoa, whereas *FXR1P*-b was not (Figure 1). *FXR1P* encodes an RNA binding protein containing 2 KH (hnRNP K-protein homology) domains and 1 RGG amino acid repeat motif box; it associates with polyribosomes, predominantly with 60S large ribosomal subunits. Huot et al (2001) investigated the mouse homolog of *FXR1P*-a by immunohistologic

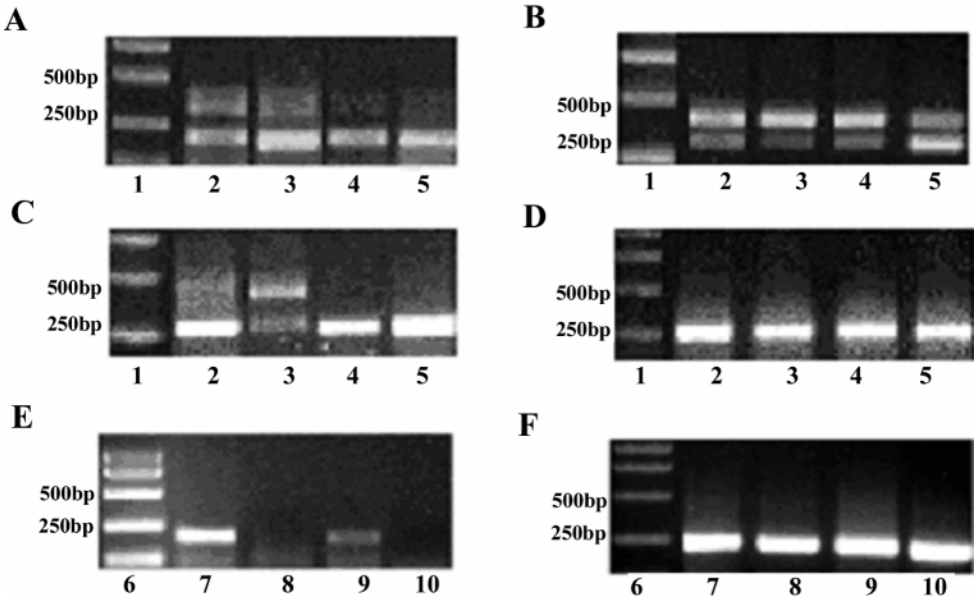

Figure 4. Developmental stage–dependent expression of the novel Unigene *NYD-SP20* and *CRISP2* (cysteine-rich secretory protein 2) in human embryonic, adult, and elderly testis tissues and spermatozoa. **(A–D)** *NYD-SP20.* Lane 1: marker; Lane 2: adult testis; Lane 3: spermatozoa; Lane 4: embryonic testis; Lane 5: elderly testis. **(A)** P1 primers could amplify all 4 forms. PCR products from spliceoform b were 200 bp, whereas the other 3 were 330 bp. **(B)** P2 primers could amplify isoforms c and d with fragments of 440 and 341 bp, respectively. **(C)** P3 primers could amplify spliceoforms a and b with fragments of 465 and 315 bp, respectively. **(D)** Positive control: amplification of human β-actin. **(E, F)** *CRISP2.* Lane 6: marker; Lane 7: human spermatozoa; Lane 8: embryonic testis; Lane 9: adult testis; Lane 10: elderly testis. **(E)** Distribution of *CRISP2* as shown by amplification of the 185-bp PCR product in human adult testis and spermatozoa. **(F)** Human β-actin was employed as the positive control.

staining of mouse testes at different developmental stages. Low-level immunoreactivity was observed in testis sections from postnatal day 6 animals. On day 14, increased staining was observed predominantly in areas containing primary spermatocytes at the early pachytene stage. Finally, in postnatal day 20 and adult testes, positive cytoplasmic signals were observed in almost all testicular cells, ranging from pachytenes to round spermatids. Surprisingly, in testes from adult mice, strong staining was also present in the luminal centers, which contained dispersed bundles of flagella from mature spermatids. Integrating this information with our data, we propose that *FXR1P*-a is highly expressed in human spermatogenic cells. In contrast, *FXR1P*-b, not detected in human spermatozoa, seems to be expressed in human testicular somatic cells, Sertoli cells, or Leydig cells. These transcripts might influence germ cell development through converging signal transduction pathways, perhaps causing posttranslational modifications of transcription factors or other proteins, or both, that regulate alternative transcript processing in spermatogenic cells (Eddy, 1998).

The identified novel alternatively spliced forms were classified into 3 groups according to their splicing types: alternative exon (including cassette exon and mutually exclusive exon), alternative intron, and alternative splice site (Table 2). We did not find an alternative intron in our report. Interestingly, we identified that 74.63% of spliceoforms employed both alternative transcription then alternative splicing because they used different promoters to initiate transcription. Among them, 70% used a downstream promoter (Table 2). This is an interesting phenomenon that has been seen in previous cases, such as the genes for angiotensin-converting enzyme (ACE; Howard et al, 1990) and calcium^{+2}/calmodulin–dependent kinase IV (CaMKIV; Means et al, 1991), both of which use a downstream promoter to produce truncated, enzymatically distinct proteins specific to male germ cells. Together, these data suggest that the complex genetics of the testis might not come from transcription of new, testis-specific genes, but rather from transcription of somatic genes from different, germ cell–specific downstream promoters that then experience alternative splicing to produce various proteins specific to male germ cells.

Spermatogenesis occurs in successive mitotic, meiotic, and postmeiotic phases, so the genes expressed during these processes must encode proteins specific to the different phases of germ cell development. Alternative splicing of pre-mRNA is important for the production of functionally diverse proteins (Lopez, 1998), and alternative transcripts can benefit male germ cells at least 4 ways. First, domain addition or replacement can expand protein function and allow addition of germ cell–specific functions. Second, spliceoform-associated changes in protein sequences can alter intracellular protein distributions. Third, long poly(A) tails, which are often found on transcripts in postmeiotic germ cells, can cause a delay of several days between transcription and translation. Fourth, alternate transcripts can arise with the use of a promoter within an intron, resulting in a protein that differs functionally in somatic cells vs male germ cells (Eddy, 2002). In support, we observed that 83.58% of the alternatively spliced transcripts had changes in their predicted ORFs. For example, the gene for testis calpastatin (accession number AF327443) showed alternative splicing in our microarray experiments. Calpain and calpastatin are involved in numerous membrane fusion events that are important in the testicular acrosome reaction. Sequence comparisons between somatic and testis calpastatin revealed 2 single amino acid changes and large-scale deletions both of domain L and of 13 amino acids between inhibitory domains I and II. However, these changes were not found to alter inhibitory activities (Takano et al, 1993), suggesting that the alternatively spliced form might have alterations in its 3-dimensional structure, inhibitory activity, subcellular location, or a combination of these factors, allowing it to influence the acrosome reaction (Zhu et al, 2002). Besides this splice, we also discovered that other novel alternative splices of various genes (accession numbers AF333336, AF327560, AF353720, AY026505, AY359880, AY313777, AY186741, AY303972, AF361054) change their predicted ORF and might be related to spermatogenesis (Cheng et al, 2002; Xiao et al, 2002; Yin et al, 2002; Zhou et al, 2002; Fang et al, 2004; Huang et al, 2004; Zhu et al, 2004; Hu et al, 2004; Zheng et al, 2004).

We further focused our attention on *NYD-SP20,* a novel Unigene cluster that showed 4 novel alternative spliceoforms in our tested microarrays. All of the spliceoforms differed in exon 1, and spliceoform b lacked exon 3, thus generating a shortened predicted protein product (Figure 3A). All 4 spliceoforms were expressed in human adult testis and spermatozoa, whereas only 3 were detected in embryonic and elderly testis tissues. Another examined gene, *CRISP2,* had 4 alternative spliceoforms in the microarrays, 3 of which were novel and 1 of which (spliceoform c) had a longer exon 8, thus encoding a longer predicted protein product (Figure 3B). All 4 *CRISP2* spliceoforms were expressed in adult testis and spermatozoa but were not detected in embryonic or elderly testis samples. Developmentally, spermatogenesis does not occur in male embryos. In adults, spermatogenic cells undergo successive mitotic, meiotic, and postmeiotic phases and then form mature sperm. In elderly males, spermatogenesis weakens and the quantity and quality of sperm decreases (Bellve et al, 1977; Eddy, 2002). We found that 3 spliceoforms of *NYD-SP20* (b, c, and d) were present in adult testis tissues and spermatozoa, but not in embryonic and elderly testes samples. In addition, the previously known alternatively

spliced form of *CRISP2* (a) was located mainly in the testis, and the mouse homolog was identified in round spermatids (Kratzschmar et al, 1996). These observations suggest that all forms of *CRISP2* could be located in germ cells and highly related to spermatogenesis. Additionally, 3 spliceoforms of *NYD-SP20* (a, c, and d) encode a different protein than form b, and 3 forms of *CRISP2* (a, b, and d) encode a different protein than form c. Taken together, these results suggest that alternative splicing could be an important mechanism for proper testis development and function, allowing genetic diversity from an array of normally somatic gene products.

We herein identified many novel development-related alternative splicings in the testis and propose that these play a role in the complicated process of testis development and spermatogenesis. Further work will be necessary to determine the regulation mechanism of alternative splicing and the function of these proteins in human testis development.

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