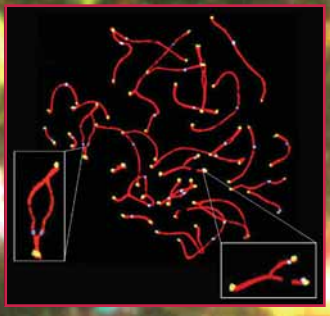
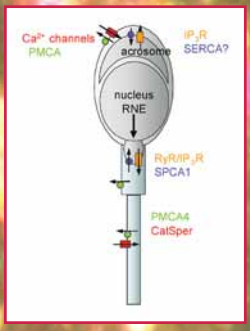
The Genetics of Male Infertility

Edited by

Douglas T. Carrell





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PREFACE

Male infertility is a common and severe health problem. Infertility not only affects one's ability to have children, but also has emotional, psychological, family, and societal effects. Despite the prevalence and significance of this health problem, resources and attention have not been sufficiently focused on this important issue.

Approximately 7% of men suffer from infertility, and the incidence may be increasing. Of those affected, roughly 40% have idiopathic infertility. It is likely that the majority of those patients have genetic abnormalities that are the cause of their infertility. However, it is important to remember that there are genetic ramifications for essentially all infertile male patients. For example, it is likely that there are genetic predispositions to pathologies such as varicoceles, and environmental factors almost certainly modulate the underlying condition. The understanding of the genes involved in spermatogenesis, sperm maturation, and normal sperm function is key, but we must also focus on better methods of accelerating advances into meaningful clinical diagnostic tests and therapies.

During the past 20 years, significant improvements in technology have advanced the treatment of male infertility. The primary advance has been intracytoplasmic sperm injection (ICSI) in conjunction with in vitro fertilization. Although this technological leap has allowed thousands of men to father a child who otherwise would have been unable to do so, the scientific study of the causes of male infertility has not kept pace. In fact, the clinical application of ICSI proceeded without sufficient scientific study of its safety to the offspring, or the future genetic ramifications.

We currently stand at a point in history in which new tools are available to evaluate genetic diseases. The completion of the Human Genome Project has ushered in an era of unprecedented momentum and ability to tackle the complex issues in the genetics of male infertility. New tools include in vitro methodologies, *in silico* technologies, and new model organisms. Together these advances portend great possibilities.

In January 2006, an international symposium was held at the University of Utah Campus in Salt Lake City to address the genetic causes of male infertility and the translation of the knowledge to the clinical realm. Twenty-one researchers and clinicians, and an international audience of

experts in the field, reviewed the study of the genetics of male infertility, the tools available in the laboratory and clinic, the current state of knowledge, and the future of research and translation into clinical diagnostics and treatments. This book is the result of the symposium. The book is intended as a review of our current understanding of genetic causes of male infertility, a guide to evidence-based clinical applications, and a preview of future possibilities.

Douglas T. Carrell, ***PhD***

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I Methods and Tools for the Study of the Genetics of Male Infertility

1 The Genetics of Male Infertility in the Era of Genomics

Tools for Progress

Douglas T. Carrell, PhD

Summary

The histories of progress in the fields of genetics and andrology are rich and include many breakthroughs. The era of genomics, initiated with the completion of the Human Genome Project, is upon us and offers many new tools for better understanding the genetics of male infertility. Genomic breakthroughs give us a better understanding of structural components of DNA, new types of genetic polymorphisms, regulation of gene expression, and the identity of genes involved in male infertility. The advances we have seen in genomics are key to facilitating some of the studies needed to gain a better understanding of the genetics of infertility, but researchers in this field can better maximize resources and tools through focused collaboration on studies of major issues.

Key Words: Male infertility; genomics; medical resequencing; consortium; gene; spermatogenesis; Human Genome Project.

1. GENETICS AND ANDROLOGY: COLLABORATION BETWEEN TWO FIELDS

With the recent passing of the 50th anniversary of the publication of Watson and Crick's elucidation of the structure of DNA, much attention has been focused on the rich history of the field of genetics. From the identification of DNA as the molecule responsible for heredity in 1944 to the completion of the Hapmap Project last year, the history of genetics is marked by regular advances in techniques and understanding that have fueled the hope of future therapies to alleviate suffering and provide a higher quality of life. Although those hopes have not been realized as quickly as desired and often predicted, recent breakthroughs, largely accelerated by the Human Genome Project (HGP), have raised

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expectations higher than ever before. It is clear that we are currently in an era of genomics, an era in which advances in genetic tools are shaping the methods and capabilities available to treat disease.

Although the term *andrology* was sporadically used as far back as 150 yr ago, the use of the term to denote the study of male reproduction and infertility was coined and commonly accepted in 1951, 2 yr before the elucidation of the structure of DNA *(1)*. Since that time, the evaluation and treatment of male infertility have evolved from simple techniques to evaluate sperm characteristics to a better understanding of underlying endocrinology to today's common use of intracytoplasmic sperm injection, chromatin evaluation, and sperm function assays, and the initiation of candidate gene evaluation. The interaction of genetics and andrology has been continual and productive throughout the past, bringing breakthroughs such as the identification of sexual differentiation abnormalities, Y-chromosome microdeletions, and DNA nicks and breaks. However, with the completion of the HGP and our entrance into the era of genomics, it is clear that many of the major concerns facing those studying male infertility will likely be solved using the techniques and tools the field of genomics is producing.

The era of genomics does not have a start date, however, it is clear that the genomics movement gained great momentum in 1990 with the planning of the HGP, and was officially ushered in by the initial publication of the sequence of the human genome in 2001 *(2–4)*. The HGP has spawned other major initiatives, such as the Hapmap Project, which is described in Section 3, and the Encyclopedia of DNA Elements project, a study that aims to identify all control mechanisms involved in a representative sample (~1%) of the genome *(5)*. Major consortia have been formed to study these and other big-issue questions, such as the role of the environment in gene function and the genetics of cancer. It is apparent that the progress made in genomics is largely a result of unprecedented collaboration of various specialties (sequencing, bioinformatics, statisticians, classical genetics, etc.) and this model of collaboration could benefit most areas of biomedical research.

Major questions in the study of male infertility include: What are the genes involved in normal spermatogenesis, sperm maturation, and sperm function? Can we identify what polymorphisms or mutations result in infertility, and if so, how can we screen and treat patients better? What are the regulators of normal gene expression during spermatogenesis? What role does abnormal meiotic recombination and segregation play in male infertility? What effect does abnormal DNA nicks and breaks have on embryogenesis? What is the role of abnormal protamines in infertility and does it relate to imprinting or epigenetic defects? What is the role of the environment, diet, and other factors in

the variation of the degree of pathology seen in different individuals (i.e., varicoceles, smoking effects, etc.)? These and many other important questions will largely be addressed through genetic studies. Proteomics, physiology, endocrinology, and other fields of study will assist in the quest, but it is likely that many of the large leaps made in the study of male infertility will be largely because of genetic advances, lessons learned, and the technologies developed from the HGP. Therefore, it is important to remember not only the advances spurred through the genomics revolution, but also the significant and unique collaborative efforts used in the process.

2. THE CONTRIBUTION OF THE HGP

The HGP was initiated with great hope that the sequencing of the human genome would yield tremendous advances in the understanding of gene function and the etiology of human diseases *(6)*. However, it is likely that, at this time, many of the major breakthroughs of the HGP are in the basic understanding of the human genome. Foremost is the identification of 20,000–25,000 genes, a number much lower than previously predicted *(2,7)*. Previous studies have estimated that at least 2000 genes may be involved in normal spermatogenesis, a strikingly high percentage of the total complement of human genes *(8)*.

Although the number of genes in the human genome is smaller than expected, the diversity of gene products is larger. It is estimated that as many as 35–60% of genes undergo alternative splicing, which increases the diversity of the proteome and the complexity of regulatory and functional mechanisms. Additionally, the data indicate a surprisingly narrow range in the number of genes found in a comparison of humans and other animals.

Another basic finding from the HGP that highlights the increased diversity of products of the genome is the common transcription of nonprotein-coding RNA. Some of these RNAs may simply be the result of alternative 5′ start sites during transcription, or may they may be involved in regulatory mechanisms, but it is obvious now that nonprotein-coding RNAs are essential to normal cellular function. More than 800 human micro-RNA “genes” have been identified and appear to be essential to normal development and metabolism *(9)*. The micro-RNAs are apparently an essential regulator of gene expression and very relevant to sperm function *(10)*. The mechanisms and functions of micro-RNAs are a current area of major research, and addressed in Chapters 3 and 4.

In addition to a better understanding of the diversity of the genome and its messenger RNA products, genomic advances have improved our understanding of several structural components of the genome. One such discovery is the presence of ultraconserved elements (UCEs;

ref. *11*). UCEs are sequences of at least 200 bp with complete homology between the human, mouse, and rat sequences. Thus far, 481 human UCEs have been identified. Their function has not been entirely worked out; however, it appears that they contain enhancer elements *(6,12)*. Given their evolutionary conservation, it seems likely that the UCEs play a vital role in gene expression regulation.

Another finding is that the genome contains "gene deserts," which are regions of 3 megabases or more that are devoid of genes *(3)*. The regions do not appear to be a result of the normal statistical distribution of the genes, which raises interesting questions as to their function. At this point, the only possible function of these regions is the possible identification of enhancers for lateral genes *(13)*. Nobrega et al. *(14)* have experimentally removed two such deserts in mice, with no apparent effect. Additionally, there is at times a clustering of functionally related genes of nonrelated origin *(15)*. It would appear that an evolutionary advantage might sometimes be found in the clustering of functionally related genes into "neighborhoods," with obvious implications for coordinated expression regulation.

Studies have found that the genome is polymorphic in a structural sense on a much larger scale than previously thought *(16)*. Using comparative microarray technology, large differences in copy number variation were shown, and it was suggested that large-scale DNA variations of up to several hundred kilobases were responsible. Several studies have since shown that these deletions and other changes are relatively common and more than 1000 such polymorphisms have been identified *(16–19)*. The studies that identified these polymorphisms used different assays and had small overlaps, indicating that the ideal assays to identify the polymorphisms are not yet known, and that there may be many more polymorphisms to be found *(20)*. This exciting find is likely to have profound implications in many areas, including a better understanding of polymorphic phenotypes, including infertility.

3. THE IDENTIFICATION AND EVALUATION OF CANDIDATE MALE INFERTILITY GENES

The great promise of the HGP is in the identification and evaluation of candidate genes in patients. Previous estimates have predicted that about 10% of the genes in the human genome may be related to spermatogenesis and fertility *(8)*. Those estimates are based largely on animal studies, with human data recently beginning to significantly add to the pool. Table 1 is a current list of genes known to affect male fertility.

Table 1
Genes That Cause Male Infertility When Targeted

Gene symbol	*Gene name*	*Reproductive phenotype*
ADAM1a	A disintegrin and metallopeptidase domain 1a	Asthenospermia, penetration defect
ADAM2	A disintegrin and metallopeptidase domain 2	Sperm–egg fusion defect
ADAM3	A disintegrin and metallopeptidase domain 3; cyritestin	Sperm–zona fusion defect
AKAP4	A kinase (PRKA) anchor protein 4	Abnormal tail morphology, asthenospermia
Acr	Acrosin	Sperm are not capable of binding and penetrating the zona pellucida
Acvr2	Activin receptor-type IIA	Small testes, delayed fertility
ACOX	Acyl-Coenzyme A oxidase 1, palmitoyl	Leydig cell hypoplasia, small testes, abnormal spermatogenesis
ADFP	Adipose differentiation -related protein	Male infertility
Arl4	ADP-ribosylation factor-like 4	Significantly reduced testicular weights and sperm counts
AFF1	AF4/FMR2 family, member 1	Male subfertility (decreased litter size)
AFF4	AF4/FMR2 family, member 4	Enlarged seminal gland, small testis, azoospermia, arrest of spermatogenesis, abnormal epididymis morphology
Man2a2	α-mannosidase IIx	Defect in adherence of spermatogenic cells to Sertoli cells; germ cells prematurely released from the testis
Amhr2	AMH receptor	Abnormal semal differentiation
Npepps	Aminopeptidase puromycin-sensitive	Asthenospermia, abnormal tubules

(Continued)

Table 1 ***(Continued)***

Gene symbol	*Gene name*	*Reproductive phenotype*
Ar; tfm	Androgen receptor; testicular feminization	Feminized external genitalia; hypogonadal; cryptorchidism with a block in spermatogenesis
ACE	Angiotensin I-converting enzyme; peptidyl-dipeptidase A 1	Presumed penetration defect; normal testicular histology, concentration, sperm morphology
Ace	Angiotesin-converting enzyme	Compromised ability of sperm to fertilize ova
AE2	Anion exchanger 2	Disrupted spermiogenesis, complete absence of spermatozoa in tubules
Amh	Anti-Mullerian hormone	Uteri development in males causes obstruction and secondary infertility
Apob	Apolipoprotein B	Decreased sperm count, motility, survival time, and ability to fertilize ova
Apaf1	Apoptotic protease-activating factor 1	Spermatogonial degeneration
Atm	Ataxia Telangiectasia	Germ cells degenerate; disruptions evident in meiosis I
Atxn7	Ataxin 7	Reduced fertility at 16 wk of age
AGTPbp1	ATP/GTP-binding protein 1	Oligospermia, teratospermia, asthenospermia
Atp2b4	ATPase, Ca^{++} transporting, plasma membrane 4	Infertile
Atp8b3	ATPase, class I, type 8B, member 3	Impaired sperm–egg interaction, reduced zona pellucida-induced acrosome reaction
Bbs2	Bardet-Biedl syndrome 2 homolog (human)	Sperm lack flagella
Bbs4	Bardet-Biedl syndrome 4 homolog (human)	Flagella are absent throughout the seminiferous tubules, even on cells with condensed sperm heads
BSG	Basigin	Azoospermia, arrest at meiosis I

(Continued)

Table 1 ***(Continued)***

Gene symbol	*Gene name*	*Reproductive phenotype*
Bsg	Basign	Block in spermatogenesis at metaphase I
Bax	Bc12-associated X protein	Premeiotic arrest of spermatogenesis
*Bc*16	B-cell leukemia/ lymphoma 16	Apoptosis in metaphase I spermatocytes
Bclw; Bc1212; Bc12-like 2	BCL2-like 2 protein apoptosis regulator *BCL-W*	Late meiotic arrest with loss of germ cells
	β 1-4-galactosyl-transferase	Male infertility; defects in sperm–egg interaction
Btrc	β-transducin repeat-containing protein	Meiotic arrest with multiple errors
bs	Blind-sterile	Small testis, oligospermia
Bmp4	Bone morphogentic protein 4	Absent primordial germ cell (PGC) population; defect in PGC development
Bmp8a	Bone morphogentic protein 8a	Degeneration of germ cells and epididymis
Bmp8b	Bone morphogentic protein 8b	Reduced or absent PGCs (developmental defect); postnatal germ cell defects and spermatocyte apoptosis
Bdnf	Brain-derived neurotrophic factor	Reduced male fertility
Brca1	Breast cancer 1	Spermatogenic arrest
BUB1B	Budding uninhibited by benzimidazoles 1 homolog β	Oligzoospermia
Camk4	Calcium/calmodulin-dependent protein kinase IV	Impaired chromatin packaging during spermiogenesis
Clgn	Calmegin	Defect in sperm-zona pellucida binding
	Cα(2)/Prkaca	cAMP-dependent protein kinase catalytic subunit 2 Males infertile, motility and fertilization affected
Crem	cAMP-responsive element modulator	Defective spermiogenesis with aberrant postmeiotic gene expression
Csnk2a2	Casein kinase Iia 1	Globozoospermia (no acrosomal cap)

(Continued)

Table 1 ***(Continued)***

Gene symbol	*Gene name*	*Reproductive phenotype*
Catsper1	Cation channel of sperm 1	Asthenospermia, normal count and testis weight
Catsper2	Cation channel of sperm 2	Capacitation defect
Cnot7	CCR4-NOT transcription complex, subunit 7	Abnormal testis morphology, testis hypoplasia
Cd59b	CD59b antigen	Teratozoospermia, oligozoospermia, asthenozoospermia
Cks2	CDC28 protein kinase regulatory subunit 2	Male and female germ cells arrest at anaphase I
Cenpb	Centromere protein B	Hypogonadal and have low sperm counts
Cldn11; Osp-11	Claudin 11	No tight junctions between Sertoli cells
Csf1	Colony-stimulating factor (macrophage)	Reduced testosterone
Gja1; C43	Connexin 43	Small ovaries and testes; decreased numbers of germ cells from E11.5
Ros1	c-ros protoncogene	Sperm motility defects
Crsp	Cryptorchidism with white spotting, deletion region	Azoospermia
Cutl1; CDP/Cux	Cut-like 1	Severely reduced fertility
Ccna1	Cyclin A1	Block in spermatogenesis before the first meiotic division
Ccnd2	Cyclin D2	Fertile with decreased testis size
p27Kip1; Cdkn1b	Cyclin-dependent inhibitor 1b	Fertile with testicular hyperplasia
p57kip2; Cdkn1c	Cyclin-dependent inhibitor 1c	Surviving mice show sexual immaturity
p18Ink4c; Cdkn2c	Cyclin-dependent inhibitor 2c	Leydig cell hyperplasia and reduced testosterone production
p19ink4d; Cdkn2d	Cyclin-dependent inhibitor 2d	Testicular atrophy and germ cell apoptosis
Ccne1	Cyclin E1	Testicular hypoplasia
Ccne2	Cyclin E2	Testicular hypoplasia

(Continued)

Table 1 ***(Continued)***

Gene symbol	*Gene name*	*Reproductive phenotype*
Cdkn2d	Cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)	Increased germ cell apoptosis, small testis
Adam3	Cyritesin	Altered sperm protein expression and adhesion defects during fertilization
CYP17	Cytochrome P450 17α-hydroxylase/ 17,20-lyase	Abnormal morphology, reduced motility, sexual behavior
Cyp11a	Cytochrome P450, 11a, cholesterol side-chain cleavage	Males feminized with female external genitalia, underdeveloped sex organs; gonads degenerate
Cyp19	Cytochrome P450, 19, aromatase	Early spermatogonial arrest, Leydig cell hyperplasia, and defects in sexual behavior
Cpeb	Cytoplasmic polyadenylation element-binding protein	Disrupted germ cell differentiation and meiosis I synaptonemal complex formation
Tial1	Cytotoxic granule-associated RNA-binding protein-like 1	PGCs lost by E13.5
Dax1 (Nr0b1)	Orphan nuclear receptor	Progressive degeneration of the germinal epithelium
Ddx4	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked (DBY) Symbol-DDX3Y, AZFa region; VASA homolog	Defective proliferation/ differentiation of PGCs
Dazl	Deleted in azoospermia-like	Reduced germ cells; differentiation failure and degeneration of germ cells
Dhh	Desert hedgehog	Complete absence of mature sperm; defects in Sertoli-to-Leydig cell signaling
Dmc1h	Disrupted meiotic cDNA 1 homolog	Defects in chromosome synapis in meiosis
	DNA polymerase λ	Asthenozoospermia

(Continued)

Table 1 ***(Continued)***

Gene symbol	*Gene name*	*Reproductive phenotype*
Dnaja1	DnaJ (Hsp40) homolog, subfamily A, member 1	Small testis, tubal degeneration
Dms	Dominant male sterility	Testicular degeneration, azoospermia
Dspd	Dominant spermiogenesis defect	Teratozoospermia, oligozoospermia
Dmrt1	Doublesex and Mab-3-related transcription factor 1	Defects in postnatal testes differentiation; disorganized seminiferous tubules and absence of germ cells
Spo11	DPO11 homolog	Defects in meiosis
Cnahc1	dynein heavy chair 7	Defects in sperm flaggelar motility
Ube2b	E2B ubiquitin-conjugating enzyme; HR6B	Alterations in sperm chromatin structure, an incomplete meiotic arrest, abnormal sperm morphology
Egr1; NGFI-A	Early growth response 1	Lack of LH
Egr4	Early growth response 4	Germ cells undergo apoptosis during pachytene stage
Esgd12d	Early spermiogenesis defective 12d	Some epididymal sperm present, asthenozoospermia, teratozoospermia
Elk1	ELK1, member of ETS oncogene family	Asthenozoospermia
Emk	Elkl motif kinase	Infertile
Emx2	Empty spiracles homolog 2	Defective development of gonads and urogenita tracts
Esr1	Estrogen receptor (ER)α	Develop disruptions of the seminiferous epithelium because of abnormal epididymal function, no ejaculations
Esr2	ERβ	Fertile, but develop prostate hyperplasia
Etv4	Ets variant gene 4 (E1A enhancer-binding protein, E1AF)	Severe oligozoospermia
Etv5	Ets variant gene 5	Early testicular degeneration
Fanc	Fanconi anemia complementation group A	Hypogonadism, reduced fertility

(Continued)

Table 1 ***(Continued)***

Gene symbol	*Gene name*	*Reproductive phenotype*
Fancc	Fanconi anemia complementation group C	Hypogonadism, compromised gametogenesis
Fancg	Fanconi anemia complementation group G	Hypogonadism, compromised gametogenesis
Adam2	Fertilin β	Altered sperm protein expression and adhesion defects during fertilization
Fgf9	Fibroblast growth factor 9	XY male-to-female sex reversal; phenotype ranges from testicular hypoplasia to complete sex reversal
Fkbp6	FK506-binding protein 6	Absence of normal pachytene spematocytes
Fmr1	Fragile-X mental retardation syndrome 1 homolog	Macroorchidism
Fishb	FSH hormone β-subunit	Decreased testis size
Fshr	FSH receptor	Decreased testis size
Gpr 106	G protein-coupled receptor 106	Crsp males homozygous for trans gene integration exhibit a high intra-abdominal position of the testes, complete sterility
Gpr64	G protein-coupled receptor 64	Enlarged testis, azoospermia
Gcl	Germ cell-less homolog (Drosophila)	Asthenozoospermia, teratozoospermia (giant heads with multiple tails), oligozoospermia
Gdnf	Glial cell line-derived neurotrophic factor	Depletion of stem cell reserves; spermatogonia differentiate
GAPDS	Glyceraldehyde 3-phosphate dehydrogenase-*S*	Severely decreased sperm motility
Cga	Glycoprotein hormone α-subunit	Hypogonadal because of FSH and LH deficiency
GRTH/ Ddx25	Gonadotropin-regulated testicular RNA helicase	Arrest of spermiogenesis, elongation failure
*iPLA(2)*β	Group VIA phospholipase A2	Reduced motility, impaired fertilization, unable to fertilize

(Continued)

Table 1 ***(Continued)***

Gene symbol	*Gene name*	*Reproductive phenotype*
Gdf7	Growth differentiation factor-7	Defects in seminal vesicle development
Ghrhr	Growth hormone-releasing hormone receptor	Idiopathic
Gdi1	Guanosine diphosphate dissociation ihibitor 1; Rho GDI α	Impaired spermatogenesis, vaculolar degeneration in males
HSFY	Heat shock factor Y	Deleted in individual with idiopathic azoospermia
Hsp70-2	Heat shock protein 70-2	Meiosis defects and germ cell apoptosis
Hfe2	Hemochromatosis type 2 (juvenile; human homolog)	Sterility
Tcf1	Hepatocyte nuclear factor (HNF-1α) transcription factor 1	Vestigial vas deferens, seminal vesicles and prostate, impaired spermatogenesis, no mating behavior
Hmga1	High mobility group AT-hook 1	Abnormal Sertoli cells, abnormal epididymis morphology
Hmgb2	High mobility group box 2	Sertoli and germ cell degeneration and immotile spermatozoa
H3f3a	Histone 3.3A	Reduced copulatory activity and fewer matings result in pregnancy
H2afx	Histone H2A family, member X	Pachytene stage arrest in spermatogenesis; defects in chromosome segregation and MLH1 foci formation
Hrb	HIV-1 Rev-binding protein	Round-headed spermatozoa lack an acrosome (Globozoospermia)
Hoxa10	Homeobox A10	Variable infertility; cryptorchidism
Hoxa11	Homeobox A11	Males have malformed vas deferens and undescended testes
HOOK1	Hook homolog 1	Teratozoospermia and decapitation
HE6/ GPR64	Human epididymal protein 6	Dysregulation of efferent ductule fluid reabsorbtion, stasis of spematozoa within the ducts

(Continued)

Table 1 ***(Continued)***

Gene symbol	*Gene name*	*Reproductive phenotype*
Bc1X; Bc121	Hypomorph	PGCs are lost by E15.5
Inha	Inhibin a	Granulosa/Sertoli tumors, gonadotropin hormone-dependent
Inpp5b	Inositol polyphosphate-5-phosphatase	Sperm have reduced motility and reduced ability to fertilize eggs; defects in fertilin β processing
Igf1	Insulin-like growth factor 1	Hypogonadal and infertile; disrupted spermatogenesis and vestigial ductal system, defects in mating behavior
Insl3	Insulin-like hormone 3	Bilateral cryptorchidism results in abnormal spermatogenesis
Izumo1	Izumo sperm–egg fusion 1	Normal zona penetration, abnormal oolema binding
JunD; Jund1	Jun D proto-oncogene	Anomalous hormone levels and sperm structural defects
Klhl10	Kelch-like 10 (Drosophila)	Sertoli cell only
Kitl	Kit ligand	Defect in PGC migration/ survival
Kit	Kit receptor	White spotting null mutation causes PGC defects
Ggtp	λ-Glutamyl transpeptidase	Hypogonadal and infertile; phenotype corrected by feeding mice *N*-acetylcysteine
LGR8 (GREAT)	Leucine-rich repeat-containing G protein-coupled receptor	Intra-abdominal cryptorchidism and sterility
Lep; ob/ob	Leptin	Obese and infertile with hypogonadotrophic hypogonadism
Lepr; db/db	Leptin receptor	Obese and infertile with hypogonadotrophic hypogonadism
Lgr7	Leucine-rich repeat-containing G protein-coupled receptor	Spermatic apoptosis at meiotic stage 12
Lipe; HSL	Lipase, hormone-sensitive	Multiple abnormalities in spermatogenesis
Lhcgr	LH receptor	Underdeveloped sex organs and infertility; spermatogenesis

(Continued)

Table 1 ***(Continued)***

Gene symbol	*Gene name*	*Reproductive phenotype*
		arrested at round spermatid stage
Smad5; Madh5	MAD homolog 5	Developing embryos lose PGCs
Smad; Madh1	MAS homolog 1	Developing embryos lose PGCs
Mell1	Mel-transforming oncogene-like 1	Decreased fertilization and embryogenesis
Mitf	Microphthalmia-associated transcription factor	Reduced male fertility
Morc	Microrchidia	Early arrest in meiosis and germ cell apoptosis
Mtap7; E-MAP-115	Microtubule-associated protein	Abnormal microtubules in germ cells and Sertoli cells
Mlh3	MutL homolog 3 (*E. coli*)	Increased sperm aneuploidy, increased arrest at pachytene
Mlh1	MutL homolog 1	Meiotic arrest and genomic instability
Msh4	MutS homolog 4	Prophase I meiotic defects apparent at the zygotene/ pachytene stage; germ cells lost within a few days postpartum
Msh5	MutS homolog 5	Zygotene/pachytene meiotic defects with aberrant chromosome synapsis and apoptosis
Myhl1; A-myb	Myeloblastosis oncogene-like 1	Germ cell meiotic arrest at the pachytene stage
NKCC1; Slc12a2	Na(+) –K(+) –2C1(–) cotransporter; solute carrier family 12, member 2	Low spermatid counts and compromised sperm transport
Nkd1	Naked cuticle 1 homolog (Drosophila)	Oligozoospermia
Nanos2	Nanos homolog 2 (Drosophila)	Azoospermia
Nanos3	Nanos homolog 3 (Drosophila)	Increased germ cell apoptosis, no germ cells were detected in the testes by E15.5

(Continued)

Table 1 ***(Continued)***

Gene symbol	*Gene name*	*Reproductive phenotype*
Neurl	Neuralized-like homolog (Drosophila)	Asthenozoospermia, missing sperm heads
Nxph1	Neurexophilin 1	Infertility appears to be an artifact of homologous recombination
Nhlh2	Neuronal helix–loop–helix 2	Infertile and hypogonadal
NIR	Neuronal insulin receptor	Hypothalamic hypogonadism; impaired spermatogenesis
Nkx3-1	NK-3 transcription factor, locus 1 (Drosophila)	Accessory gland deformation
Nmp4	Nuclear matrix protein 4	Abnormal seminiferous tubule morphology, decreased spermatocytes
Nr5a1	Nuclear receptor subfamily 5, group A, member 1	Prostate hypoplasia, seminal gland hypoplasia, germ cell depletion
Ncoal; SRC1	Nuclear receptor co-activator; steroid receptor coactivator-1	Decreased responsiveness to steroid hormones in testes and prostate
Nr0b1	Nuclear receptor subfamily 0, group B, member 1	Early testicular degeneration
Nr2c2	Nuclear receptor subfamily 2, group C, member 2	Oligozoospermia, cells arrest in meiotic prophase stage/ pachytene spermatocyte stage resulting in an increase in the ratio of stage X to stage XII tubules
Nr5a1; SF-1	Nuclear receptor subfamily 5, group A, member 1; steroidogenic factor-1	Gonadal agenesis in both sexes
Ovo	Ovo protein (Drosophila melanogaster homolog)	Reduced fertility and underdeveloped genitalia
P2rx1	P2X1 receptor	Oligospermia and defective vas deferens contraction
Wip1	p53-induced phosphatase	Runting and testicular atrophy
PLCdelta4	Phospholipase C δ 4	Sperm fail to activate eggs, no calcium transients
Pi3k	Phosphatidylinositol 3′-kinase	Defects in proliferation and increased apoptosis of spermatogonia

(Continued)

Table 1 ***(Continued)***

Gene symbol	*Gene name*	*Reproductive phenotype*
Piga	Phosphatidylinositol glycan, class A	Abnormal testes, epididymis and seminal vesicles
Pss2/ Ptdss2	Phosphatidylserine synthase 2	Reduced testis weigth, some infertile males
Styx	Phosphoserine/threonine/ tyrosine interaction protein	Defects in round and elongating spermatid development
mili/piwil2	Piwi-like homolog 2	Spermatogenesis arrested in early prophase I
Pafah1b1	Platelet-activating factor acetylhydrolase, isoform 1b, β1 subunit	Azoospermia, abnormal testicular morphology
Nectin-2/ Pvrl2	Poliovirus receptor-related 2	Abnormal morphology, males are sterile
TPAP/ Papolb	Polymerase β (testis-specific)	Sperm arrest during spermiogenesis
Pea3	Polyomavirus enhancer activator 3	Normal mating behavior, but males do not set plugs or release sperm
Pms2	Postmeiotic segregation increase 2	Abnormal chromosome synapsis in meiosis
Doppel/ Prnd	Prion protein dublet	Reduced counts, motility and morphology
Adamts2	Procollagen *N*-proteinase	Defects in spermatogenesis; marked decrease in sperm within testes tubules
Prlr	Prolactin receptor	Variability in infertiity and subfertility
Prm1	Protamine 1	Protamine haploinsufficiency; abnormal spermatogenesis
Prm2	Protamine 2	Protamine haploinsufficiency; abnormal spermatogenesis
PN-1	Protease inhibitor protease nexin-1; serpine2	Abnormal seminal vesicle morphology and altered semen protein composition
Ppp1cc	Protein kinase A, catalytic subunit λ	Defects in spermiogenesis
P2rx1	Purinergic receptor P2X, ligand-gated ion channel 1	Impaired neurogenic vas deferens contraction, azoospermia
CatSper	Putative sperm cation channel	Defects in motility and fertilization

(Continued)

Table 1 ***(Continued)***

Gene symbol	*Gene name*	*Reproductive phenotype*
Rsn/ CLIP-170	Reed-Steinberg cell-expressed intermediate filament-associated protein	Abnormal head morphology
Rara	Retinoic acid receptor α	Complete arrest and degeneration or germ cell depletion
Rarg	Retinoic acid receptor λ	Squamous metaplasia of the seminal vesicles and prostate
Rxrb	Retinoid X receptors	Germ cell maturation defects and tubular degeneration
RNF17	Ring finger protein 17	Component of granuales, arrest as round spermatids
Sept4	Septin 4	Mice sterile because of defective motility, tail morphology
Serac1	Serine active site containing 1	Male sterility
Serpina5	Serine proteinase inhibitor A 5; protein C inhibitor	Sertoli cell destruction
SH2-B	SH2-B homolog	Small testes and reduced sperm count
NHEt	Sodium hydrogen exchanger-testis	Normal morphology and count, but severely reduced motility
sAC	Soluble adenylyl cyclase	Total loss of forward motility
SLC19A2	Solute carrier family 19 (thiamine transporter), member 2	Germ cells arrest as primary spermatocytes
Sp4	Sp4 *trans*-acting transcription factor	Defects in reproductive behavior
PF20/ Spag16	Sperm-associated antigen 16	Significant loss of sperm cells at round spermatid stage, disorganized axoneme structure
SMCP	Sperm mitochondria-associated cysteine-rich protein	Reduced motility, impaired fertilization
SMCP	Sperm mitochondrion-associated cysteine-rich protein	Defects in sperm motility and migration into the oviduct; defects in fertilization
	Sperm-1	Defect in haploid sperm function

(Continued)

Table 1 ***(Continued)***

Gene symbol	*Gene name*	*Reproductive phenotype*
Spnr	Spermatid perinuclear RNA-binding protein	Defects in seminiferous epithelium and spermatogenesis
Sycp1	Synaptonemal complex protein 1	Ensures formation of crossovers, spermatocytes arrest in pachynema
Sycp3	Synaptonemal complex protein 3	Defects in chromosome synapsis during meiosis; germ cell apoptosis
Tlp;TRF2	TATA-binding protein-like protein	Postmeiotic spermiogenesis block (defective acrosome formation in early stage spermatids)
TLF	TBP-like factor	Complete arrest of spermiogenesis at round spermatid stage
Tektin-t	Tektin-t	Abnormal morphology, bent tails, reduced motility
Tert	Telomerase reverse transcriptase	Progressive infertility
PC4; Pcsk4	Testicular germ cell protease	Sperm have impaired fertilizaiton ability
Tenr	Testis nuclear RNA binding protein	Infertile males with reduced sperm count, motility, and morphology
SSTK	Testis-specific serine kinase	Profound impairment of motility and morphology
Theg; Kisimo	THEG homolog isoform 2	Abnormal elongated spermatids; asthenospermia
Tnp1	Transition protein 1	Abnormal chromosome condensation, sperm motility
Tnp2	Transition protein 2	Abnormal chromosome condensation
Fus1	Translocated in liposarcoma; TLS	Defects in spermatocyte chromosome pairing
Utp14b	U3 small nucleolar ribonucleoprotein, homolog B	Type A spermatogonia fail to differentiate
UTP14c	U3 small nucleolar ribonucleoprotein, homolog C	In frame stop codon identified in infertile males
Siah1a	Ubiquitin protein ligase seven in absentia 1A	Block in spermatogenesis and germ cell apoptosis; failure to complete transition to telophase

(Continued)

Table 1 ***(Continued)***

Gene symbol	*Gene name*	*Reproductive phenotype*
		of meiosis I
Ube3a	Ubiquitin proteinligase E3A; E6-AP ubiquitin protein ligase	Testicular hypoplasia, defects in spermatogenesis and prostate gland development
USP26	Ubiquitin-specific protease 26	Mutations identified in infertile males
USP9Y; DFFRY	Ubiquitin-specific protease 9; AZFa region	Azoospermia
Ube2b	Ubiquitin-conjugating enzyme E2B, RAD6 homology	Increased apoptosis of primary spermatocytes, infertile males
HR23B; Rad23b	Ubiquitin-like DNA repair gene	Most knockouts die during development or shortly after birth; surviving mice have multiple abnormalities and male sterility
Vdr	Vitamin D receptor	Defects in estrogen biosynthesis, elevated serum gonadotropins
VDAC3	Voltage-dependent anion channel 3	Normal counts, abnormal axonemes, loss of a single microtubule doublet
Vdac3	Voltage-dependent anion channel type 3	Immotile sperm; axonemal defets with sperm maturation
Wt1	Wilms tumor homolog	Gonadal agenesis
Wnt7a	Wingless-type MMTV integration site family member 7A	Do not have Mullerian duct regression
MSY2/ YBX2	Y box protein 2	Spermatogenesis disrupted in postmeiotic null germ cells with misshapen spermatids, no sperm in epididymis
Synj2	Ynaptojanin 2	Male sterility
Zfp148	Zinc finger protein 148	Required for development of fetal germ cells
Zfx	Zinc finger protein X-linked	Reduced germ cell numbers; males have reduced sperm, but are fertile
Dax1 (Nr0b1)	Orphan nuclear receptor	Progressive degeneration of the germinal epithelium

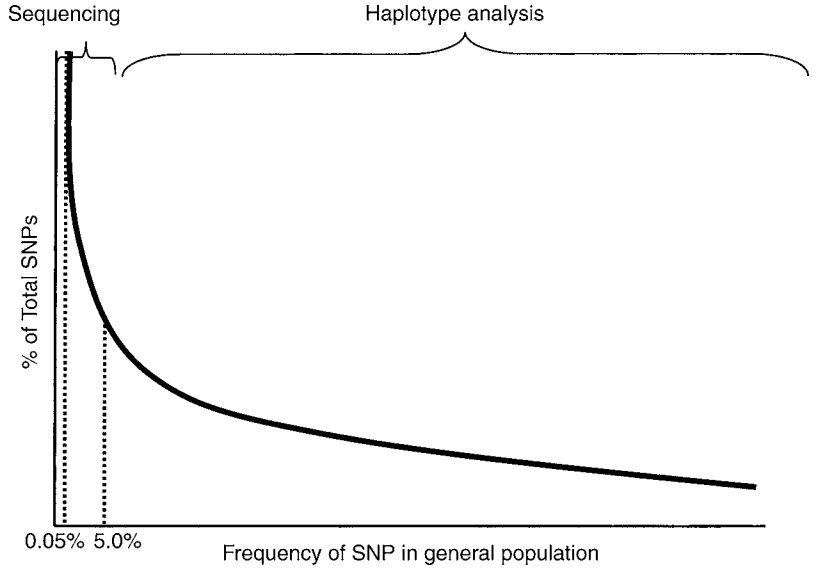

Fig. 1. A plot of the relationship between gene variants and overall polymorphisms. It is expected that many disease causing variants are found in low frequency and will likely require gene sequency studies to identify relevant polymorphisms. On the other hand, common variants can be evaluated using linkage disequilibrium studies, such as haplotype analysis.

The list has been updated from an original listing of both male and female infertility genes by Matzuk and Lamb 4 yr ago *(21)*. Since that time, the number of "male infertility genes" identified has more than doubled.

The strategy used to identify and analyze novel candidate genes in infertile men will be largely dependent on information available about the gene from animal studies, the resources of the laboratory, and the frequency of the variants (polymorphism/mutation) that cause the altered phenotype. Figure 1 demonstrates the hypothetical distribution of gene variants causing disease in relation to the overall frequency of polymorphisms. Common variants, those seen in more than 5% of the population, lend themselves to be analyzed differently than rare variants. Many common diseases may be caused by common variants/polygenic effects (the common disease/common variant hypothesis) such as is expected for heart disease and other common diseases. These types of common diseases may be more easily studied through classical linkage disequilibrium studies and using haplotypes analysis. Alternatively, many diseases are caused by rare (<5% frequency) or very rare (<0.05% frequency) variants. These variants cannot be as easily identified through linkage disequilibrium, rather will likely necessitate

large-scale medical resequencing studies. The causes of male infertility are likely many, and it is possible that numerous strategies will be used to identify and analyze relevant gene defects. Therefore, it is incumbent that new techniques be monitored and analyzed for their potential assistance.

Last year the International Hapmap Consortium released the first draft of the human hapmap *(22)*. The hapmap sequenced more than 1 million single-nucleotide polymorphisms (SNPs) in 269 individuals from four ethnic populations. One SNP was evaluated for approximately every 3 Kb of the genome, and eventually SNPs will be evaluated for every 1 Kb. The usefulness of the hapmap in identifying disease-causing genes is that nearby SNPs generally remain associated with the target SNP because of linkage disequilibrium. Therefore, rather than genotyping each SNP in the genome, targeted SNPs can be evaluated in patients and controls to evaluate haplotypes associated with a given phenotype, which can then be narrowed and further analyzed to identify disease causing polymorphisms *(23)*. Although this technique will undoubtedly be a great tool in identifying some disease-related polymorphisms, drug discovery, and other areas, it is uncertain how beneficial it will be in aiding the study of male infertility. However, it is apparent that such studies must be undertaken in the near future.

Because many causes of infertility are likely caused by rare functional variants not suitable for analysis using hapmap data, identification and analysis of those genes will be continue to be dependent on direct genotyping, or medical resequencing. Medical resequencing has already been performed on a number of candidate genes, largely identified by animal transgenic and knockout studies in which the targeted genes resulted in altered spermatogenesis without other severe phenotypic effects *(24)*. Medical resequencing has been used to identify polymorphisms in several genes affecting male fertility, including the estrogen receptor gene, protamine genes, transition nuclear protein genes, and genes related to globozoospermia *(25–28)*.

Because gene sequencing is key to future studies, advances in this area are of importance. The Sanger method of gene sequencing remains the gold standard for the evaluation of candidate genes in study populations. However, some reports highlight the potential of alternative strategies to more cheaply perform large screens. Current sequencing has been estimated to cost approx $1 per 1000 bases (sequencing costs only), or roughly $3.1 million per human genome *(5)*. The long-term prediction of the eventual ability to sequence a genome for $1000 highlights the predicted advances in improved sequencing capabilities. Whether the ideal sequencing technology

will ultimately rely on modifications to the currently used Sanger technique, such as the application of microfluidic technology to improve injection and separation times, or new sequencing techniques will replace the Sanger method remains to be seen *(29)*. Several alternative sequencing techniques have been proposed and are yet to be refined to the point of practical application *(30)*. Additionally, some new techniques allow the pooling of study samples to identify low-variance polymorphisms and mutations, which could potentially reduce costs dramatically *(31)*.

4. THE FUTURE OF THE STUDY OF THE GENETICS OF MALE INFERTILITY

In 2003, Francis Collins wrote a perceptive evaluation of the future of genomics research *(4)*. Collins related the future to the building of a house, and proposed three levels of future development, all resting on the foundation of the HGP. The first level, genomics to biology, is dependent on three key needs: the development of a comprehensive catalog of all components encoded by the genome, the determination of how those components function in an integrated manner to maintain normal cellular functions, and the need to understand how genomes change. The second level, genomics to health, is dependent on the identification of gene pathways with a role in disease and a determination of how they interact with environmental factors, the development of genome-based diagnostic methods and evaluation of drug response, and the development of translational therapeutic advances. The third and final level, genomics to society, relies on the analysis of genomics, societal factors, and health, and the use of such data to define policy options and ethical guidelines. Six pillars relevant to each of the levels was proposed: resources; technology development; computational biology; training; ethical, legal, and social implications; and education.

Although an understanding of the genetics of male infertility rests on the foundation of areas other than just genomics, this model is of benefit in the development of a vision of the future of the study of the genetic causes and treatments of male infertility. In our field, the three levels are similar to those identified by Collins, and dependent on the same needs and approaches. For example, on the biological level an understanding of not only fertility-related genes is needed, but, as mentioned earlier, we also need to understand the role of micro-RNA, enhancers, promoters, and other regulators. The level of health is dependent on improved assays and treatments, including a stronger regard to the safety of the offspring.

Last, society will benefit by improved safety, lower costs, and broader coverage of assistance to those needing treatment.

Although each of the six pillars proposed by Collins are relevant to our field, focus should be made on three key areas. First, the prudent use of limited resources. With the lack of sufficient funding to assure completion of all relevant areas of study, prioritization and collaboration are key. Identification of a major goal, such as genotyping relevant candidate genes in a large population of well-characterized patients with an equally suitable control group would be an obvious undertaking for a collaborative consortium of researchers. This goal highlights a major area of need for most studies: quality DNA and tissue from patients and controls. Although most laboratories have small repositories of tissue available, pools are usually inadequate for large-scale studies that will be needed. Even more critical, phenotyping is usually inconsistent and incomplete. Last, many studies lack proper controls, both in quantity, diversity, and quality (phenotyping). The establishment of a funded collaborative research tissue repository with community sharing would greatly facilitate studies.

Education is needed on all levels, including interaction between basic scientists and clinicians. Progress is being made in this area as seen by this book and the underlying International Symposium on the Genetics of Male Infertility. However, more is needed. Proper clinical techniques and interpretation of assay results are lacking on the level of the clinician, the patient, and in the decisions society makes regarding health care; these problems can only be solved through education.

The third pillar that we must be especially cognizant of is technology development. Advances are made using proper techniques and utilizing new technologies to their fullest. In addition to equipment and procedures, the proper use of technology includes the use of other model organisms, sometimes more efficient for studies. Examples would be the exciting possibilities for a better understanding of the interaction of proteins with messenger RNA to regulate translation, a regulatory mechanism key to spermatogenesis, which can now be better studied using techniques such as the yeast three-hybrid system and the ability to use RNAi techniques in vivo to better understand protein function *(32,33)*. An example of technological advances involving other species, which can be exploited in the study of male infertility, is the use of the drosophila catalog of more than 2000 induced mutations related to male infertility *(34)*. Most medical and scientific meetings and symposia that deal directly with the diagnosis and treatment of male infertility deal very little with technology. Future meetings must emphasize technology more, so those with a broad spectrum of backgrounds can more fully utilize available tools.

5. CONCLUSIONS

Genomics has brought many tools beneficial to the future study of male infertility. It is clear that the HGP and Hapmap Project could not have been completed without major collaboration and pooling of resources. The study of male infertility greatly benefits from the altruistic collaborative spirit of those pioneers, and will continue to progress using traditional approaches. However, it is clear that the concept of major collaborative efforts in the banking of study tissues, the careful and thorough phenotyping of controls and study subjects, the consistent and careful physiological evaluation of samples, and the genotyping of large study groups and controls are ideas that may hasten the breakthroughs needed to better understand the genetics of male infertility.

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2 The Use of cDNA Libraries to Demonstrate a Linkage Between Transcription and Translation in Male Germ Cells

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Summary

cDNA libraries have played a prominent role in developing the extensive database of gene expression in germ cells and somatic cells of the mammalian testis. Differential screening of cDNA libraries has allowed investigators to determine the temporal up- and downregulation of many genes. This chapter discusses how suppressive subtraction hybridization and cDNA sequencing have been used to define populations of messenger RNAs (mRNAs) that selectively bind, or do not bind, to the germ cell-specific Y-box protein, MSY2. MSY2 is an abundant DNA/RNA-binding protein that in vitro binds to all mRNAs, but shows selective binding to a subset of male germ cell mRNAs in cells. This specificity is regulated by MSY2 binding to a conserved sequence in gene promoters, which facilitates MSY2 binding to the transcripts from these promoters in the nucleus and coordinates the transport, storage, and translational suppression of these mRNAs in the cytoplasm.

Key Words: DNA/RNA-binding proteins; spermatogenesis; germ cells; transcription; translation; mRNA storage.

1. INTRODUCTION

The differentiation of male germ cells from diploid spermatogonia to haploid spermatozoa requires the precise temporal expression of a large number of genes. Based on the National Center for Biotechnology Information Mouse Unigene database, gene profiling, and analyses of testicular cDNA libraries, nearly 20,000 different sequence clusters have been identified *(1–4)*. Because many of the sequence clusters are

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derived from short expressed sequence tag sequences, and because splice variants are common in germ cells, the exact number of messenger RNAs (mRNAs) expressed in the diverse cell types of the mammalian testis is unknown. However, estimates that at least 3000 of these sequence clusters are expressed in the germ cells appear reasonable.

This chapter aims to first present a historical description of how cDNA libraries have been used to develop and build our current database of gene expression during spermatogenesis, and then discuss how cDNA libraries can be used to answer relevant current questions. Many different investigators have contributed to our understanding of testicular gene expression and in defining the cellular expression patterns of their favorite proteins using cDNA cloning procedures. Because of space limitations, I apologize in advance for my inability to cite the research of many important contributors.

2. HISTORICAL BACKGROUND

In the beginning, investigators isolated testis RNA and prepared cDNA libraries in vectors such as pBR322, λ gt10, and λ gt11. These libraries were usually screened with DNA or RNA probes, often designed from known sequences of genes expressed in other tissues or extrapolated from gene sequences from related species. cDNA protein expression libraries in vectors such as λ gt11 were an improvement and provided a means to isolate cDNAs encoding proteins or specific domains of proteins by allowing individual colonies of cDNAs to be screened with monoclonal or polyclonal antibodies. These approaches helped to characterize the temporal expression of many testicular proteins. Moreover, they also identified numerous germ cell-specific proteins whose isoforms are expressed in somatic cells.

Defining the cellular sites of expression of the testis-expressed proteins has proven both exciting and frustrating, largely because of the large number of different somatic and germ cells in the adult testis. Before the isolation of individual populations of male germ cells became a routine procedure in laboratories, researchers utilized the differentiating prepuberal testis as a means to relate protein expression and cell type(s). In the first wave of spermatogenesis, meiotic cells such as pachytene spermatocytes are present in testes of 17-d-old mice and spermiogenesis initiates with round spermatids in the testes of 22-d-old mice. However, analyzing changes in gene expression during this first prepuberal wave of differentiation had limitations in defining cell type expression because it only allowed cellular correlations to be made. With the development of testicular cell dissociation and cell separation

procedures, such as unit gravity sedimentation *(5)* and centrifugal elutriation *(6)*, researchers in reproduction gained a powerful new means to better define temporal (cell type) expression patterns of specific proteins, often using testis cDNAs as probes.

3. DIFFERENTIAL SCREENING OF cDNA LIBRARIES

The utilization of testicular cell dissociation and separation and differential cDNA library screening made it possible to critically test hypotheses, such as whether haploid gene expression occurred in the mammalian testis. Autoradiographic studies had demonstrated a large amount of RNA synthesis occurs during meiosis and hinted at low levels of postmeiotic RNA synthesis in mammals *(7)*. To determine whether the RNAs transcribed during spermiogenesis were just a continued "leakage" of meiotic transcripts or represented additional and perhaps a novel distinct population of postmeiotic transcripts, an adult testis cDNA library was differentially screened with probes derived from cytoplasmic poly(A)+ RNA prepared from isolated populations of meiotic pachytene spermatocytes or postmeiotic round spermatids *(8)*. A number of cDNA clones were identified that hybridized more strongly with the round spermatid cDNAs than pachytene spermatocyte cDNAs. These cDNAs did not hybridize with cytoplasmic poly(A)+ from testes of 17-d-old mice, total RNA from cultured Sertoli cells, adult liver or brain, nonpolyadenylated mRNA from adult testis, or mouse mitochondrial DNA, suggesting selective expression in the haploid germ cells. Sequencing some of the cDNAs identified protamine 1 and 2 and transition protein 1, three genes now known to be first and solely expressed in spermatids. Differential cDNA screens were also successfully used with mRNAs isolated from mice carrying the sex-reversed and testicular-feminization mutations to identify mRNAs expressed during spermiogenesis *(9)*.

4. APPLICATIONS OF cDNA LIBRARY SCREENING

In a larger screening effort, Nishimune and colleagues utilized stepwise subtraction hybridization to identify mouse genes whose transcription is upregulated during spermiogenesis *(10)*. They prepared a cDNA library from adult mouse testis (35-d-old mice) and subtracted it from a cDNA library from prepuberal mouse testis (17-d-old mice). The testes of 17-d-old mice contain spermatogonia and spermatocytes as well as the somatic Leydig, myoid, and Sertoli cells, but lack significant numbers of postmeiotic haploid cell types. Of the approx 30% cDNA clones that were found to be expressing more intensely in the

adult testes, the clones were grouped into three categories. Type 1 cDNA clones were not expressed in the prepuberal testes, type 2 cDNA clones showed an increased expression in the adult testis, and type 3 cDNA clones often detected multiple mRNAs with one size mRNA being present in equal amounts in prepuberal and adult testes and one being predominantly found in adult testis. The detection of mRNAs with different sizes in postmeiotic male germ cells compared with somatic cell types is common and often reflects the use of novel promoters and/or truncation of transcripts in round spermatids (reviewed in refs. *1* and *11*). The stepwise subtraction hybridization of Fujii et al. *(10)* was highly successful, identifying 153 mouse genes whose expression is upregulated during spermiogenesis. Eighty appear to be highly specific to the testis and upon sequencing many encoded novel uncharacterized genes. The stepwise subtraction hybridization methodology can be valuable for both defining temporal expression as well as for gene discovery. Subsequent detailed analyses of cDNAs isolated in this screen helped to identify and provide valuable insight into the expression timing of a number of novel testicular mRNAs encoding proteins such as Hils 1, Hanp 1, Tektin-t, t-actin 1 and 2, and Haprin (reviewed in ref. *12*).

Differential screening of cDNA libraries can be a valuable tool to identify molecular targets of proteins. For instance, cells that rely heavily on posttranscriptional regulation express many different RNA-binding proteins. To define the functions of these RNA-binding proteins, it is useful to determine their RNA target molecules. We have used suppressive subtractive hybridization (SSH) to understand how a very abundant male and female germ cell-specific DNA/RNA-binding protein, MSY2, selects which mRNAs it binds and have found it marks specific transcripts for transport in the nucleus and then helps stabilize them in the cytoplasm *(13)*.

MSY2 is a member of a widely expressed and highly conserved (bacteria to humans) family of nucleic acid-binding proteins. Y-box proteins serve as coactivators of transcription in the nucleus recognizing the DNA motif, CTGATTGGC/TC/TAA *(14)*. In the cytoplasm, they stabilize/store/suppress the translation of specific mRNAs. Y-box proteins are expressed in both somatic cells and germ cells in vertebrates. One member of this family, MSY2, is a 360 amino acid mouse protein encoded by a single-copy gene, which is solely expressed in meiotic and postmeiotic cells of the mouse *(15)*. It is an abundant protein, constituting about 0.7% of total protein in male germ cells *(16)* and 2% of total protein in oocytes *(17,18)*. Gene targeting of MSY2 has confirmed it is solely expressed in germ cells and demonstrated it is essential for male and female fertility *(16)*. Contrin, its highly conserved

human ortholog, is also expressed in meiotic and postmeiotic germ cells *(19)*. Based on its abundance in germ cells *(16)* and from in vitro binding assays that indicate sequence-independent RNA-binding of MSY2, we wondered how germ cells manage to selectively synthesize certain proteins while avoiding random translational inactivation of all their mRNAs by MSY2. Suppressive subtractive hybridization and cDNA cloning gave us the answer by identifying the in vivo mRNA targets and non-targets of MSY2.

Using an affinity-purified antibody to recombinant *MSY2*, immunoprecipitation followed by SSH were used to fractionate MSY2-bound and -nonbound mRNAs. cDNA inserts from randomly chosen clones of the *MSY2*-bound subtracted cDNA library (MSY2-bound) and a nonbound (polysomal) mRNA cDNA library were amplified and sequenced to confirm the differential screening. Genes not expressed in germ cells, such as clusterin, which is expressed in Sertoli cells in the testis, served as valuable controls for binding specificity. A total of 98 clones (48 clones encoding *MSY2*-bound mRNAs and 50 clones of *MSY2*-nonbound mRNAs) were analyzed. Quantitative real time reverse transcriptase-polymerase chain reaction confirmed that the immunoprecipitation and cloning successfully differentiated between two distinct populations of mRNAs.

Using cluster analyses to identify related functions of the bound and non-bound mRNAs, many of the *MSY2*-bound mRNAs were shown to be gamete-specific transcripts expressed in meiotic and postmeiotic cells. Moreover, many of the *MSY2*-bound mRNAs were translationally delayed and specifically critical for male gamete development. In contrast, many nonbound mRNAs were involved in constitutive cellular processes such as cell growth and general metabolism and were translated immediately upon transcription.

Although SSH produced two distinct populations of *MSY2*-bound or -nonbound mRNAs, the molecular mechanism whereby MSY2 recognized specific mRNAs, but not others, was unknown. Analyses of sequence and secondary structures of the mRNA populations failed to detect obvious differences between the bound and non-bound mRNA populations. However, many of the *MSY2*-bound mRNAs contained Y-box sequences in the promoters of their genes, whereas nonbound mRNAs often were transcribed from promoters lacking Y-boxes. To test for a possible linkage between transcription and translation, we compared whether exogenous green fluorescent protein (GFP) transcripts from transgenic mice fractionated in the *MSY2*-bound or -nonbound populations when they were transcribed from promoters containing or lacking the Y-box sequence *(13)*. When GFP was expressed from SP-10

promoters containing or lacking the *MSY2* DNA-binding sequence, GFP mRNAs were either bound or not bound by MSY2, respectively. These experiments demonstrated selective marking by MSY2 of specific mRNAs transcribed from promoters containing Y-box sequences. Chromatin immunoprecipitation assays confirmed that the promoters of this population of mRNAs were preferentially bound by MSY2 (13). Thus, our use of cDNA libraries and SSH allowed us to dissect a mechanism whereby nuclear events associated with transcription were linked to selective mRNA storage and translational regulation in the cytoplasm of male germ cells.

5. cDNA SCREENING BY COMPUTER

The field of reproductive biology is fortunate to have attracted scientists such as M. Eddy, M. Griswold, J. McCarrey, M. Primig, and their colleagues who are willing to prepare and readily share their valuable databases. Through their efforts they have made available to the scientific community detailed expression profiling analyses *(2–4)* and valuable cDNA libraries prepared against many different types of highly purified testicular germ cells (6-d primitive type A spermatogonia, type A spermatogonia, type B spermatogonia, 18-d pre-leptotene spermatocytes, 18-d leptotene and zygotene spermatocytes, spermatocytes, and round spermatids) and from 20-d Sertoli cells and the mouse embryonal carinoma cell line F9 (www.ncbi.nlm.nih.gov/UniGene/library.cgi). With such resources, it is now possible to clone unknown novel genes and define their temporal expression pattern without leaving one's office.

6. CONCLUSION

In summary, cDNA libraries have been instrumental in establishing the extensive databases of gene expression during spermatogenesis. Early efforts at cDNA screening were labor intensive, whereas current technologies provide a rapid means or *in silico* gene identification. Such approaches will greatly enhance our abilities to define the molecular bases of many human male infertilities as well as provide valuable insights for the control of mammalian fertility.

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3 Considerations When Using Array Technologies for Male Factor Assessment

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Summary

Expression profiles from sets of genes are currently being explored as candidate diagnostics to assess male fertility status and as surrogate makers of paternal toxicological exposure. In this chapter, we describe considerations for their design when using microarrays to create a clinical diagnostic tool. Two commercially available oligonucleotide-based platforms were compared. The results are then referenced against an expressed sequence tag data set and a cDNA array. The concordance between the different platforms for genes indicated as present with high confidence and absent with high confidence when provided with the same pool of RNA is presented. Based on these data, the capacity for this technology to develop into a robust diagnostic for male factor fertility is discussed.

Key Words: Microarray; sperm mRNA; expressed sequence tags; EST; cDNA.

1. INTRODUCTION

Two methods have principally been adopted for high-throughput transcriptome analysis. These are the transcript sequencing approaches that include serial analysis of gene expression (SAGE) and expressed sequence tag (EST) library sequencing and hybridization-based microarray approaches *(1–4)*. Familiarity and cost have led many investigators to adopt the microarray technology. Inherently, the array approach assumes that the informative transcripts in a given tissue have been characterized. This assumption may require further evaluation in light of recent data from high-resolution transcript mapping *(5)*. Conversely, the EST or SAGE strategies are independent of *a priori* assumptions regarding the

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presence or absence of transcripts. This approach can have a higher discovery rate, but the statistical confidence of the resulting analysis is dependent on the level of the sequencing or depth of coverage that the tags provide. SAGE is likely to remain a discovery tool for the time being, not generally suited to the clinical setting. This reflects the cost constraints imposed by the technology.

Various microarray platforms holding the promise of designer-based medicine *(6–9)* are beginning to be adopted as clinical diagnostics *(10–15)*. Implementation of array technologies in the clinical setting will need to be considered with careful reference to cross-platform and biological reproducibility of the results *(16)*. Some comparative studies have been undertaken *(17,18)*, but have tended not to address whether a robust set of platform-independent probes can be identified. In this chapter, we examine the high-confidence data from two commercially available oligonuceotide microarray platforms to define the human testis transcriptome. This is compared to a cDNA microarray platform and as a function of the EST data available from the National Center for Biotechnology Information (NCBI) dbEST repository *(19)*.

2. PLATFORMS

The advent of a variety of high-throughput technologies that are accessible to many laboratories has spawned an interest in developing diagnostics based on these technologies. One of the foci of our laboratories is to develop DNA microarrays for characterizing sperm RNA content and assessing the genetic and environmental basis of male fertility status *(20–22)*. Several strategies have been implemented to address this task of creating a diagnostics tool. They include both *in silico* and wet-bench approaches. For example, *in silico* tools have been built to combine public data from numerous projects. This has been made possible by the research community primarily adopting two standards: first, a uniform means to describe a microarray experiment and data (i.e., the minimum information about a microarray experiment; refs. *23* and *24*) and second, extensible markup language (XML) protocols describing how high-throughput data can be represented (e.g., microarray and gene expression markup language [MAGE-ML]; ref. *25*). With these standards in place, a range of databases have been constructed to warehouse microarray data. These include subject area-specific repositories, such as the mammalianreproductive genetics (MRG) database (http://mrg.genetics.washington.edu/), and super-repositories, such as the NCBI Gene Expression Omnibus *(26)* and European Molecular Biology Laboratory-European Bioinformatics Institute

ArrayExpress *(27)* projects, that archive data from all high-throughput platforms. Parallel databases derived from transcript sequencing, such as the NCBI dbEST, complement these assets. These harbor a wealth of information that have enabled data mining and various meta-analyses. However, caution must be exercised, because the primary data represent a variety of perspectives that were each tailored to address the initial primary research goal.

Although the *in silico* avenue will provide a guide *(28)*, wet-bench laboratory analysis is required to develop and put a clinical diagnostic into practice. The platforms discussed in this chapter reflect some of the technologies that may soon be considered or have even entered the pipeline towards clinical application, including the Illumina Sentrix® 6: Human Whole Genome BeadChip array *(29)* and the Affymetrix Human U133+2 GeneChip® array *(30)*. Both assess a wide distribution of transcripts, many of which overlap between the platforms. The Illumina BeadChip interrogates 47,300 segments using a series of 50 basepair (bp) probes. A subset of 23,259 was mapped directly through GenBank to known gene transcripts. In comparison, the Affymetrix Human U133+2 array interrogates 54,613 segments using a series of 25 bp probesets of which 41,282 were mapped to known gene transcripts representing 38,572 distinct genes.

3. EXPERIMENTAL CONSIDERATIONS AND ANALYTICAL PROCEDURES

Many excellent texts have already been devoted to the various statistical aspects of analyzing microarray-based experiments *(31–33)*. Approaches and array technologies continue to evolve. A host of tools are being made available online, where they are frequently updated. Their URLs are best found by searching the Internet, or where this fails by using www.archive.org to identify deprecated tools. Similarly, when seeking the most recent statistical perspectives, a search of PubMed for microarray analysis provides a good starting point.

Table 1 lists some of the most frequently cited primary analysis software tools that are commercially or freely available. These include GeneSpring *(34)*, which provides a generic turnkey analysis solution with limited data mining capabilities; the oligonucleotide centric DChip *(35)*; and others, such as BeadStudio, that is array platform- and manufacturer-specific.

3.1. Experimental Design

Most microarray studies are comparative rather than absolute and are designed as time series, dose–response, or disease state comparisons.

Table 1
Examples of Microarray Analysis Software Suites With Their Literature Citation Frequency

Analysis suite	*Citations*[a]	*Platform specificity*
TIGR suite (www.tigr.org)	1890	Primarily aimed at cDNA platforms
GeneSpring (Silicon Genetics)	1440	All platforms
Imagene	938	Generic one- and two-color arrays
Bioconductor/R	690	All platforms
DChip (www.dchip.org)	546	Primarily oligonucleotide, although import is possible for other platforms
GCOS/MAS5 (Affymetrix)	513	Affymetrix array analysis software tools
BeadStudio	1	Illumina Sentrix® BeadChip analysis software

[a]As of January 1, 2006.

They do not seek to quantify a gene's expression in absolute terms, but rather demonstrate whether a significant change in the level of the transcript has occurred between states. This may be assessed relative to a single comparator (e.g., an initial time point or a "normal" state or between different states).

The design of these differential experiments generally reflects the question being addressed and the technological solution adopted. Multichannel platforms are capable of simultaneously measuring two states. The test condition can thus be associated with one channel, whereas the second channel can be dedicated to a control that is common among all arrays. This approach permits the relative expression from all experiments to be evaluated with one intermediate array at most. Alternatively, when a single-channel platform is employed, a second array provides a control for each condition. The cost can prove prohibitive when multiple conditions are explored. A compromise of cross-referencing the samples, by comparing sample 1 to sample 2, sample 2 to sample 3, and so on, is frequently employed.

Whether exploring differential or absolute expression, the underlying biological differences between the conditions tested are only one source that contributes to the differential signal. Sources of signal variance can

arise through subject differences, sampling differences, or through the technical variance that is introduced by small differences in target preparation. There are many experimental designs that can be adopted to identify this variance. Selection of the appropriate design depends on a number of factors, including whether there is a need to identify all sources of variance or merely to characterize variance with respect to test variables and a subset of candidate nuisance elements. Factorial designs, typified by the Latin-square, are the most complete experimental designs. At least one array is devoted to each combination of parameters. This optimizes the potential to quantitatively link the nuisance and tested contributions to the measured outcomes through, for example, analysis of variance (ANOVA). Unfortunately, the Latin-square can prove cost-prohibitive for complex protocols. Consequently, several variables are usually clustered together to define groups of factors rather than individual factors.

The primary source of analytical discordance between differential analyses is encountered in the chip-normalization procedure *(36)*. This step is necessitated when sources of variation cannot be adequately addressed by the experimental design even when the same target is independently hybridized to an identical array. Slight differences in hybridization conditions, chip preparation, processing, and data capture lead to global differences between the two arrays. As shown in Fig. 1, these differences are usually observed as broad changes to the chip's envelope, i.e., the histogram of the signal from all of the chip's probes. In some cases, one chip might be brighter than the other, exhibiting a greater dynamic range of signal while having the same rank order of genes. Without compensation, some probes can exhibit substantially different signal levels between chips where no transcript differential is biologically present.

For many platforms, the initial chip normalization will be the median normalization. This simple adjustment globally factors the signal levels across a chip such that the median signals between chips are equalized. However, this approach is unable to compensate for changes in the shape of the envelope that leave the median signal unchanged. Hence, more advanced envelope-shaping normalizations such as invariant-set and percentile normalizations may be considered. Additional pre-analyses that model the signal and noise of the individual probes have become available to determine which probes are inherently noisy and which probes are stable. A range of models are available in Bioconductor *(37)* and GeneSpring, including the Robust Multichip Average (RMA) and the basepair-adjusted extension gcRMA. In comparison DChip *(35)* uses the model-based expression index approach. The relative merits of each have been compared *(38)*.

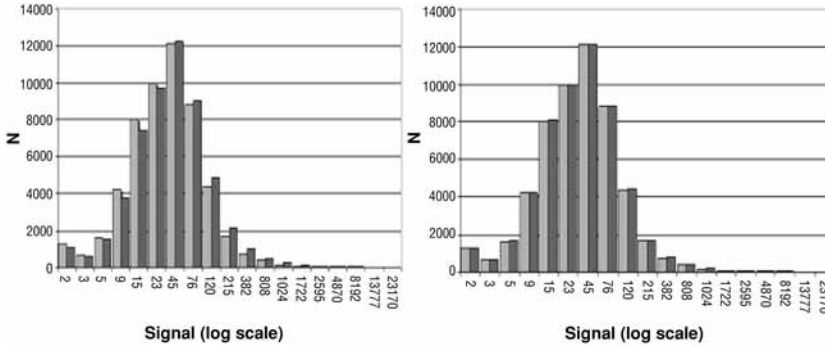

Fig. 1. Two histograms showing the distribution of signal intensity illustrating the signal envelope of two arrays before and after normalization. **(Left panel)** Before normalization signal intensities greater than 45 show a clear skew towards the darker bars. A similar skew is observed toward the lower signal intensity values less than 45 as illustrated by the lighter bars. **(Right panel)** After normalization, the level of hybridization between the arrays is similar.

The final stage of preanalysis typically identifies the genes that exhibit the largest quantifiable changes with respect to the test variables while determining which changes are likely significant and which likely result from stochastic processes. Approaches can be graphical and simple (like volcano plots, in which the fold log of the change in signal is plotted as a function of the log of significance or representations of the coefficient of variation [CV]). Alternatively approaches can be complex and graphically involved, like hierarchical clustering or principle component analyses. They are now routinely utilized to identify groups of genes that appear to be responding in a coordinated manner. Within each group regression, approaches that model the causes and outcomes of variance, such as ANOVA or multivariate ANOVA, are used to indicate whether a result is likely to be by chance alone. The decision of what to accept and what to reject is complex, given that an array can contain tens of thousands of probes. If a p value of 0.05 is adopted as the significance criteria for a 50,000-probe array, then in any given experiment 2500 genes could be reported by chance alone. This multiple comparison problem can be reconciled by the strict Bonferroni correction. However, this will reduce type I (false-positive) error, at the expense of a significant penalty to type II (false-negative) error. Other approaches are available to optimize the balance between type I and II errors. These include data-independent step-down procedures as well as data- and prior-based approaches, such as Storey's Q *(39)* value and the permutation-based Westfall and Young adjustment *(40)*.

4. ORIGINS OF DIFFERENCES BETWEEN PLATFORMS

Platforms exhibit different capacities to measure a broad range of signal. One or more probe sequences are available for hybridization for each gene detected on a given platform. The characteristics of each probe are determined by their corresponding sequence and system design. Thus, although a platform can be generally characterized, there are probes that differ from these general trends. Other innate factors brought about by the platform's design impact concordance among probes, including probe length, the data analysis strategy used to generate high-specificity probes, the distribution of probes with respect to translation initiation and termination signals, the number of times each probe is represented on the chip, the approach to measuring background signal and spurious hybridization, and the experimental technologies and protocols employed. Misannotation is inevitable, in these rapidly evolving platforms that incorporate tens of thousands of probes. This is usually reflective of genes that are not well understood or gene predictions. Generally, subjecting the probe sequences to another round of BLAST analysis will resolve this issue. This is particularly useful when comparing the results from older versions of arrays because genome builds and locations during their respective development are subject to change.

4.1. Platforms and Analytical Approach

A common reference standard of commercially available pooled testes RNA has been used to define amplification, hybridization, and image capture differences within two platforms and inclusivity and sensitivity characteristics between platforms. Data from each platform was analyzed based on our past experience with each platform. The specific approaches undertaken for this study were:

- Affymetrix. GeneChip images were processed with GCOS 1.1 (Affymetrix) and normalized relative to each other using DChip 2006 for invariant set normalization. Signal consistency was then assessed using the DChip model-based expression index approach in PM-MM mode.
- Illumina. The Illumina Sentrix array was imaged, validated, and then signal derived using the custom BeadStudio software.
- Platform-specific normalization within platforms containing duplicates. Only the probes corresponding to well characterized genes and well annotated within Genbank were compared between arrays.
- Rank invariant normalization between platforms. This approach was conducted using the National Institute of Aging online ANOVA system *(41)*.

4.2. Platform Performance in Limited Technical Replicates

Before assessing the extent to which platforms accord, it is useful to explore concordance within platforms. Because each platform has a preparation protocol that may bring about discordant results, reproducibility with respect to independent target preparation from the same biological specimen was assessed. These constitute technical replicates intended to incorporate elements of preparation protocol variance *(42)*, rather than simply technical repeats.

4.2.1. Affymetrix U133+2 Platform

Two samples of Clontech-pooled human testes RNA were independently amplified using the Ambion Message Amp™ amplification system according to the manufacturer's protocol. The RNA was hybridized to the arrays and the signal preprocessed as described. Affymetrix technologies report a signal value that is linearly related to RNA concentration across a broad range beyond which it becomes nonlinear *(43)*. The platform also uses signal stability across probe pairs relative to a mismatched background probe to deliver a "call" or confidence of detection. Assigning a confidence of detection above 0.96 (one-sided Wilcoxon rank sum divided by the signal discrimination score) leads to a P or present denotation, between 0.96 and 0.94, an M or marginal denotation, and below 0.94, an A or absent denotation. Because previous research has largely shown good cross-platform concordance only with the assignment of presence or absence *(44)*, it is useful to explore the extent to which the Affymetrix platform is concordant among technical replicates with respect to both signal and the assignment of presence or absence. This permits us to validate a set of clinical analysis parameters well-suited to the data from this platform.

As illustrated in Fig. 2A, the Affymetrix technical replicate signal values concord well. Signal-dependent variance that reduces signal correspondence among replicates is observed at lower signal intensity values. A least squares fit validates the data normalization approach, indicating a scaling M of 0.99 between chips and r^2 of 0.9895 [Log(I) r^2 0.9]. Analysis of the CV, with respect to signal, pinpoints signals below 100 units as the primary origin of spurious covariance. When assessed as a function of the signal being deemed present or absent, 89%, (i.e., 49,062/54,613) of probesets on the arrays were concordant between replicates. The remaining 11% were principally located in the lower quartile signals. Implementing a variable lower signal threshold cutoff yielded 95% presence–absence concordance, once the signals below 110 units were excluded from the analysis. This suggests a lower boundary of reliable, validated present signal of between 50 and 150 signal units. The lower bound is dependent on the user's preference for inclusivity or reproducibility.

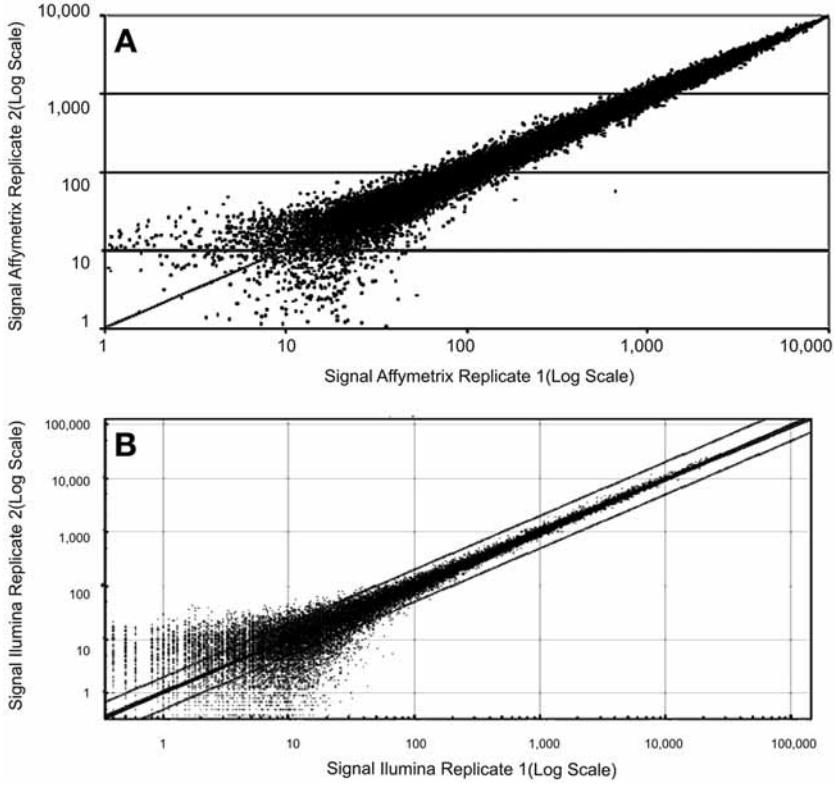

Fig. 2. Log_{10}–Log_{10} dot plot technical replicates illustrating excellent co-linearity and good signal correspondence above a threshold. **(A)** Affymetrix data. **(B)** Illumina data, after cubic-spline normalization. Normalization corrects for signal skew between replicates but introduces some noise below the lower signal threshold.

4.2.2. Illumina Sentrix 6: Human Whole Genome BeadChip Platform

An additional sample of Clontech-pooled human testes RNA was independently amplified using the Ambion Message Amp amplification system. Signal from the Illumina Sentrix 6 BeadChip arrays was first validated for quality using the insuite array validation function and then further analyzed in the Illumina BeadStudio software suite. BeadStudio offers several options for array normalization. These include background, invariant set, and average normalizations. Although this normalization will not affect the confidence of a signal being assigned present or absent between platforms, it is key to attaining optimal signal concordance. Comparison of the various normalization strategies revealed little difference. Each did not substantially improve the signal correlation between the two arrays. However, spline normalization did correct a slight non-linearity at higher signal strengths to the linear relationship as

shown in Fig. 2B. Signal intensity correlation determined by least squares fit generated r^2 0.9899 [log(I) r^2 0.988] with an M of 0.99 after normalization. The Pearson product moment of 0.994 indicates that 99% of the detected signal can be correlated between the replicates. Approximately 99% of the CoV was below 40 signal units and 80% below 25 signal units. Of the 47,300 probes on the platform, 28,455 were identified as definitely present ($p > 0.99$) or definitely absent ($p < 0.75$). These exhibited 100% concordance between experiments. The remaining 18,845 probes were of marginal intensity and not assigned as being present or absent and were not considered further. This reflects our conservative choice of $p < 0.75$ when assigning definitely absent. It contrasts the more inclusive value of $p < 0.94$ that is typically used when Affymetrix arrays are analyzed but marginally impacts the concordance of the assignment of presence or absence.

4.3. Comparing Oligonucleotide Platforms

Describing the Affymetrix and Illumina technologies jointly as only oligonucleotide platforms is a significant oversimplification. There exist a number of substantial differences between the technologies. The Affymetrix U133+2 platform has multiple probesets for some well-characterized genes, derived through the subclustering of EST data within 600 bp of the 3′-end of the transcript. Signal is measured through sets of 25-mer probes (probesets) aligned to a sequential region of the transcript with correction for nonspecific hybridization made relative to a parallel set of 1-bp centrally mismatched probes. This is key to the successful use of shorter oligonucleotide probes. By contrast, the Illumina Sentrix 6 platform uses longer 50-mer oligonucleotide-coated beads randomly dispersed across a very high-density array of pits with up to 60 identically coated oligonucleotide beads for each probe. This larger number of repeat measurements yields a very stable signal for each longer oligonucletide probe, lessening the need for probesets. Unlike the Illumina platform, the Affymetrix platform often queries a gene using several independent probesets that are offset from one another. This may have the advantage of simultaneously examining isoforms. Therefore, we can anticipate that only a subset of the Affymetrix probesets targeted to a gene will report the same biological behavior as the Illumina probe(s). Addressing this difference, albeit at the risk of biasing the assessment, can be achieved by either screening the Affymetrix probes for those that best correlate with the expression for that gene on the Illumina platform or by masking those reporters that are aligned to spatially distinct genomic locations. Of the 23,259 probes on the Sentrix Whole Genome GeneChip arrays that were

Table 2
Distribution of Known Transcripts Identified as Present

Platform	*Cross-referenced probes*	*Total probes identified*	*Common transcripts identified*	*Total probes identified (%)*
Affymetrix	25,635	9598	5265	37
Illumina	18,916	6837	5265	36

annotated relative to GenBank sequences, 18,916 could be matched through their Entrez Gene IDs to the Affymetrix arrays. These subsets of probes were normalized to each other using the National Institute of Aging normalization tool.

As shown in Table 2, 5265 unique platform-independent probesets (i.e., transcripts) were identified as common between the platforms. Both platforms possess a similar ability to detect probes (i.e., 37 and 36%, for the Affymetrix and Illumina platforms, respectively). The concordance of those genes defined as present is 77%. Although the platforms generally accord with respect to transcript identification, only 7% concordance is observed for the relative level of the signal. This can arise from at least two independent sources of interplatform discordance. On the one hand, the inherent differences in platform design (e.g., oligonucleotide length and probeset composition) could lead to different measurements even when the probes are assessing the same region. On the other hand, differences in the probe sequence may discern different isoforms leading to their independent measurement. These differences that arise from sequence selection may be assessed by restricting the probes included in the analysis. Restricting the data set to those probes that target sequences in closer proximity allows one to begin to identify the contribution of reporter location to platform concordance. The sequence alignments from the group of well annotated genes from each platform has already been undertaken by others and the results reported *(45)*. There is an evident leap in agreement to 21% when only the closest matching Affymetrix probeset is compared with its Illumina counterpart for each gene. Beyond this, the further thinning of the data sets to include only those probes targeted to within 200 bp or less of each other only reconciles 31% of the signal between the platforms, which is similar to the value obtained (27%) when the subsets of probesets that show a similar relative expression are compared. Accordingly, approx 60% of the interplatform signal variance may arise from causes beyond simple probe choice. This is consistent with the view that signal

Table 3
Number of Transcripts Sequenced in a Combined Testis Expressed Sequence Tag Library That Were Also Detected Present on Each of Three Microarray Platforms

Platform	*Unigenes represented on the platform*	*Probes identified*	*Unigenes identified*	*Unigenes identified (%)*	χ^2 *Unigenes identified*[a]
dbEST	11,784	52,000	11,784	100	
cDNA	6563	4226	2817	43	5 ($p < 0.05$)
Affymetrix	7468	12,306	6431	86	1411 ($p < 0.005$)
Illumina	7326	4887	4887	67	1648 ($p < 0.005$)

[a]The χ^2 statistical correlation of array platforms with the dbEST data set is given. The corresponding *p* value is parenthesized.

strength comparison among platforms is, at best, poorly correlated even when inclusivity is compromised.

5. DATA CONCORDANCE WITH A REFERENCE TECHNOLOGY

Four EST libraries were aggregated from the NCBI dbEST totaling approx 52,000 sequence reads (libraries 18,517, 16,441, 18,476, 1752). These were combined to form a single external reference library of 11,784 distinct unigene clusters. Because many platforms, Affymetrix most notably, derive their hybridization sequences and calculate cross-hybridization resilience primarily from dbEST, it might be anticipated that a platform with probes so constructed would yield results in good agreement with dbEST. This has been reported for large SAGE projects *(46)*. Table 3 illustrates the correlation between a gene being identified as present on the Affymetrix and Illumina oligonucleotide platforms together with a cDNA platform *(2)* alongside being identified in the combined dbEST library. The percentage of genes discovered by sequencing that are also discovered on the array platforms is informative. This suggests that the Affymetrix platform can identify a larger number of the transcripts present. The apparent enhanced performance of the Affymetrix platform may reflect the concurrent probesets that cover the same gene region, each being independently considered.

The ability to model the data can also be considered using the χ^2 statistic to inform on the level of agreement and disagreement and the significance of such agreement. As shown in Table 3, this analysis

indicates that the cDNA platform does not model the EST data well. Both the Affymetrix and Illumina platforms concord to a greater extent with the EST data. However, the higher number of Affymetrix probesets directed toward the same gene region that increased its ability to identify a given probe reduces the corresponding χ^2 statistic when compared with the Illumina single multireiterated probe platform.

6. DISCUSSION

Previous studies reporting expression differences across platforms have drawn vastly different conclusions. Some have found the level and direction of differential expression to be somewhat concordant *(18,47–49)*. Others have found little in common between platforms in terms of their absolute signal *(50)*. Similarly, the microarray platforms tested did not strongly reflect the EST discovery frequency, a proxy value for expression level that is measured by EST sequence count per million averaged across the libraries. To an extent the differences and similarities reported are as much functions of the analytical pathway adopted *(51)* as they are functions of differences and similarities between the platforms themselves. Nevertheless, there is encouraging but not absolute agreement between the platforms when the presence or absence of a transcript is assessed relative to an external EST data set of known transcripts.

From the series of hybridizations conducted, the data suggests that the oligonucleotide platforms have excellent internal consistency. The exceptionally high signal and reproducibility of presence or absence assignment on the Illumina platform is statistically similar to that observed when real-time polymerase chain reaction techniques are employed. However, it is not possible to assert that one oligonucleotide platform outperforms another. Both are likely capable of drawing similar conclusions when given the task of identifying whether a broad set of genes are, as a group, expressed or silent. This directs us to explore larger and not smaller sets or individual genes when using these technologies. What is the number of transcripts that must be monitored to enable diagnosis? It has recently been shown by others *(13)* that as few as 70 probes can reliably inform on breast cancer prognosis. This may provide a lower bound, but it is not clear whether this would have sufficient power to distinguish among the various forms of male factor infertility.

Although still in its infancy *(10,14,21,52–54)*, the application of these technologies to assessing male factor fertility status holds considerable promise. Compared with the criteria currently in use that includes the well-accepted World Health Organization standards for normal

semen analysis *(55)*, these new strategies may provide for the objective evaluation of male factor status. Analogous to a heterozygous CREM$^-$ oligospermic individual, the ability of oligonucleotide-based micorarrays to convincingly discern differences among "normal" and CREM$^-$ mouse testis transcripts has been demonstrated *(56)*. In our own laboratories, we have used microarrays to identify differences in testicular gene expression in mouse genetic models and human cases of infertility *(57)*, as well as from mice exposed to testicular toxicants resulting in infertility *(58,59)*. Although these are only a few examples of male factor infertility, it is reasonable to assume that the implementation of these arrays or other genomic technologies in the clinical setting will also enable the rapid identification and classification of what are currently cases of "idiopathic" infertility. This would constitute a significant advance for both our clinical and our basic understanding of this disease.

7. ABBREVIATIONS/TECHNICAL REFERENCES

ANOVA: analysis of variance (http://en.wikipedia.org/wiki/ANOVA).

CoV, coefficient of variation 100 × standard deviation/mean (http://en.wikipedia.org/wiki/Coefficient_of_variation).

dbEST, NCBI database of expressed sequence tags (http://www.ncbi.nlm.nih.gov/dbEST/).

EMBL-EBI, European Molecular Biology Laboratory-European Bioinformatics Institute (http://www.ebi.ac.uk/).

gcRMA, nucleotide (G/C) enrichment-adjusted robust multichip average (http://www.bioconductor.org/repository/devel/vignette/gcrma.pdf).

MAGE-ML, MicroArray Gene Expression-Markup Language (http://www.mged.org/Workgroups/MAGE/mage-ml.html).

MANOVA, multivariate ANOVA (http://en.wikipedia.org/wiki/MANOVA).

NCBI, National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Presence Significance on the Affymetrix Platform (http://www.affymetrix.com/support/technical/technotes/statistical_reference_guide.pdf).

Probe, an element, usually a sequence, that is printed onto an array to which a labeled target sequence in the sample can hybridize (note that *probe* and *target* are sometimes interchanged in the literature).

Probeset, a set of probes that investigate a near-sequential sequence of a gene. The signal from perfectly matched sequences is generally

assessed relative to a 1-bp centrally mismatched sequence to determine the level of spurious hybridization.

RMA, robust multichip average (http://stat-www.berkeley.edu/users/bolstad/RMAExpress/RMAExpress.html).

Target, usually a mixture of labeled RNA, DNA, or protein elements from a tissue or other source that can hybridize to a probe.

XML, extensible markup language (http://www.xml.org/).

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4 Microarray Analysis of a Large Number of Single-Nucleotide Polymorphisms in Individual Human Spermatozoa

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Summary

Genetic studies in humans have been limited by various factors, including small family size and diploidy of the human genome. The ability to use individual spermatozoa as subjects has significantly facilitated these studies. However, because each sperm usually

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contains only one copy of the genome and the sensitivity of the available detection methods was low, single sperm could not be used for large-scale genetic analysis. After a series of enhancements, a high-throughput genotyping system has been developed. With this system, more than 1000 genetic markers consisting of single-nucleotide polymorphisms (SNPs) in a single sperm genome can be amplified to a detectable amount in a single tube. Sequences amplified from different polymorphic loci are then resolved by hybridizing these sequences to the corresponding probes spotted on a microarray. The allelic sequences of each SNP are then discriminated by the commonly used allele-specific single-base-extension assay with dideoxyribonucleotides labeled with different fluorescent dyes. Using single sperm cells as subjects, the highly sensitive and high-throughput multiplex genotyping system was used to analyze the physical structure of the human immunoglobulin heavy chain variable region that contains highly repetitive sequences and may not be easily analyzed with conventional methods. This system was also used to understand the contribution of meiotic recombination to haplotype structure formation in the human genome. The system may have various applications including study of genetic factors underlying male infertility.

Key Words: Single sperm; single-nucleotide polymorphisms; genetic analysis; high-throughput genotyping; microarray analysis.

1. INTRODUCTION

Genetic studies involve analysis of genetic material that are informative about the behavior of genetic markers. The resulting information can then be used for various purposes, such as determination of gene locations, recombination rates in given chromosomal regions, and parental origins of certain genetic variants. In mammals, including humans, individual gametes are ideal subjects for these studies because their genomes are simple and contain abundant information about the behavior of genetic markers. Unfortunately, because individual gametes are haploid and contain only one genome copy, genetic information in gametes is usually deduced by study of individual organisms that are the result of fusion of gametes from two sexes. Unlike other organisms in which the number of individuals with interesting phenotypes/genotypes can be expanded by breeding, in humans, individuals of interest for study have to be identified in the population, which involves an unusual amount of effort. The human families are so small that the conventional methods based on scoring the individuals with different phenotypes/genotypes from certain mating arrangements is not practical. Therefore, these analyses in humans usually require pedigree information from at least two generations *(1)*.

Like other higher organisms, the diploidy of the human genome also complicates genetic analysis. Spermatozoa (sperm) and oocytes are both gametes, ideal subjects for genetic analysis. Because these cells are haploid, when used for genetic studies the complications associated with

diploid cells can be avoided. However, acquiring and using oocytes are practically much more difficult than using sperm and the number of oocytes from a donor is usually limited. In contrast, sperm cells are much easier to obtain. From each donor, a practically unlimited number of sperm can be obtained. If sperm can be used as subjects, genetic analysis may be performed by direct scoring and may not need pedigree information and complicated statistical analysis. A sufficient number of sperm may be collected from any voluntary doners or interest. However, because sperm are haploid, each sperm usually contains one genome copy, which cannot be analyzed by most available genotyping methods.

2. EFFORTS IN THE DETECTION OF GENETIC MARKERS IN INDIVIDUAL SPERM

The advent of DNA amplification technology, namely polymerase chain reaction (PCR) *(2,3)*, made it possible to detect DNA sequences from a very small amount of material. Detection of polymorphic sequences at single-nucleotide polymorphism (SNP) in single sperm loci enhanced the sensitivity to the high end *(4)*.

In the early stages, most single sperm were prepared by micromanipulation. The most commonly used method was the spread-scraping method. With this method, a small aliquot of the semen sample was first washed and suspended in 1% low-melting-point agarose to a concentration at which sperm can be well separated when a thin layer of the suspension is spread on a glass slide. Single sperm can then be picked up by scraping the areas of the thin layer containing well separated sperm with a fine needle *(5,6)*. Later, the manual method was replaced by flow cytometric separation *(7–9)*. Although it is very efficient, flow sorting of single sperm cells requires access to a flow cytometer and well-trained personnel. Correct alignment and sorting setup of the instrument to ensure single sperm delivered into wells of a microtiter plate at a high percentage can be time-consuming and rather challenging. Once the instrument is correctly aligned, a large number of single sperm can be rapidly sorted. Success in single sperm analysis also relied on the development of sperm lysis solutions that allows DNA to be released from sperm and is suitable for the subsequence PCR amplification of high efficiency *(10)*.

The earliest sperm typing method involved a two-round PCR protocol. In the first round, two to three polymorphic sequences were coamplified in a single tube. Small aliquots were then used to amplify individual sequences separately in different tubes. The amplified products were then analyzed by a method called allele-specific

oligonucleotide hybridization. With this method, amplified sequences were immobilized onto nylon filters followed by hybridization with radioisotope-labeled probes *(11,12)*. For each SNP, two probes were designed. Each probe perfectly matched one of the allelic sequences but differed from the other by a single base. Under optimized conditions, each probe mainly hybridized to the perfectly matched template allowing allelic state of a sample at the SNP locus to be determined by analyzing the signal on X-ray films. Such a strategy allowed determination of genetic distance between two genetic markers, *parathyroid hormone*, and *^{G}g hemoglobin* on chromosome 11 *(10)* for the first time utilizing single sperm analysis.

The genotyping system was later further simplified *(13)*. With the simplified method, the allelic sequences at each marker locus were amplified with a pair of allele-specific primers of different lengths. Each of these primers had its sequence perfectly matched to one of the two allelic sequences but mismatched the other at its 3′-end. Therefore, the primers were preferentially extended only when they annealed to the perfectly matched allelic templates. Because the resulting PCR products from the two alleles differed in length, they could be easily separated by gel electrophoresis. When the lengths of the PCR products from different loci were also taken into consideration, PCR products generated from the alleles of several SNP loci could be analyzed in the same gel lane. Using gel electrophoresis to separate sequences amplified from different loci and from different alleles of each locus not only simplified the experimental procedure, but was also nonradioactive. This method was used for simultaneously typing three loci in single sperm cells *(13)* and for several genetic linkage studies with single sperm *(14–16)*.

To increase the number of markers that can be detected from a single sperm, a whole genome amplification (WGA) procedure was developed in Norman Arnheim's laboratory. The method is called primer-extension preamplification (PEP) *(17)*. With PEP, the entire sperm genome is preamplified with 15mers consisting of degenerated sequences. Aliquots of the PEP product are then used for later PCR amplification with individual primer pairs. With the WGA procedure, single sperm samples could be genotyped with up to 12 markers *(17,18)*. WGA is a linear increasing process. The probability of amplifying any sequence in the genome to a minimum of 30 copies is not less than 0.78 *(17)*. Because of the poisson distribution, it is impossible to separate every individual copy of the amplified sequences into different tubes. Therefor, a WGA product may only be used for approx 25 PCR amplifications.

Table 1
Interacting Pairs as a Function of the Number of Primers

Number of primer pair(s)	*Interacting pairs*
1	3
2	10
3	21
4	36
5	55
10	210
20	820
30	1830
n	$n(1 + 2n)$

By using a single set of family-specific PCR primers, we were able to amplify more than 30 sequences in the human *immunoglobulin heavy chain* variable *(IGHV)* region from single sperm. Because the differences among the amplified sequences were minor, these sequences could be separated by denaturing gradient gel electrophoresis *(19–22)*. With this approach, we determined the organization of these sequences on individual chromosomes *(5,23,24)*.

As the demand for analyzing a large number of SNPs increases and high-throughput genotyping approaches become available, PCR amplification has becomes an expensive and rate-limiting step. However, increasing multiplex PCR capacity is limited by primer dimerization. Primer dimers are very deleterious during PCR not only because they possess perfect primer anchoring sequences, but also because they are usually much shorter than the amplicons and therefore amplify easily. PCR becomes difficult when more then three pairs of primers are multiplexed. As shown in Table 1, the number of interacting pairs increases exponentially when the number of primer pairs increases. For this reason, the capacity of multiplex PCR was once a bottleneck in high-throughput genotyping.

To address this issue, a three-round amplification protocol (Fig. 1) was developed in our laboratory *(25)*. With this protocol, multiplex amplification is limited to the early stage of PCR, and is converted into "singleplex" later by using primers with universal tails. Experimentally, the target sequence at each locus is first amplified with a regular primer and a primer with a 20-base "tail" (tail 1) at its 5′-end. This tail sequence is universal to one of the primers used for each SNP. Amplification with the tailed primers attaches tail 1 to all amplified sequences. In the second round, an aliquot of the first-round PCR product is reamplified. All tailed primers are replaced by only one

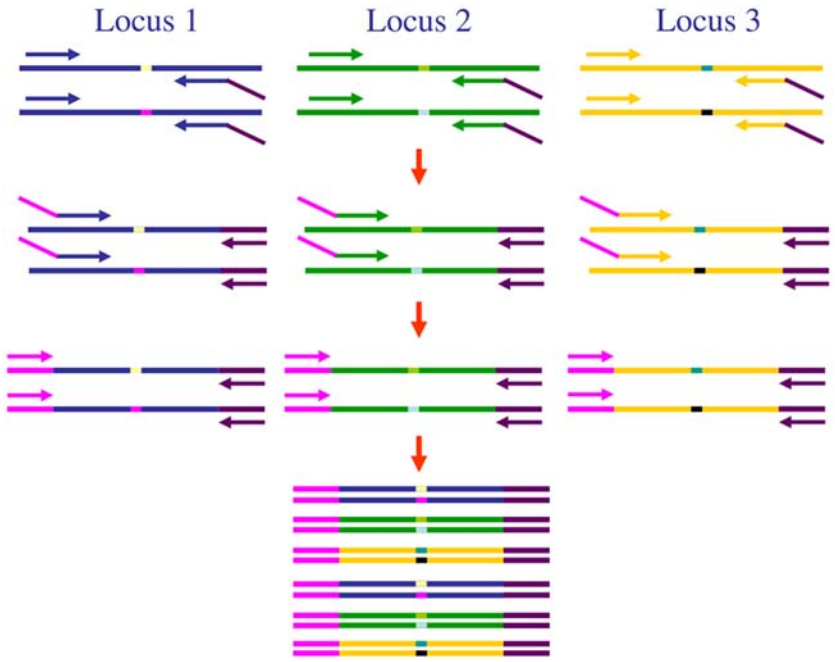

Fig. 1. Schematic illustration of the three-round multiplex amplification. Only three loci are shown. Regular primers, universal tails, and polymorphic sites are indicated as short arrowed lines, short diagonal lines attached to primers, and very short bars of different colors, respectively. *See* text in section 2 for more details.

primer that is identical to tail 1. The regular primer for each locus is then replaced by a primer containing another universal 5′-tail (tail 2). The second tailed primer is an internal (nested) primer with respect to the replaced regular primer to enhance the amplification specificity and yield. In the third round, only two primers identical to tails 1 and 2 are needed to amplify the target sequences to the detectable amounts. The number of PCR cycles in the first and second round is minimized so that amplification in the third round with only two primers significantly reduces primer–primer interaction. This protocol allowed us to amplify 26 loci simultaneously (Fig. 2; ref. *25*), compared with approx 15 loci that could be amplified in a multiplex way in other publications *(26,27)* at that time.

By further optimizing PCR conditions including replacing the regular *Taq* DNA polymerase with a “hot-start” enzyme, the multiplex amplification protocol was simplified from three rounds to two rounds.

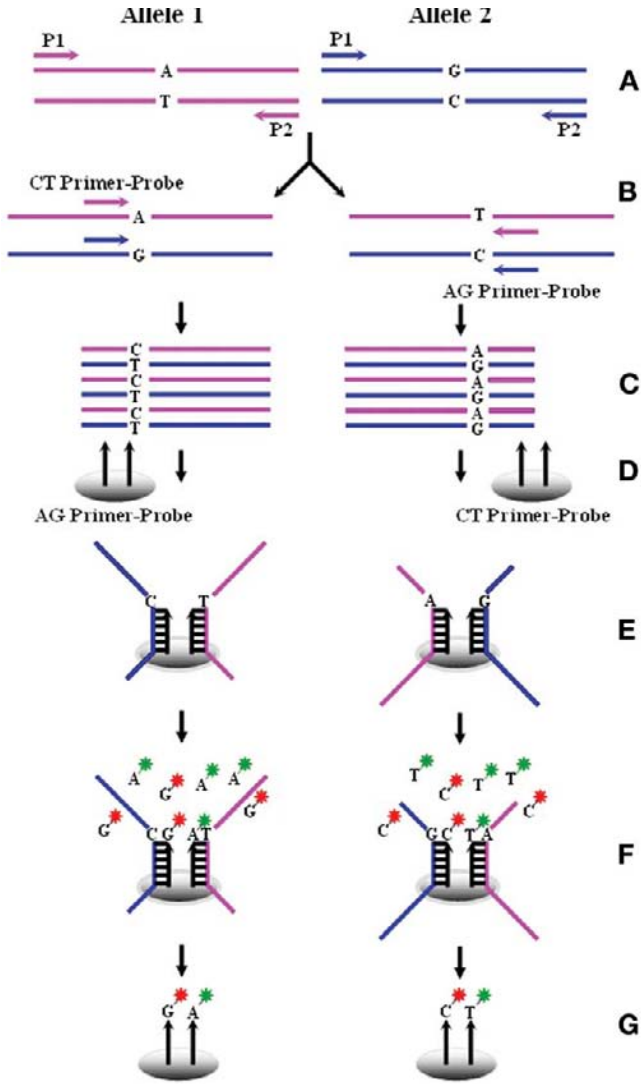

Fig. 2. Schematic illustration of the multiplex genotyping procedure. Only one polymorphism is shown. Primers and probes are shown as arrowed lines. Microarray spots are indicated as ellipsoids. **(A)** Amplification of the polymorphic sequence. Two alleles using the same set of primers, P1 and P2, are shown. **(B)** Generation of single-stranded DNA (ssDNA) by using the primer-probes in both directions in separate tubes. Only the two allelic template strands in each reaction are shown. **(C)** ssDNA generated from B. **(D)** Addition of the ssDNA to the respective microarrays containing probes in different directions. **(E)** ssDNA templates hybridized to their probes on the microarrays. **(F)** Labeling probes by incorporating fluorescently labeled ddNTPs. **(G)** Labeled probes on the microarray after washing off all other reagents. *See* text in section 2 for more details. (Adapted from ref. *36*.)

The procedure for the simplified protocol is generally the same as that for the first two rounds of the three-round PCR protocol described earlier. However, because the third round is not necessary, one primer (instead of two) with the universal tail is needed for each locus. With this protocol, 41 loci could be multiplexed in a single tube (Cui and Li, 1997, unpublished data).

More recently, the universal tail concept was applied to a few newly developed systems *(28–32)*. With these systems, universal sequences are attached to the amplicons by different approaches before PCR allowing more than 1000 SNP-containing sequences to be amplified in a single reaction. This advance has significantly facilitated high-throughput genotyping. The technique initially described by Yeakley et al. *(28)* has been commercialized by Illumina and used for completion of approx 65% of the International HapMap Project (www.hapmap.org). The technologies described by Hardenbol et al. and Kennedy et al. *(29,30)* have also been commercialized and used in large-scale genetic analyses *(33–35)*. However, attaching universal tails to the amplified sequences requires additional experimental steps in comparison with amplification with regular PCR primers, limiting the detection sensitivity. As a result, hundreds of nanograms to 2 µg of genomic DNA are required for each multiplex reaction. Some of these procedures also require pooling of individually amplified PCR products from multiple tubes, followed by column purification. Requirements of specialized probes and detection platforms and long oligonucleotides also limit the flexibility and cost-effectiveness of these systems.

3. DEVELOPMENT OF A HIGH-THROUGHPUT GENOTYPING SYSTEM WITH HIGH SENSITIVITY/DETECTION OF MORE THAN 1000 SNPs IN INDIVIDUAL SPERM CELLS

We have developed a simple genotyping system that requires a single round of multiplex PCR followed by a single step to generate single-stranded DNA (ssDNA) before genotype determination, with a sensitivity for detection of more than 1000 SNPs from a single sperm *(7,36)*. With our new system, instead of attaching universal tails, primers with no predictable productive interaction (i.e., lacking significant complementarity that might cause primer dimerization) are used. With these primers, all experimental effort toward minimizing and avoiding primer–primer interaction becomes unnecessary. A computer program was written to accomplish this task. The candidate sequence frames were first selected based on a user-defined melting temperature range

within a sequence span flanking the polymorphic sites (usually 150 bp on each side). The qualified frames were then further selected based on the following criteria: (1) fewer than four consecutively complementary bases between the 3′-ends of any two frames; (2) fewer than eight but one consecutively complementary base between the 3′-ends of any two frames; (3) fewer than 10 consecutively complementary bases between the 3′-end of any frame and anywhere in all others; (4) fewer than 12 but 1 consecutively complementary base between the 3′-end of any frame and anywhere in all others; (5) complementary bases fewer than 75% anywhere between any two frames; and (6) fewer than 13 complementary bases between the 3′-end of any frame and any amplicon sequence. Results from our simulation study indicate that for 1200 or more SNPs selected from the National Center for Biotechnology Information dbSNP, primers could be designed for genotyping approx 90% of SNPs.

The high-throughput genotyping process is illustrated in Fig. 2. The polymorphic sequences of more than 1000 SNPs can be simultaneously amplified in a single tube with all primers (Fig. 2A). To generate ssDNA, a 1- to 2-µL aliquot of the amplified product is used as the template under the same conditions used in PCR. However, only one primer for each SNP is used. The primers used for ssDNA synthesis are designed in such a way that after annealing, their 3′-ends will be next to the polymorphic sites (Fig. 2B). Therefore, they could also be used as probes (primer-probes) on the microarray for genotyping (Fig. 2). For each SNP, two such primer-probes are designed in opposite directions so that they could be used as primers to generate ssDNA and as probes for genotyping in different directions. When used as primers, because they are internal (nested) with respect to primers used in multiplex PCR, the specificity and yield is increased for ssDNA production. The relative correspondence between these primer-probes and the regular primers is shown in Fig. 2. By hybridizing the resulting ssDNA to the probes arrayed onto a glass slide, sequences from different SNP loci are resolved (Fig. 2E).

To determine the allelic state of the sequences hybridized to their probes on the microarray, the commonly used single-base-extension assay *(37–41)* is used. As mentioned earlier, the 3′-ends of the primer-probes hybridizing to the ssDNA are immediately next to the polymorphic sites (Fig. 2E). Using ssDNA as a template, each probe is extended by a single ddNTP conjugated to a fluorescent chromophore (Cy3 or Cy5) in an allele-specific way. As a result, the allelic sequences of each SNP are labeled with different fluorescent colors (Fig. 2F). After labeling, everything but the labeled probes is washed off and the microarray is ready for scanning.

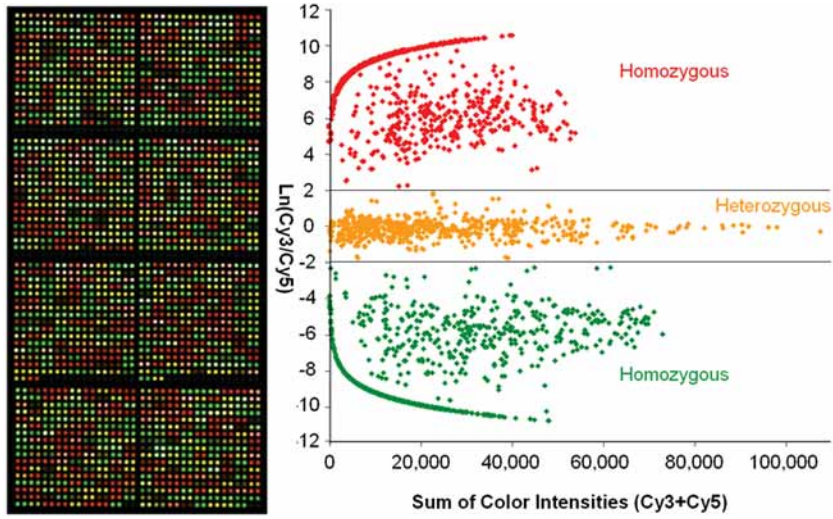

Fig. 3. Typical result from genotyping a genomic DNA from a human individual. (Left panel) A microarray image with a panel of 1172 SNPs. Each probe was printed twice and shown as neighboring spots. Red and green, homozygous; yellow, heterozygous; white, pink, and light green, spots with strong signal that have exceeded the linear range; and dark, low signal but does not necessarily mean no signal or too low for genotype calls. (Right panel) Scatter plot based on the color intensities from the microarray image on the left. Two horizontal lines are the cutoffs (natural logarithm ratios [Cy3/Cy5] of 2 and –2) dividing the spots into three genotype groups. (Adapted from ref. *36*.)

Because DNA sequences are double stranded, ssDNA can be generated in two directions with corresponding primers and can be used for independent genotyping with corresponding probes. Results generated with such a dual-probe method can be compared. Inconsistent genotypes can then be discarded to ensure a very high level of genotyping accuracy. A typical microarray image is shown in the left panel of Fig. 3. A scatter plot of the signal intensities in this image is shown in the right panel of Fig. 3.

Theoretically, when the template ssDNA is from a homozygous individual, a probe should predominantly incorporate one color (signal color that is specific) over the other (background color), whereas a probe hybridizing to ssDNA from a heterozygous individual should incorporate both colors equally. Experimentally, the color intensity is affected by various factors such as nonspecific hybridization, the bandwidth of the light filters, and the ratio between photomultiplier gains selected for each wavelength during scanning. Because of the impact of these experimental factors, three major issues need to be addressed

when determining genotypes from the digitized data produced from a microarray image: normalization of the two color intensities, background subtraction of each color, and genotype determination.

We have taken advantage of our ability to analyze a large number of SNPs in a single assay and use homozygous SNPs as internal controls. Our computer program first sorts the SNPs based on the ratio of the two color intensities. For each human subject, the maximal fraction of heterozygous SNPs is expected to be 50%, and the other 50% would be homozygous with 25% for each of the two alleles. To be safe, we treat 20% of SNPs with the highest ratio and 20% with lowest ratio as homozygous.

A given homozygous SNP has two color intensities, the background color intensity and the signal color intensity. The background color intensity can be used for background subtraction. For a heterozygous SNP, the intensities of the two signal colors should be at a 1:1 ratio, but often deviate from this ratio because of experimental variables. Such a difference can be calibrated based on the signal color intensities of the two groups of homozygous SNPs.

After normalization and background subtraction, the genotypes are determined based on the natural logarithms of ratios (Ln[R]s) between the two normalized color intensities by using empirical linear values as cutoffs. These divide SNPs into three groups, two homozygous and one heterozygous for a diploid sample. The cutoff values were validated by comparing the microarray results obtained by using the dual-probe method and from with those obtained by using independent genotyping methods. For sperm samples, usually no SNPs should be in a heterozygous state, and therefore, the Ln(R)s for SNPs labeled predominantly with different colors are usually well separated on the scatter plots (Fig. 4).

4. APPLICATIONS OF HIGH-THROUGHPUT GENETIC ANALYSIS OF INDIVIDUAL SPERM

Because of the high level of sensitivity of our genotyping system, robust results can be obtained from 5 ng of human genomic DNA. With genomic DNA, the detection rate is usually higher than 97% with an accuracy of higher than 99.6%. When single sperm samples are used, the detection rate is usually approx 90% and the accuracy is comparable with that of genomic DNA. Very recently, we have shown that more than 2000 SNPs can be analyzed in a single assay with a detection rate and accuracy similar to the results for multiplexing approx 1000 SNPs (Luo and Li, unpublished data, 2004). The upper limit of the capacity for our genotyping system still needs to be tested. Using our high-throughput

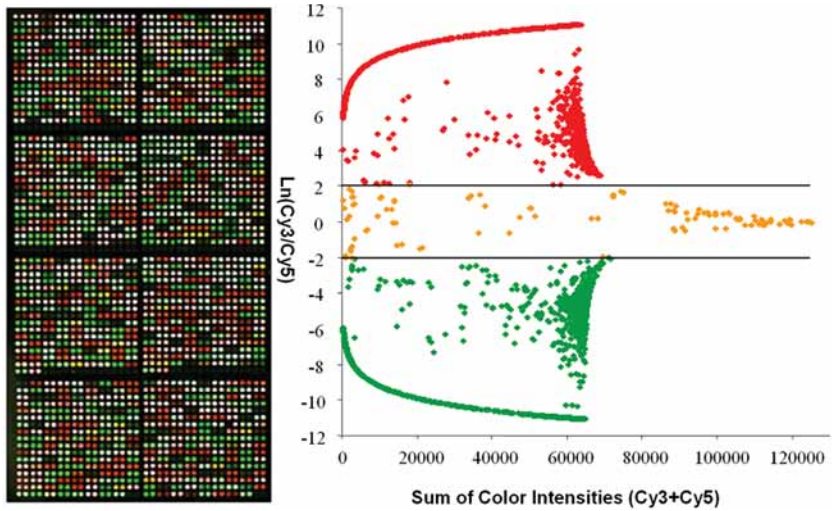

Fig. 4. Typical result from genotyping a single sperm. (Left panel) A microarray image with the panel of 1172 SNPs shown in Fig. 3. Each probe was printed twice and shown as neighboring spots. Red and green, homozygous; yellow, heterozygous; white, pink, and light green, spots with strong signal that have exceeded the linear range; and dark, low signal but does not necessarily mean no signal or too low for genotype calls. (Right panel) Scatter plot based on the color intensities from the microarray image on the left. Two horizontal lines are the cutoffs (natural logarithm ratios [Cy3/Cy5] of 2 and –2) dividing the spots into three genotype groups. Yellow spots are either from SNPs that were not real because of the presences of a small portion of SNPs consisting of paralogous sequence variants in the databases *(87,88)* or from a low level (3.91%) of contamination as demonstrated in the previous studies *(87,88)*, which has been shown to be from oligonucleotides synthesized by the current semi-open oligonucleotide synthesis system. Note: heterozygous SNPs are treated as noninformative in genetic analysis with single sperm.

genotyping system with single sperm as subjects, several studies have been completed in our laboratory (refs. *7*, *9*, and *36*; Luo et al., unpublished data, 2005; Cui et al., unpublished data, 2005; Pramanik et al, unpublished data, 2005. Here, we review two studies that have been recently reported.

4.1. Determination of the Physical Structure of Chromosomal Regions With Highly Repetitive Sequences on Single Chromosomes

Microdeletion on the Y chromosome is the most common cause of human male infertility *(42–44)*. Precise and exhaustive identification of microdeletions requires screening with a high-marker density. With the conventional one-marker-per-assay approaches, such a screening is

very expensive and unaffordable. With our high-throughput genotyping methods, a large number of markers can be included in the screening, making the exhaustive screen possible and practical.

Although no analysis on male infertility has been performed in our laboratory, the microarray approach that we use for analyzing the human *IGHV* region may be easily adapted for such purpose. Human *IGHV* region is a very complex chromosomal region. The region contains 123–129 gene segments *(45–51)*. Of the 123 *IGHV* gene segments described by Matsuda et al. *(50)*, 79 were pseudogenes and 44 gene segments had open reading frames. Of the 44 genes, 39 were expressed as heavy chain proteins and one as messenger RNA, while the remaining 4 were not found among cDNAs. However, the functional *IGHV* gene segments may vary from 38 to 46 depending on the haplotypes *(46)*. *IGHV* segments are also found at two other chromosomal locations, 15q11 and 16p11 *(45,52–54)*. In total, each human haploid genome contains approx 150 distinct immunoglobulin *IGHV* sequences *(45,47,49,52–54)* that are subdivided into seven families *(50,55–64)*. *IGHV* sequences in each family share more than 80% identity. Human *IGHV* segments of different families are highly interspersed *(47,49, 50,56,65–67)*.

Determination of the *IGHV* segment organization has been a slow process. The difficulty stems from the large number of *IGHV* sequences in each family sharing a high degree of identity; extensive polymorphisms with respect to the *IGHV* segment sequences, number, and composition; different chromosomal locations; and diploidy of the human genome. Completion of a physical map with complete sequence determined for the *IGHV* region is only the beginning for thorough understanding of the physical structure of the *IGHV* region because (1) if the map was constructed by using diploid materials, the organization of the *IGHV* gene segment in such a map may not represent the organization in any actual haplotype; (2) even if the map was constructed by using the materials from a single haplotype, because the *IGHV* region is highly diversified, a map for a single haplotype is far from sufficient for understanding the physical structure of the *IGHV* region; and (3) gene order is not the only aspect of understanding the physical structure of the *IGHV* region. Many other aspects, such as gene segment number and composition of the haplotypes, the content of sequence variation among the allelic sequences, and insertion/deletion and duplication polymorphisms among the haplotypes also need to be examined.

However, with single sperm as subjects, the experimental problems caused by the complexity of the *IGHV* region can be significantly

simplified. This is because sperm cells only have one copy of the genome. Therefore, variations in the *IGHV* region among the haplotypes can be studied on an individual haplotype basis. The parental origins of the two haplotypes among the sperm from a single donor can also be easily determined by analysis of a few SNPs in a chromosome region. For study of highly repetitive regions, one of the major difficulties is discriminating between the allelic and interlocus differences, which become confusing in the effort to determine the physical structure of such a region. With sperm, interlocus differences can be found by analyzing multiple sperm of the same parental origins. Allelic differences can only be found between sperm groups of different parental origins. Before development of the microarray-based high-throughput genotyping methods, significant progress was made in using the early version of the multiplex amplification and genotyping methods to analyze the *IGHV* region *(5,8,23,24,68)*.

The microarray-based approach later allowed us to analyze the *IGHV* region in great detail by using a high-marker density along the length of the region *(9)*. For each *IGHV* gene, a segment of sequence was selected as a marker for detection. Marker sequences were amplified by a two-round PCR procedure as described earlier. Instead of arraying probes on to glass slides, multiplex PCR products from a group of single sperm samples from five individuals were spotted onto each microarray, allowing a large number of sperm to be analyzed simultaneously on each array. Two probes labeled with different fluorescent dyes, Cy3 and Cy5, were hybridized to a microarray. *IGHV* genes were detected based on the microarray signal. The parental origin of the haplotype in each sperm was determined by analysis of multiple SNPs in the region.

By analyzing 374 single sperm samples from five Caucasian males, three deletion/insertion polymorphisms (Del I–Del III) with deletion allele frequencies ranging from 0.1 to 0.3 were identified. Del I was reported in our previous publications as 35–40 Kb in size and affecting three *IGHV* genes (*IGHV1-8*, *IGHV3-9*, and *IGHV2-10*; ref. *8*). Del II affects a region 2–18 Kb containing two pseudogenes *IGHV(II)-28.1* and *IGHV3-29*, and Del III spans approx 21–53 Kb involving genes *IGHV4-39*, *IGHV7-40*, *IGHV(II)-40-1*, and *IGHV3-41*. Deletion alleles of both Dels II and III were found in a heterozygous state, and therefore, could not be easily detected if haploid samples had not been used in the study. Results of the present study indicate that deletions/insertions together with other possible chromosomal rearrangements may play an important role in forming the genetic structure of the *IGHV* region, and

may significantly contribute to antibody diversity. Because these three polymorphisms are located within or next to the 3′-half of the *IGHV* region, they may have an important role in the expressed *IGHV* gene repertoire during immune response.

4.2. Understanding the Contribution of Meiotic Recombination to Haplotype Structure Formation

It is known that in the human population, certain alleles of genetic markers within a short distance are in tight association (linkage disequilibrium [LD]) and LD becomes weak or disappears when the markers are located farther apart *(69)*. Chromosomal segments containing markers in LD are called haplotype blocks *(70)*. Haplotype blocks in the human genome were first described on a large scale for a 500-Kb region of chromosome 5q31 *(71)* and the entire chromosome 21 *(72)*, and subsequently in other regions of the genome *(73–76)*. Information on the haplotype structure of the human genome is of great interest because it can be used to significantly reduce the number of markers necessary for localizing genes responsible for complex diseases *(77–79)*. The progress of the International HapMap Project has resulted in the mapping of haplotype blocks across the entire human genome *(80)*. However, very little is known about the mechanisms underlying the formation of haplotype blocks. There is a strong belief that meiotic recombination plays a primary role in shaping LD observed in the human genome and therefore has a direct effect on the haplotype structure found in the human *(18,71,73,76,81–85)*. However, proving such a correlation requires direct evidence of the contribution of recombination on haplotype block formation. By using pooled sperm, a 216-Kb segment in the class II region of the major histocompatibility complex was studied *(82)*. Six recombination hotspots were precisely located within regions where LD breaks down.

To address the issues whether the human genome contains recombination hotspots in a similar pattern and/or density; and whether the hotspots fall between haplotype blocks, another region on chromosome 1q42.3 was analyzed *(81)*. The authors' approach allows them to focus on small subregions to learn a great deal about the mechanisms underlying meiotic recombination and its impact on the genetic structure of the human genome during evolution. However, by analysis of small regions, it is difficult to learn the distribution of recombination crossovers at levels higher than individual hotspots. In-depth study of a large chromosomal region is also necessary for this purpose. However, this is especially challenging because haplotype blocks are usually very

small, ranging from several hundred bases to several hundred kilobases, and meaningful information cannot be obtained until a large number of meiotic products are scored with a high-marker density. The diploid nature of the human genome and the difficulty in gathering pedigrees large enough to study make this analysis very challenging.

Our high-throughput genotyping allowed us to study a much larger chromosomal region with a high-marker density. In the study, we included a panel of 578 SNPs in a 2.5-Mb region on the long arm of chromosome 21 (from 38.01 to 40.51 Mb) with an average intermarker distance of 4323 bp. The markers were incorporated into our high-throughput genotyping system. Three Caucasian donors (D-8, D-11, and D-12) heterozygous at 131, 193, and 209 SNP loci, respectively, were selected for analysis. After lysis, the polymorphic DNA sequences at all 578 SNP loci in each sperm were amplified with our multiplex genotyping procedure. The resulting PCR product was used as a template for genotype determination on microarray by a single-base-extension assay as described previously *(36)*. In total, 662 single sperm samples, 472 from D-11, 115 from D-12, and 75 from D-8 were genotyped at all 578 marker loci. Forty-one recombinants were identified from the 662 single sperm samples, each containing a single crossover. The crossovers identified represent a 6.19% recombination rate, 1.41 times the male average for chromosome 21, and 2.57 times the genomic average *(86)*. The size of these regions containing the crossovers ranged from 2.6 to 98 Kb, depending on the availability of informative markers, except one, that was 306 Kb. All of the crossovers, except one, fall in areas where the haplotype structure exhibits breakdown, displaying a strong statistically positive association between crossovers and haplotype block breaks. The data also indicate a particular clustered distribution of recombination hotspots within the region. This finding supports the hypothesis that meiotic recombination has a primary contribution to haplotype block formation in the human genome.

5. CONCLUSION

A major breakthrough toward increasing the number of detectable markers from single sperm was made recently in our laboratory. By developing a high-throughput and highly sensitive multiplex genotyping system, we have shown that more than 1000 genetic markers can be typed simultaneously in a single assay by using single sperm as subjects. The ability to analyze a large number of SNPs in single sperm has a number of applications including construction of detailed genetic maps for large chromosomal regions, understanding the contribution of

meiotic recombination to genetic structure formation of the human genome, and learning the physical and genetic structure of chromosomal region containing highly repetitive sequences. Many of these studies can be used in understanding male infertility. To further increase the number of markers in single sperm analysis, we have used WGA products from single sperm for multiplex analysis. We have shown that an aliquot of 2.5 μL of WGA product could be used for genotyping more than 1000 SNPs. The detection rate and accuracy were comparable with those obtained by directly typing single sperm samples, indicating that at least 20,000 SNPs can be analyzed from a 50 mL WGA product of a single sperm. WGA products from single sperm have been used successfully for analyzing meiotic recombination along the length of chromosomes 6 and 18 (Cui et al., unpublished data, 2005).

Although offspring may be produced in many higher organisms other than man at a researcher's willing, some organisms, such as farm animals and fruit trees, may not be easily cultivated. Sperm (or pollen) analysis allows for very efficient and highly cost-effective genetic analysis in these organisms.

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5 Physiological and Proteomic Approaches to Understanding Human Sperm Function

Prefertilization Events

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Summary

Sperm dysfunction is the single most common defined cause of infertility. Approximately 1 in 15 men are subfertile and the condition is increasing in frequency. However, the diagnosis is poor and, excluding assisted conception, there is no treatment because of our limited understanding of the cellular, biochemical, and molecular functioning of the spermatozoon. The underlying premise of our research program is to establish a rudimentary understanding of the processes necessary for successful fertilization. We detail advances in our understanding of calcium signaling in the cell and outline genetic and proteomic technologies that are being used to improve the diagnosis of the condition.

Key Words: Proteomics; calcium signaling; zona pellucida; capacitation.

1. INTRODUCTION

This chapter discusses the premise that there is a clear need to significantly improve our understanding of the cellular basis of normal sperm function. This knowledge is fundamental for two key developments in

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male fertility: first, to provide the basis for effective diagnostic tools and, second, to facilitate the study of the physiology of abnormal/dysfunctional cells that is central to developing rational, non-assisted reproduction technology (non-ART) therapy. We discuss the role of calcium in sperm function and question the exclusive use of in vitro experiments with no reference to the in vivo situation. Additionally, we highlight how our understanding of sperm zona binding has been questioned with the discovery of the "four zona proteins" model. Finally, we examine strategies to determine the dysfunction of spermatozoa, in particular the use of proteomics.

Epidemiological data shows that one in seven couples are classed as subfertile *(1,2)*. Sperm dysfunction is the single most common cause of infertility and affects approx 1 in 15 men *(3)*. Studies using semen assessment as the criteria for subfertility (sperm concentration $<20 \times 10^6$/mL) show that one in five 18 yr olds are classed as subfertile *(4)*. This is a high proportion of the population compared with other prevalent diseases, such as diabetes (2.8% of the population; ref. *5*). Thus, male subfertility is a very significant global problem and reports suggest that its prevalence is increasing *(6)*.

2. IMPROVING THE DIAGNOSIS OF MALE FERTILITY

There is a clear requirement to produce new and robust tests of sperm function to diagnose male infertility. The value of traditional semen parameters (concentration, motility, and morphology) in the diagnosis and prognosis of male infertility has been debated for almost 60 yr and, perhaps not surprisingly, the debate continues *(7)*. Suffice it to say traditional semen parameters provide some degree of prognostic and diagnostic information for the infertile couple *(8,9)*. However, it is only at the lower ranges of the spectrum that these parameters are most useful and even then they can only be used as guidance for couples and do not represent absolute values *(10)*.

Traditional semen analysis will therefore only be a limited first-line tool in the diagnosis of male infertility. Consequently, the emphasis has been on developing simple, robust, and effective tests of sperm function. Yet despite the plethora of potential assays available, results have been very disappointing *(11)*. Recent data suggest that only three potential tests of sperm function have sufficient data to support their routine use: penetration into cervical mucus (or substitutes; ref. *12*), measurement of reactive oxygen species production *(13)*, and estimate of sperm DNA damage *(14)*, however, promising initial data for the latter is now being questioned *(15,16)*.

After decades of research why are more effective and robust estimates of sperm function unavailable? The primary reason is our limited basic understanding of the functioning of the spermatozoon.

3. DEVELOPMENT OF EFFECTIVE DRUG-BASED NON-ART THERAPY

With such an important health issue as male infertility, we would expect a number of rational and effective treatments to be available. However, there are no drug treatments to enhance sperm function that have been shown to be effective in randomized controlled trials *(17,18)*. Thus, remarkably, the only treatment option for the subfertile man is in vitro fertilization (IVF) or intracytoplasmic sperm injection (collectively termed "assisted conception"). Assisted conception is very expensive, invasive, has limited success, a number of side effects, is not widely available, and poses significant concerns about the long-term health of children *(19,20)*. However, the number of patients treated with assisted conception is continually increasing. For example, in the United States there was a 78% increase in ART treatments from 1996 to 2002 *(21)* and currently, up to 4% of births are a result of ART *(4)*. Put simply, this means that, as a result of our ignorance of the causes of sperm dysfunction, we are currently subjecting an increasing number of women to inappropriate invasive therapy to treat their partners.

In summary, we need to understand in cellular, biochemical, genetic, and molecular terms how a sperm cell works to address the issues discussed previously *(22)*. Because the functioning of a spermatozoon is critically dependent on the tight regulation of calcium that, when disrupted, results in fertilization failure *(23)*, our research is focused on this area. We are attempting to study the regulation of calcium within the context of the journey a sperm makes in the female tract in the belief that this will lead us to a more comprehensive understanding.

4. UNDERSTANDING THE CALCIUM TOOLKIT AS A BASIC FUNCTION OF THE SPERMATOZOON

4.1. Functional Importance of Sperm Intracellular Calcium Concentration Signaling

Although there is little doubt that spermatozoa use a range of cell messengers, (e.g., cyclic adenosine monophosphate), data accumulated over the last few years have shown that intracellular calcium concentration ($[Ca^{2+}]_i$) plays a major role in all important sperm functions that occur after ejaculation *(24)*. However, in contrast to somatic cells

(25,26), an understanding of Ca^{2+}-signaling in the sperm cell (despite its great importance) is only now developing *(24,27)*. The simple pump-leak model is initially attractive for spermatozoa. Because of the small size of the cell, diffusion is unlikely to be a limiting factor and it is reasonable to assume that Ca^{2+}-influx from the extracellular compartment can effect a rapid rise in $[Ca^{2+}]_i$ in any part of the cytoplasm. However direct evidence for Ca^{2+} store mobilization in mammalian spermatozoa is now available (probably the acrosome) and activation of capacitative Ca^{2+} influx *(28–30)*. It has become apparent that a second, separately regulated store may exist, which functions primarily to regulate flagellar beat *(31,32)*. Thus sperm may possess a relatively complex Ca^{2+}-signaling apparatus including pump-leak and multiple stores (Fig. 1).

In the last few years there have been several developments in our understanding of calcium regulation by the spermatozoon *(33)*. In our laboratories, we have recently concentrated on role of Ca^{2+} clearance mechanisms in sperm. In most cells, Ca^{2+} clearance is undertaken to a great extent by adenosine 5′-triphosphate (ATP)-requiring Ca^{2+} pumps (Ca^{2+}-ATPases) or Na^+-Ca^{2+} exchangers, which extrude Ca^{2+} either out of the cell, or into intracellular Ca^{2+} stores *(34)*. Analysis of Ca^{2+} clearance in mouse sperm suggests that both Ca^{2+} pumps and Ca^{2+} exchangers are important contributors to Ca^{2+} clearance in mammalian sperm, although the relative importance of each is yet to be determined *(24)*.

4.2. Ca^{2+} Pumps

To date, three types of ATP-utilizing Ca^{2+} pumps have been identified: plasma membrane Ca^{2+} ATPase (PMCA), sarcoplasmic-endoplasmic Ca^{2+} ATPase (SERCA), and secretory pathway Ca^{2+} ATPase (SPCA; refs. *34* and *35*).

4.2.1. The Role of PMCA in Sperm

PMCA protein is present in rat spermatids, mouse spermatozoa *(36,37)*, and sea urchin sperm *(38)*. PMCA4 is the main isoform present (>90% of the PMCA protein in sperm is PMCA4 *[39]*) and is primarily confined to the principal piece of the sperm flagellum *(37,39,40)*. Null mutants for PMCA isoforms have been created *(41)*. The major phenotype observed in PMCA4-null mice was male infertility, yet they showed normal spermatogenesis and mating behavior. The sperm, although appearing normal before capacitation, failed to respond to conditions that induce hyperactivated motility. After 90 min, most cells were non-motile, with a few showing very weak hyperactivated motility compared to wild-type *(39,41)*. Measurement of $[Ca^{2+}]_i$ showed that, after 60 min of incubation in capacitating medium, resting $[Ca^{2+}]_i$

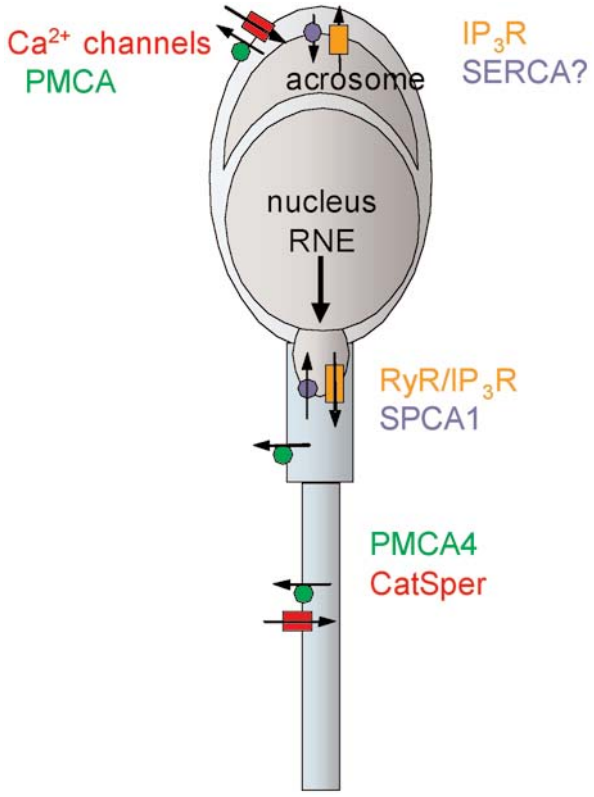


Fig. 1. Two-store model for Ca^{2+}-signaling in human spermatozoa. Plasma membrane Ca^{2+} adenosine 5′-triphosphatases (ATPases) and Na^{+}-Ca^{2+} exchangers are shown in green; Ca^{2+} channels in the plasma membrane are shown in red; sarcoplasmic-endoplasmic reticulum ATPases on intracellular stores are shown in blue, and channels for mobilization of stored Ca^{2+} are shown in orange. Identified or putative components of the Ca^{2+}-signaling toolkit are labeled (using the same color coding) adjacent to their localization *(24,103)*.

was increased from 157 to 370 n*M* in PMCA4-deficient sperm *(40)*. This effect could be mimicked using the PMCA inhibitor 5-(and -6)-carboxyeosin diacetate succinimidyl ester on wild-type mice. A similar failure of hyperactivated motility was observed.

4.2.2. The Role of SERCA in Sperm

In contrast to the persuasive evidence for the importance of PMCA in sperm function, the activity of SERCA in mature sperm is controversial. In our laboratory, using an anti-SERCA antibody (which recognized all known mammalian SERCA isoforms), no cross-reactivity was detected in Western blots using human sperm *(42)*. Furthermore, thapsigargin only

induced Ca^{2+}-mobilization and disruption of Ca^{2+}-signaling in sperm in the 1–10 μ*M* range, which is far higher than concentrations used to specifically inhibit SERCA *(43,44)*. Earlier studies on the effects of thapsigargin on the acrosome reaction and on $[Ca^{2+}]_i$ in sperm also showed that between 0.5 μ*M* and more than 20 μ*M* thapsigargin concentrations were required to induce this response. Thus, treatment of sperm with thapsigargin at concentrations sufficient to inhibit SERCAs is largely without effect, significant actions occurring only at high, "nonspecific" doses. Recently, Gunarante and Vaquier showed that sea urchin SERCA was present in the testis but not mature sperm, further questioning the role of SERCA in mature spermatozoa *(38)*.

4.2.3. The Role of SPCA in Sperm

In somatic cells, SPCA are found located on the Golgi apparatus or secretory vesicles *(45,46)* and are believed to control the levels of both Ca^{2+} and Mn^{2+} within the Golgi to regulate its function *(34)*. We have shown that rat germ cells (spermatids) express the messenger RNA for SPCA1 *(46)*. SPCA1 is also present in mature human sperm and is localized to the anterior midpiece, extending into the rear of the head *(42)*, perhaps reflecting expression in the putative Ca^{2+} store of the redundant nuclear envelope (RNE; ref. *47*).

In summary, there is strong evidence to indicate that sperm express both PMCA and SPCA and that these Ca^{2+} pumps play a major role in controlling sperm Ca^{2+} homeostasis. The role for SERCA in mature sperm is more tenuous, but there appears to be some evidence that it may play a role during spermatogenesis.

5. ACTIONS OF CA^{2+}-MOBILIZING AGONISTS: PROGESTERONE AS AN EXAMPLE

In human spermatozoa the $[Ca^{2+}]_i$ response to progesterone has been studied in great detail. In fact, human spermatozoa appear to be unusually sensitive to progesterone *(48)*. When stimulated with 3 μ*M* progesterone, believed to be representative of concentrations present in the vicinity of the oocyte-cumulus, human sperm generate a biphasic $[Ca^{2+}]_i$ response consisting of a transient (lasting 1–2 min) followed by a sustained elevation. Both parts of the response involve influx of extracellular Ca^{2+} and presumably reflect gating of membrane Ca^{2+}-permeable channels. However, the nature of the channels involved is largely unknown. We have suggested previously that the response to progesterone may be similar to that induced by zona pellucida (ZP), activating a voltage-operated calcium channel (VOCC; although probably not

T-type *[49,50]*) and possibly converging with the ZP-activated pathway on activation of store-operated influx *(22)*. Extracellular La^{3+} can completely inhibit the response to progesterone *(51)*, confirming the importance of membrane Ca^{2+} channels, and a late component of the initial $[Ca^{2+}]_i$ transient (that is particularly sensitive to occlusion by prior progesterone stimulation *[32]*) is sensitive to nifedipine *(48)*. However, the balance of evidence from studies that have specifically attempted to demonstrate a role for VOCCs in the response to progesterone does not support this model *(49,52–54)*. Stimulation of sperm from PLCδ4-knockout (KO) mice with 50–100 μ*M* progesterone generates a response of reduced amplitude and greatly reduced duration compared with that of wild-type cells *(55)*, consistent with a requirement for emptying of an inositol 1,4,5 triphosphate (IP_3)-sensitive store, although the high doses required to evoke large responses in murine sperm may be acting by a different pathway than that normally studied in human spermatozoa, which saturates at approx 300 n*M* progesterone *(32,56)*. Attempts to demonstrate pharmacologically that the sustained elevation of $[Ca^{2+}]_i$ is a result of activation of store-operated channels have produced equivocal data *(32,42,49)*.

It has been shown that progesterone also activates repeated $[Ca^{2+}]_i$ oscillations in human spermatozoa that are the result of store mobilization *(32,57)*. If progesterone is applied as a gradient (to represent more closely the stimulus encountered as a spermatozoon approaches the oocyte) then the initial $[Ca^{2+}]_i$ transient, a characteristic of all previous studies, does not occur, but $[Ca^{2+}]_i$ oscillations occur in a large portion of cells *(32)*. Although IP_3 receptors (IP_3Rs) have been localized to this area of the sperm, the $[Ca^{2+}]_i$ oscillations are resistant to pharmacological treatments designed to inhibit phospholipase C (PLC) or IP_3Rs, suggesting that IP_3 generation is not required for their generation *(32)*. Instead, Ca^{2+} influx induced by progesterone apparently activates a ryanodine-like receptor located in the sperm neck/midpiece (probably on the RNE) leading to repetitive bursts of Ca^{2+}-induced Ca^{2+} release. Reuptake of Ca^{2+} during oscillations is thapsigargin-insensitive and apparently is dependent (at least in part) on activity of SPCA1 *(42)*.

6. SUMMARY: CA^{2+} SIGNALING TOOLKIT IN SPERM

On the basis of the data summarized previously, a complex model for sperm Ca^{2+}-homeostasis involving several types of Ca^{2+}-permeable channels in the plasma membrane and at least two stores is appropriate (Fig. 1). Furthermore, it is clear that these toolkit components are distributed to allow localization of $[Ca^{2+}]_i$ signals. In addition to a range

of VOCCs, which are clearly localized to sperm regions, the CatSpers, which are essential for activation of hyperactivated mobility, are expressed specifically in the principal piece of the sperm tail, as is PMCA4. It appears that the acrosome functions as an IP_3-releasable store, activated by agonists linked to PLC. Recent studies suggest mobilization of acrosomal Ca^{2+} is intimately involved in activation of acrosome reaction *(58,59)*. A separate store, probably the RNE, exists in the neck region of the sperm and plays a key role in regulation of flagellar beat mode.

7. THE INTERACTION BETWEEN HUMAN SPERMATOZOA AND THE FEMALE REPRODUCTIVE TRACT: WHAT CAN WE LEARN?

We already know that the journey of the sperm cell from the site of deposition to the site of fertilization is both dynamic (on the part of the sperm and the female tract) and highly complex *(60–62)*, with the female tract clearly regulating the function of the sperm cell. However, the details of these interactions, especially in the human, are unknown.

There are a number of elegant in vivo investigations studying the transport of sperm in several species (e.g., pigs, hamsters, cows), which show that the female tract sequesters spermatozoa in a functional reservoir (primarily in the oviduct; ref. *61*). However, the situation in the human may be somewhat different, because an oviductal reservoir in the human has yet to be discovered *(63)*. Additionally, in vitro experiments using a series of oviductal tissues preparations (including explants) have shown that human sperm will bind to these tissues but with noticeably less tenacity than in animals. In fact, human spermatozoon will bind, release, then bind again, potentially repeating this cycle several times *(64)*. In animals, binding to the oviduct acts to prolong the survival of the sperm cell, perhaps keeping it in a state of suspended animation.

In the human, it is not only the oviduct that may influence the physiological state of the sperm cell. Several experiments in the 1970s and 1980s have shown that cervical mucus acts to rapidly activate the sperm yet maintain it in a state ready for fertilization for several days *(60)*. Such experiments reveal the strong influence of the female reproductive tract but at the same time make us aware of gaping holes in our understanding of the true biological basis of sperm activation (capacitation). For example, how is the process arrested/suspended? In fact, there is almost a complete divorce in thinking between researchers

studying capacitation (in vitro) and what is likely to be happening in vivo. For our understanding to progress there needs to be a reconnection of the two schools. For example, it does not take 6–24 h to fully capacitate a human sperm cell. A sperm may, however, begin the process of capacitation very rapidly (e.g., when in cervical mucus), but then be prevented from further activation. The IVF system, specifically the culture medium, is designed to almost immediately maximize the fertilizing potential of the sperm, switching on a series of capacitation-related events very rapidly with no intention to put a break in the system *(65)*. Consequently, the differences in the systems (in vivo, in vitro in a research laboratory and IVF) need to be realized and used to complement our understanding. Almost all our knowledge about human sperm function has been gained from experiments in vitro with almost no regard to how the cell may behave in vivo. Perhaps this is why our understanding has remained at a low level. Probably we have been studying the spermatozoon in the wrong environment, in the wrong way, and at the wrong time.

8. HOW DO SPERM INTERACT WITH THE HUMAN ZP? THE "FOUR ZONA PROTEINS" MODEL

A number of studies have shown the importance of sperm zona binding as a prerequisite of normal sperm function *(66)*. However, the molecular details of this interaction remain a mystery. One reason for this is that we may have been using the wrong paradigm to study these interactions. Almost all our knowledge is based on the three zona protein model of the mouse (ZP1, ZP2, and ZP3). Using this model, ZP1 is thought to contribute to the structural integrity of the ZP matrix acting as a linker molecule between ZP filaments *(67)*. ZP2 has been found to be involved in the secondary binding for acrosome-reacted spermatozoa *(68–70)* and ZP3 is accepted to be the primary sperm receptor responsible for binding to intact capacitated spermatozoa and induction of the acrosome reaction *(71)*.

There are four ZP genes and four ZP proteins in the human *(72,73)*. This is very different from the mouse, in which only three proteins are present *(74)*. Accumulating evidence supports the four protein model as the prevalent structure for the ZP across vertebrates (Table 1). Mass spectrometry suggests that ZP4 levels in the human are equivalent to those of ZP3 and ZP2 with ZP1 being a more minor component *(72)*. Experiments have shown that mouse zonae humanized to express human ZP2 and ZP3 can bind mouse sperm but are unable to bind

Table 1
Expression of ZP Genes in Vertebrate Species Commonly Used in Research

	ZP1	*ZP2*	*ZP3*	*ZP4*
Human	•	•	•	•
Macaque	•	•	•	•
Pig		•	•	•
Cow		•	•	•
Rabbit		•	•	•
Mouse	•	•	•	Non-functional
Rat	•	•	•	•
Hamster			•	
Chicken	•	•	•	•

Blank boxes indicate that no gene has yet been identified but that the gene is not necessarily absent. Accession numbers: *human ZP1*, XM_172861; *human ZP2*, M90366; *human ZP3*, NM_007155; *human ZP4*, NM_021186; *macaque ZP1*, Y10381; *macaque ZP2*, Y10690; *macaque ZP3*, X82639; *macaque ZP4*, AY222647; *pig ZP2*, D45064; *pig ZP3*, NM_213893; *pig ZP4*, NM_214045; *cow ZP2*, NM_173973; *cow ZP3* NM_173974; *cow ZP4*, NM_173975; *rabbit ZP2*, L12167; *rabbit ZP3*, U05782; *rabbit ZP4*, M58160; *mouse ZP1*, NM_009580; *mouse ZP2*, NM_011775; *mouse ZP3*, NM_011776; *rat ZP1*, NM_053509; *rat ZP2*, NM_031150; *rat ZP3*, NM_053762; *rat ZP4*, NM_172330; *hamster ZP3*, M63629; *chicken ZP1*, NM_204683; *chicken ZP2*, AY268034; *chicken ZP3*, NM_204389; *chicken ZP4*, NM_204879 *(73)*.

human sperm *(75)*. It is possible that this failure to bind is to the result of a requirement for species-specific glycosylation *(76)*. Alternatively, this result may reflect human sperm having evolved to bind to a ZP consisting of four ZP proteins rather than three. Further to this is the possibility that ZP4 is required for direct interaction as part of the sperm receptor on the ZP. Recombinant forms of human ZP3 and ZP4 have both been shown to induce human sperm to undergo the acrosome reaction, seemingly by independent pathways *(77,78)*. There is also data from a number of other species of mammal (macaque, cow, and rabbit) that supports the hypothesis that ZP4 has sperm binding activity *(79–81)*. In the pig, for example, the primary sperm receptor is a heterocomplex of ZP3 and ZP4 *(82)*.

Interestingly, four zona proteins are expressed in the rat ZP *(83)*. The rat may therefore represent a better animal model for human fertilization than the mouse. Whatever the model, it is clear that we need to understand how the four protein model relates to sperm interaction as a prerequisite to understanding sperm zona interaction in the human. Without acquiring this knowledge we are unlikely to make any real progress in studying sperm function/dysfunction.

9. METHODS TO DETERMINE THE PATHOLOGY OF SPERM DYSFUNCTION

9.1. Mouse Models for Male Infertility: The Role of KO Mice

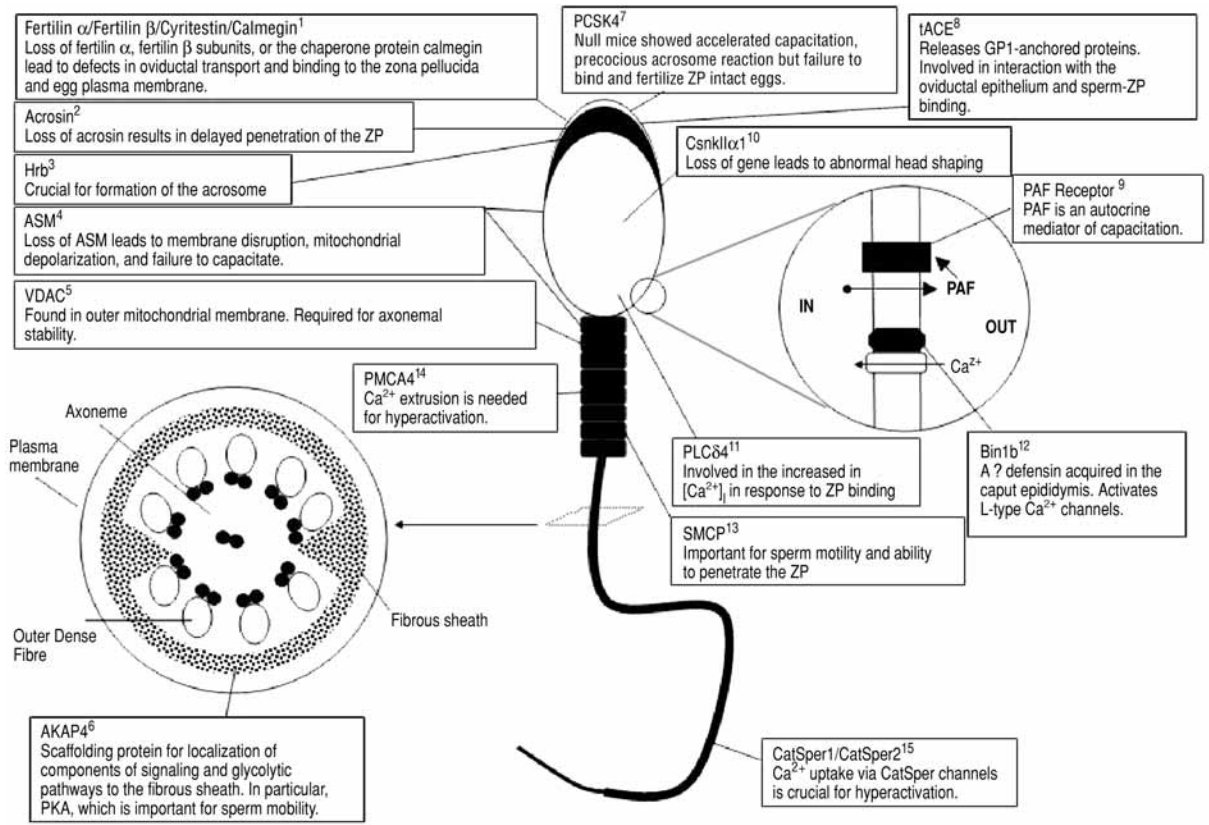
With the increasing number of KO mice being generated with a male infertility phenotype (Fig. 2; refs. *84–86*) and the relative ease of screening men, we should expect that the causes of sperm dysfunction would be well known. However, although KO animals are very useful, there are specific difficulties translating findings in mice to men: (1) there is significant redundancy in the reproductive process, (2) the pathology, although similar, is not the same and very detailed studies are needed on both the mouse and man to determine the real differences, and (3) fertilization in humans has a number of very specific differences from that in mice *(22)*. Consequently, successful examples of identifying gene defects in subfertile men by screening for genes knocked out in mice are rare *(87)*. The usual case is that no mutations are found and much effort has been wasted (e.g., examination of men with globozoospermia for mutations in casein kinase IIα [encoded by *Csnk2a2* gene]; ref. *88*).

Thus alternative/complementary strategies to determine the defects in men with sperm dysfunction are required. Simplistically, differences between normal and dysfunctional cells can be examined using transcript or proteomic profiling. Whether sperm have functional messenger RNA is open to debate and thus a transcriptome approach may be limited *(89)*. However, spermatozoa, because they are transcriptionally inactive, are ideal cells for proteomics to examine normal cell function and changes associated with defined correlates of fertilization success *(90)*.

9.2. Proteomics for Understanding the Sperm Cell and Diagnosis of Sperm Dysfunction

Comprehensive and systematic identification and quantification of proteins expressed in cells and tissues are providing important and fascinating insights into the dynamics of cell function. For example, there has been a wealth of detailed proteomic studies to identify molecular signatures of disease states (e.g., phospho-protein networks in cancer cells; ref. *91*).

Although spermatozoa are ideal to study from a proteomic perspective, there have been relatively few studies examining the proteome of human spermatozoa *(90)*. Initial studies have used antisperm antibody sera in an attempt to detect potential sperm targets for male contraception. This is a logical approach because antisperm antibodies are associated with sterility, albeit in a very limited number of cases. Unfortunately, however, this rational approach has met with limited


Fertilin α/Fertilin β/Cyritestin/Calmegin[1]
Loss of fertilin α, fertilin β subunits, or the chaperone protein calmegin lead to defects in oviductal transport and binding to the zona pellucida and egg plasma membrane.
Acrosin[2]
Loss of acrosin results in delayed penetration of the ZP
Hrb[3]
Crucial for formation of the acrosome
ASM[4]
Loss of ASM leads to membrane disruption, mitochondrial depolarization, and failure to capacitate.
VDAC[5]
Found in outer mitochondrial membrane. Required for axonemal stability.
PCSK4[7]
Null mice showed accelerated capacitation, precocious acrosome reaction but failure to bind and fertilize ZP intact eggs.
tACE[8]
Releases GP1-anchored proteins. Involved in interaction with the oviductal epithelium and sperm-ZP binding.
Csnklα1[10]
Loss of gene leads to abnormal head shaping
PAF Receptor[9]
PAF is an autocrine mediator of capacitation.
IN
PAF
OUT
Ca2+
PMCA4[14]
Ca2+ extrusion is needed for hyperactivation.
Axoneme
Plasma membrane
Outer Dense Fibre
Fibrous sheath
PLCδ4[11]
Involved in the increased in [Ca2+]i in response to ZP binding
Bin1b[12]
A ? defensin acquired in the caput epididymis. Activates L-type Ca2+ channels.
SMCP[13]
Important for sperm motility and ability to penetrate the ZP
AKAP4[6]
Scaffolding protein for localization of components of signaling and glycolytic pathways to the fibrous sheath. In particular, PKA, which is important for sperm mobility.
CatSper1/CatSper2[15]
Ca2+ uptake via CatSper channels is crucial for hyperactivation.

success *(92–94)* with very few robust candidate proteins being identified *(95)*.

Other than those previously mentioned, there have been very few studies that have employed proteomic approaches to examine male infertility. A small number of studies have attempted initial characterization of the sperm plasma membrane *(96)*. Further studies have examined specific processes, for example calcium-binding proteins and proteins that are tyrosine phosphorylated *(97,98)*. Interestingly, it has been more than 10 yr since the discovery of tyrosine phosphorylation as a putative marker of capacitation, yet the role of the proteins and their sequence of activation is very sketchy and, with the exception of the A kinase anchor proteins (AKAPs) (AKAP3 and AKAP4), only a small number of candidate proteins have been identified *(99)*. We are still a long way from obtaining even a minimal "picture" of events.

The slow progress/application of the proteomic revolution in human spermatozoa contrasts markedly with other fields. A relevant example is the large-scale proteomic studies on human cilia *(100)*. Estimates from *Chlamydomonas* suggest there are at least 250 flagellar proteins. In close agreement, Ostrowski and colleagues were able to identify 200 ciliary axonemal proteins, some of which were sperm/testis-specific (e.g., Sp17). However a combination of proteomic approaches is often required. For example, Ostrowski and colleagues *(100)* only identified 38 potential proteins using two-dimensional polyacrylamide gel electrophoresis. A number of proteins were not resolved (e.g., dynein heavy chains, which have a large molecular mass) and complementary approaches were needed to provide a detailed picture. One-dimensional gels identified another 110 proteins. A second approach involved isolated axonemes, followed by digestion and analysis directly by liquid chromatography/mass spectrometry (MS)/MS or multidimensional liquid chromatography/MS/MS. This led to the identification of a further 66 proteins.

In our laboratory, we have been using proteomic strategies to identify defects in sperm function responsible for fertilization *(101,102)*. Specifically we are interested in identifying differences in sperm protein expression between control (fertile) men and patients with spermatozoa that failed to fertilize oocytes in vitro. Our initial studies have focused

Fig. 2. *(Opposite page)* A diagrammatic representation of a spermatozoon illustrating the site of expression and effect of gene knockout experiments in the mouse. Particular emphasis is placed on the process of sperm capacitation, transport in the female tract and interaction with the zona pellucida. [1]*104–109*, [2]*110*, [3]*111*, [4]*112*, [5]*113*, [6]*114*, [7]*115*, [8]*116,117*, [9]*118*, [10]*119*, [11]*120*, [12]*121*, [13]*122*, [14]*39,40*, [15]*123–125*. (Adapted from refs. *126* and *127*.)

on a two-dimensional gel-based approach and developing a series of fertile controls (with several ejaculates) to determine if any differences observed in the patient samples are real. Initial results are interesting. To our surprise, there was relatively little intra- and interdonor variation (1.4 and 1.8% of the total number of spots identified, respectively; ref. *102*). However, differences between gels occur and when accounting for this, we have categorized one man *(102)* in which we have identified 20 differences from the control that we are confident represent true differences.

10. CONCLUSION

There is an urgent need to develop a more detailed understanding of the physiological, biochemical, and molecular functioning of the human sperm cell. We can use this knowledge as a platform to improve the diagnosis of male infertility and importantly to develop potential non-ART-based therapies. The tools at our disposal have never been more sophisticated and it is likely that rapid progress will be made in this area within the next 5 yr. Perhaps then we will see a decrease in the use of inappropriate ART treatment.

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DISCLAIMER

Part of this chapter contains previous arguments and adaptations from manuscripts produced by our group in the last year *(24,62,73,103)*.

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6 Genetics of Idiopathic Male Infertility

The Power of a Cross-Species Approach

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Summary

Nearly 7% of men suffer from male factor infertility. In one-fourth of infertile males, the etiology remains unexplained. Unlike other multifactorial disorders, gene–gene and gene–environment interactions in the regulation of male fertility have been poorly characterized. A candidate-gene approach that incorporates biological information from model organisms is likely to be critical in deciphering the genetic basis of idiopathic male fertility. Genes that fulfill essential roles in spermatogenesis often have orthologs in several species wherein they serve similar functions. By using a comparative cross-species approach, major susceptibility genes underlying male infertility can be identified in association studies. With a better understanding of the molecular regulation of spermatogenesis, proper diagnosis and treatment of male infertility should be realized in the foreseeable future.

Key Words: Infertility; idiopathic; candidate gene; spermatogenesis; *in silico*.

1. INTRODUCTION

Infertility is a major global health problem with wide-ranging socioeconomic ramifications for the affected couple and the society at large. Clinically defined as an inability to conceive after 1 yr of unprotected intercourse, the worldwide estimates for the prevalence of infertility range from 10 to 15% *(1,2)*; significantly, in a 1982–1985 multicenter study conducted by the World Health Organization, the male partner was observed to have an abnormal semen analysis in approx 50% of the infertile couples investigated *(1)*. Whereas endocrine defects (e.g., gonadotropin deficiency or hyperprolactinemia), systemic disorders,

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erectile dysfunction, and ejaculatory inadequacy were present in a minor fraction of infertile males, approx 22% were classified as idiopathic, with idiopathic oligozoospermia (<20 $\times$ 10^6 sperm/mL of semen) being the single most common diagnosis (11.2%) for a semen abnormality *(1)*.

A routine semen analysis *(3)* constitutes the mandatory first step in the evaluation of an infertile male. An initial semen evaluation, a detailed personal and family history, and a physical examination are usually followed by a work-up for specialized tests for sperm physiology, semen quality, and endocrine abnormalities, where appropriate *(4)*. If indicated, karyotyping for aneuploidies and screening for Y-chromosome microdeletions is also considered in selected patients *(4)*. Although novel tests of semen quality *(4,5)* have been introduced over the past decade to better characterize the heterogeneous nature of infertility, the prevalence of idiopathic infertility has remained more or less static.

As our understanding of the genetic regulation of gametogenesis and fertilization develops in the postgenomics era, it becomes intuitively apparent that a significant proportion of idiopathic cases are of genetic etiology. It has long been recognized that fertility or fecundity is a function of an individual's genetic makeup and his/her interaction with the environment. Subfertility is a multifactorial trait in which environmental toxins (e.g., lead, radiation, ethylene oxide) directly disrupt genes involved in gametogenesis or interact with multiple alleles that increase susceptibility to environmental modulation *(6)*. Association studies to detect familial segregation of male subfertility *(7,8)* have shown that male siblings of infertile males more often fail to sire offspring than control populations; segregation analysis has suggested an autosomal-recessive mode of inheritance for a majority of cases *(7)*. In addition, male infertility has also been identified as part of monogenic syndromes *(9)*, wherein it is a direct consequence of absence of gene function during spermatogenesis; however, idiopathic infertility is by definition nonsyndromic and is probably unrelated to these rare disorders.

More relevant have been the identification of microdeletions on the long arm of the Y chromosome (Yq dels) in azoospermic and severely oligozoospermic males. Since Tiepolo and Zuffardi *(10)* presented evidence in 1976 suggesting an association between Yq microdeletions and spermatogenic failure, an impressive body of evidence has accumulated *(11)* indicating that these lesions are present in approx 18% of males with idiopathic azoospermia *(12)*. Further evidence for a genetic basis of infertility comes from molecular genetic analysis of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene; approx 25% of patients with obstructive azoospermia have congenital bilateral absence of the vas deferens *(13)* and occasionally the unilateral form, anatomical defects that are associated with mutations in the *CFTR* gene in approx 80% of cases *(14)*.

The most conclusive evidence for the genetic basis of male idiopathic infertility or subfertility comes from the vast number of mouse knockouts (KOs) with defects in fertility *(2,15)*. Several mouse models have been generated with isolated azoospermia (absence of sperm), oligozoospermia, asthenozoospermia (<50% motile sperm), or a combination of these defects. In this chapter, we discuss the relevance and importance of a cross-species approach to understanding the molecular genetics of idiopathic male infertility in humans, with particular reference to the laboratory mouse, *Mus musculus*. We exclude from our discussion forms of male infertility that are present as part of nonendocrine syndromes (e.g., Kartagener and Kallmann syndromes), endocrine disorders (pituitary failure, hyperprolactinemia, sex steroid deficiencies), and chromosomal defects (e.g., Yq deletions). Also excluded are congenital bilateral absence of the vas deferens and congenital unilateral absence of the vas deferens, which cause obstructive azoospermia primarily because of mutations in the *CFTR* gene, as described previously. We instead highlight a few studies in the mouse that have provided a basis for investigating humans with idiopathic male infertility and conclude with a general discussion on the vast array of novel technical and informatics tools that allow us to manipulate model organisms with greater ease and higher specificity.

2. GENETICS OF MALE IDIOPATHIC INFERTILITY: A CANDIDATE-GENE APPROACH

Classical genetic approaches have focused on linkage and association between markers and traits in large multigenerational pedigrees to map gene loci involved in the etiology of disease. This positional cloning approach, although tedious, has served researchers well in identifying causative genes for several monogenic disorders. For complex disorders such as infertility, in which several common variants are believed to modestly influence disease risk, traditional linkage analyses are less powerful *(16)* and have rarely been successfully used. In addition, large families that segregate the infertility trait are seldom obtained, making linkage an ineffective tool *(17)* in studying infertility. The recent completion of phase I of the International HapMap Project *(18)* and the development of low-cost high-throughput genotyping technologies may eventually lead to genome-wide association studies in the future *(16)*; however, an additional deterrent to this approach in the study of male infertility lies with the genetic heterogeneity in sample populations with infertility, which requires collection of large sample sizes for achieving sufficient statistical power *(17)*.

Resequencing of candidate genes to detect allele frequency differences between cases and controls therefore remains the most practical approach to identifying infertility-associated disease genes in humans *(17)*. This candidate-gene approach has been fairly successful in other complex disorders but has several limitations for infertility research: the requirement for prior knowledge of the function and expression of the candidate gene so that biological plausibility of the role of the gene in causing the phenotype can be inferred and the effect of population stratification or ethnic admixture in cases and controls. Whereas the latter can be tackled through additional marker typing *(19)*, resolution of the former is exclusively dependent on our understanding of spermatogenesis in model organisms, particularly mice.

Thus, studies that have generated approx 100 mouse models of nonsyndromic male infertility with isolated defects in either sperm production, motility, or maturation *(2)* provide us with sufficient biological information to undertake a candidate approach in humans. Although single gene mutations in mice on homogeneous genetic backgrounds are not the best representative models for human infertility, which is supposedly complex in nature with multiple gene–environment interactions on essentially heterogeneous genetic backgrounds, these studies nevertheless uncover excellent candidate genes that serve essential and nonredundant roles at different steps of spermatogenesis.

3. MUTATIONS IN INFERTILE MALE PATIENTS: LESSONS FROM SELECTED MOUSE MODELS

Several comparative studies have illustrated the utility of choosing candidate genes for association studies in humans based on a defined function of the ortholog in mice. Homozygous deficiency for the synaptonemal complex protein 3 (SYCP3) leads to meiotic arrest and sterility in male mice. Failure of synaptonemal complex formation produces massive apoptotic cell death during the meiotic prophase in spermatocytes *(20)*. Interestingly, in a study of 19 infertile male patients with nonobstructive azoospermia, Miyamoto et al. *(21)* identified 2 unrelated patients of different ethnicity (2/19) with a 1-bp heterozygous deletion (643delA) in the human *SYCP3* gene that resulted in a frameshift mutation and a premature termination codon; the mutation was absent in 75 fertile control males. The authors also demonstrated defective binding of the mutant protein to the wild-type protein in an in vitro interaction assay. Although heterozygosity for a null mutation in *Sycp3* does not cause meiotic arrest in mice, the authors concluded that the mutant protein in the patients was producing a dominant negative effect on the

wild-type protein. An alternative explanation could be the presence of multiple loci that interact with this allele to produce the phenotype in these patients. Regardless, the study demonstrates the feasibility of a candidate-gene approach to analyzing male infertility in humans.

The deleted in azoospermia-like (*Dazl*) gene is essential for differentiation of male germ cells; male mice that lack functional DAZL protein undergo germ cell loss and are sterile *(22)*. In an extensive study of 102 infertile men with oligozoospermia and/or asthenozoospermia, Tung et al. *(23)* sought to study the relationship between variants in the human *DAZL* gene and idiopathic oligoasthenozoospermia. The authors identified seven variants, six single-nucleotide polymorphisms (SNPs) in the 3′ untranslated region (UTR) and one nonsynonymous-coding SNP that were associated with the defects in the infertile men across different ethnic backgrounds. Surprisingly, the nonreference alleles of these SNPs showed positive association with increased sperm counts in these patients compared with controls, leading the authors to conclude that these UTR SNPs affect the function of the *DAZL* gene in a complex manner. Notably, however, the 200 controls chosen in this study represented a sample of the general population without characterization of their semen parameters, which might have led to the confounding effect.

Similar examples of correlation between mouse KOs and male patients relate to the basic nuclear proteins that compact DNA during spermiogenesis. After meiosis, histones are first replaced with transition nuclear protein (TNP)1 and TNP2 and then protamine (PRM)1 and PRM2 in two major chromatin remodeling steps designed to condense the sperm nucleus. Haploinsufficiency for either *Prm1* and *Prm2* in mice leads to male infertility in high-percentage chimeras *(24)*; heterozygous deficiency of either protamine leads to defective nuclear condensation and male sterility. Interestingly, PRM2 deficiency had been previously described in infertile men *(25)*; more recent studies have uncovered association of SNPs in human *PRM2* and male sterility. In one study *(26)*, a single missense mutation that introduced a premature termination codon in the *PRM2* gene was identified in 1 of 226 (0.4%) sterile male patients and not in 270 proven-fertile male controls. The resultant nonsense transcript was inferred to lead to haploinsufficiency for *PRM2*. The low frequency of the mutant allele in cases is probably due to the result of the flawed design of the association study: it included male sterile patients as a single group of cases without classifying them into different subgroups based on their semen analysis. Using more detailed semen analysis, including specialized tests to detect sperm DNA fragmentation and sperm morphology analysis, another group *(27)*

studied only infertile males with normal sperm counts and increased DNA fragmentation and defective sperm morphology. Significantly, they found the association of a *PRM1* SNP in 3 of 30 (10%) cases, highlighting the critical value of proper phenotypic definition of patients and controls. However, this study used an extremely small control set ($n = 10$) of ethnically unmatched fertile males and lacked statistical verification of significance.

A similar study was designed to investigate the association between the human *TNP1* and *TNP2* loci and male infertility. In mice, loss of either *Tnp1* or *Tnp2* genes was found to cause male subfertility *(28,29)*. In a group of 282 infertile patients as cases, several SNPs were identified that were similar in frequency in cases and controls. However, a single patient with azoospermia was found to have a deletion in the promoter region that constitutes the binding site for the transcription factor cyclic adenosine monophosphate responsive element modulator *(30)*; this deletion in the cyclic adenosine monophosphate responsive element-binding promoter was absent from 266 controls.

Although these studies indicate the feasibility of association studies with a candidate-gene approach, most of these studies are underpowered to detect all the relevant causative alleles because of modest sample sizes or poor characterization of cases and controls. Future studies should focus on proper phenotypic classification of patients based on detailed semen analyses and a high-throughput strategy to study multilocus association, similar to one recent study that used selected markers to study association between components of estrogen signaling pathway and male idiopathic infertility *(31)*.

4. IDENTIFICATION OF NEW CANDIDATES CONSERVED ACROSS DIFFERENT VERTEBRATE LINEAGES USING TESTIS-SPECIFIC EXPRESSED SEQUENCE TAG LIBRARY SEARCHES

Although the studies of male reproductive genes conserved between humans and mice helped us to characterize their roles in male infertility, expanding the scope of cross-species analysis to include various mammalian lineages and beyond can provide further insights into the roles of conserved components in germ cell physiology. Because the continuation of life in higher organisms depends on the success of sexual reproduction, crucial gene functions and gene interactions during gametogenesis have been conserved under selective pressures that lead to the generation of functional gametes *(32,33)*. In other words, evolution has tested the dispensability of core

reproductive genes, and the results are waiting to be deciphered by cross-species comparative genomics.

The cross-species comparative genomics approach was made possible by the methodological progress in high-throughput sequencing and the constantly improving bioinformatics tools tailored for the analysis of the vast amount of data. Expressed sequence tag (EST) libraries have been extensively used as the first step of large-scale gene discovery processes by cataloging the transcriptomes with short end-sequencing data of the cloned cDNA fragments *(34–36)*. After partitioning ESTs into nonredundant sets of gene-oriented clusters, such as UniGene entries, *in silico* subtraction can be used to extract genes preferentially expressed in target tissues from tissue-specific EST libraries *(37–40)*. Among them, testis-specific EST libraries catalog the genes actively expressed during spermatogenesis, and those from nonmammalian species are especially useful for identifying highly conserved reproductive genes (Table 1). Recently, abundant fathead minnow (*Pimephales promelas*) transcripts were deposited into the EST database as an effort to allow government and industry to develop tools to improve environmental toxicity monitoring in aquatic ecosystems. In fact, the large collections in the testis-specific EST library from fathead minnow (Table 1) also provided an excellent reference to identify conserved male reproductive genes showing preferential expression in testis. Compared to the approx 92 million yr of divergence within the mammalian lineage, the divergence time between mammal-bird, mammal-frog, and mammal-fish groups are 3.5–5 times longer (~310, ~360, and ~450 million yr, respectively; ref. *41*). If the preferential expression of orthologous genes in testis is conserved beyond the mammalian lineage, it would strongly suggest that these genes are indispensable during gametogenesis and their malfunction may cause male infertility.

Combining our accessibility to databases and other Internet-based tools, we can now easily identify new candidate genes and revisit known players involved in male reproduction. Using KO mice as models to mimic the disruption of male reproductive genes and to functionally dissect the genetic components of male infertility, our current approach begins with the identification of conserved testis-specific genes *(42)*. Here, we report the identification and computational analysis of *Klhl10 (43)*, a germ cell-specific gene identified in mice through *in silico* subtraction and validated through traditional expression analysis, as a step-by-step example to explain our cross-species gene characterization approach.

Table 1
Testis-Specific EST Libraries

Chicken (*Gallus gallus;* 21,447 total UniGene entries; refs. *48*,*49*)
- Lib.15562 WLtestis library (4891 sequences/2473 UniGene entries)
- Lib.15563 RJtestis library (5516 sequences/2294 UniGene entries)
- Lib.16173 Korean native chicken testis cDNA library (5069 sequences/2159 UniGene entries)

Xenopus laevis (24,738 total UniGene entries)
- Lib.12882 NICHD_XGC_Te1 (2407 sequences/1095 UniGene entries)
- Lib.15412 NICHD_XGC_Te2N, normalized (11,700 sequences/3897 UniGene entries)
- Lib.15418 NICHD_XGC_Te2 (12,231 sequences/2877 UniGene entries)

Xenopus tropicalis (15,440 total UniGene entries)
- Lib.16859 NIH_XGC_tropTe3 (21,384 sequences/3961 UniGene entries)
- Lib.16860 NIH_XGC_tropTe4 (22,173 sequences/4547 UniGene entries)
- Lib.16861 NIH_XGC_tropTe5 (21,812 sequences/4949 UniGene entries)

Rainbow trout (*Oncorhynchus mykiss*; 14,340 total UniGene entries)
- Lib.12452 AGENAE Rainbow trout normalized testis library (1039 sequences/708 UniGene entries)
- Lib.15060 AGENAE Rainbow trout normalized testis library (11,503 sequences/5906 UniGene entries)

Zebrafish (*Danio rerio*; 24,009 total UniGene entries; ref. *50*)
- Lib.9768 Gong zebrafish testis (8964 sequences/2531 UniGene entries)
- Lib.15929 Adult testis full-length (TLL) (2173 sequences/1127 UniGene entries)
- Lib.15931 Adult testis normalized (TLL) (1680 sequences/938 UniGene entries)
- Lib.15936 Adult testis ORESTES (TLL) (2646 sequences/708 UniGene entries)

Fathead Minnow (*Pimephales promelas*; 249,938 total sequences; not incorporated in UniGene database yet)
- 7–8 mo adult testis (60,617 sequences/~11,500 initial clusters)

Ciona intestinalis (14,370 total UniGene entries; ref. *51*)
- Lib.10379 K. Inaba unpublished testis cDNA (9380 sequences/2112 UniGene entries)

Testis-specific EST libraries (with >1000 sequences) available from species outside mammalian lineage as of January 2006. UniGene entry numbers, the nonredundant set of gene-oriented clusters, may be used for assessment of the coverage of the testis transcriptomes. Detailed information (except fathead minnow) can be found using library browser on the species-specific webpages of UniGene database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene).

1. Obtain the full-length cDNA sequence. After validation of the germ cell-specific expression of mouse *Klhl10*, we examined the 3′-end of *Klhl10* transcript for poly-A signal as an indication of completeness and used 5′-rapid amplification of 5′ complementary DNA ends (RACE) to obtain the 5′-UTR and the full coding region. We deposited the identified full-length sequence into GenBank database as nucleotide entries AY495337. The full-length cDNA sequences are crucial in the correct interpretation of gene structure for further analysis.
2. Identify the human ortholog. The translated *Klhl10* amino acid sequence was first used for the translated BLAST search against nucleotide database (tBLASTn) while limiting the search to *Mus musculus* to check for closely related genes in mouse transcriptome. The results showed that the closest homolog, *Klhl20*, shared only 37% protein identity in the matched region and it should not confuse further BLAST searches. After repeating tBLASTn search with *Klhl10* amino acid sequence limited to *Homo sapiens*, the matches with human cDNAs and ESTs suggested the presence of human ortholog of *Klhl10*. After aligning mouse *Klhl10* with all available cDNAs, ESTs, and genomic sequences, human *KLHL10* sequence was confirmed and deposited as AY495339. Similarly, searching with human *KLHL10* sequence returned matches with mouse *Klhl10*. Besides fulfilling the mutually best similarity criteria, the orthologous relationship was further confirmed by the shared gene order synteny around *Klhl10* (*Nt5c3l-Klhl10-Klhl11*), which can be visualized in Genome Browser (http://genome.ucsc.edu; ref. *44*).
3. Determine domain structures. The domain structures were examined by searching Pfam database (http://www.sanger.ac.uk/Software/Pfam/; ref. *45*) or Simple Modular Architecture Research Tool database (http://smart.embl-heidelberg.de/; ref. *46*). The searches with both mouse and human KLHL10 protein sequences identified BTB, BACK, and six Kelch repeats.
4. Extend searches in other species. To find *Klhl10* in other species, we began with translated mouse *Klhl10* amino acid sequence for tBLASTn to search the EST subset excluding mouse and human entries ([est_others] selected from the "CHOOSE DATABASE" option). After ruling out low-score matches by careful examination of the listed matches, the high-score matched ESTs can be grouped by species and assembled into longer contigs. The BLAST search process can then be repeated with assembled *Klhl10* contigs from other species until all EST matches were retrieved and assembled. These assembled *Klhl10* contigs can then be used computationally in gene prediction and experimentally in RACE-PCR cloning to obtain full-length sequences in those species. Interestingly, in species that have testis-specific EST

libraries, *Klhl10* mostly matched to testis-derived ESTs, suggesting conserved preferential expression in testis.

After retrieving all the available orthologs across distant lineages, the final prize lies in the information unlocked by cross-species comparison. Not only the alignment of amino acid sequences of conserved orthologs from distantly divergent species can show us the functionally constrained domains even in the absence of known protein domains, but the conserved orthologs can also provide common grounds to integrate the information from studies of male reproduction in a wide variety of species. Starting from these conserved reproductive genes, two-hybrid screening may build up the protein–protein interaction network and microarray gene profiling of KO animals may reveal the underlying genetic network.

5. CONCLUSIONS

Advances in assisted reproductive techniques (e.g., in vitro fertilization and intracytoplasmic sperm injection) have dramatically increased the chances of a successful pregnancy in a hitherto infertile couple *(47)*. However, several studies have raised questions regarding the safety of these procedures, particularly related to the transmissibility of undetected molecular defects to the offspring born from these techniques. The combination of a lack of informative tools and a slowly emerging understanding of the molecular basis of spermatogenesis has meant that the genetic basis of male factor infertility remains obscure. Although the drive to further improve assisted reproductive techniques carries on, emphasis also needs to be placed on developing newer methodologies to understand the molecular etiopathogenesis of infertility. The gap between the laboratory bench (and the mouse) and the patient can be effectively bridged by induction of diagnostic methods (semen analysis, imaging techniques, sperm RNA profiling, and so on) that harness the power of cutting-edge molecular genetic research with the simplicity of application that can be widely implemented in the clinic. Equipped with better knowledge, proper diagnosis and treatment of male infertility should be realized in the very near future.

6. REVIEW

Infertility is a genetically heterogeneous disorder with a multifactorial etiology. Using classical genetic strategies of linkage analysis to identify genes causing infertility is limited by the small family sizes in inherited forms of the disease. A candidate-gene approach to identify

genes associated with disease relies heavily on biological information regarding the expression and function of the gene in model organisms. This strategy has been successfully used to identify several human genes (*SYCP3*, *DAZL*, *PRM1*, *PRM2*, and *TNP1*) that show association with several forms of idiopathic male infertility, as illustrated in this chapter. More than 100 genes can cause isolated infertility and subfertility in mice when mutated, suggesting that several more candidate genes should be tested in humans for association with male infertility. Furthermore, comparative analysis of genes in several model organisms can identify highly conserved genes that share similar spatiotemporal expression profiles and serve analogous roles, thereby making them the best candidate genes for analysis in humans. We review this cross-species approach in this chapter with examples highlighting the strengths and shortcomings of this strategy and conclude with a discussion of novel tools that aid the researcher in identifying conserved genes across multiple mammalian and nonmammalian species.

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II Meiosis and Errors of Meiosis

7 The Immunocytogenetics of Human Male Meiosis

A Progress Report

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Summary

Recently, several components of the mammalian meiotic recombination pathway have been identified and new immunofluorescence approaches to the analysis of human meiosis have been developed. This has made it possible to directly examine the dynamics of chromosome behavior in meiosis I spermatocytes and address previously intractable questions, such as: How do chromosomes pair and synapse with one another? How does recombination occur? What is the relationship—if any—between abnormalities in meiosis and cases of unexplained male infertility? In this chapter, we discuss results from immunocytogenetic studies of human males, summarizing how they have contributed to our understanding of both normal and abnormal spermatogenesis.

Key Words: Meiosis; pachytene; synaptonemal complex; recombination; aneuploidy.

1. INTRODUCTION

The contribution of meiotic chromosome abnormalities to human male infertility has long been recognized. As early as 1959, human cytogeneticists documented the association between numerical sex chromosome abnormalities (i.e., 47,XXY) and failure to produce sperm *(1)*. Subsequently, cytogenetic analyses of males attending infertility clinics demonstrated that structural chromosome abnormalities (e.g., translocations) were also an important contributor to infertility *(2)*. The reason for these effects was revealed by conventional light and electron microscopic analyses of testicular biopsies in aneuploid or translocation-carrying individuals in studies conducted during the 1970s and 1980s. Specifically, germ cells with additional or rearranged chromosomes

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were found to have severe defects in orientation and movement of chromosomes during the earliest stages of meiosis, frequently resulting in meiotic arrest and germ cell death. Further, infertile individuals with apparently normal chromosome constitutions but with defects in homologous chromosome pairing and/or synapsis were also identified, suggesting that gene mutations as well as chromosome abnormalities might contribute to arrest at meiosis I (MI).

During the intervening two decades, we have witnessed remarkable advances in our understanding of the molecular basis for male infertility (e.g., the identification of *DAZ* mutations; refs. *3* and *4*), but there has been surprisingly little new information on the possible contribution of meiotic abnormalities to infertility. In large part, this reflects technical limitations: although we have been able to conduct routine karyotypic analyses and, occasionally, analyses of meiotic chromosome synapsis and pairing in infertile men, it has not been possible to simultaneously analyze meiotic chromosomes and chromosome-associated proteins.

Fortunately, this situation is now changing. The identification of mammalian homologs of meiosis-acting proteins of lower organisms and the introduction of novel immunofluorescence technology to meiotic analysis has made it possible to directly analyze protein localization patterns in meiocytes, leading to renewed interest in human meiosis research. Thus, beginning approx 10 yr ago, several groups initiated studies to characterize protein–DNA interactions in human male meiosis, using testicular biopsy material obtained from infertility clinics. In this chapter, we summarize some of the most important of these studies, first discussing the impact they have had on our understanding of normal male meiosis, and second their relevance to the etiology of idiopathic male infertility.

2. WHAT IMMUNOCYTOGENETICS HAS TAUGHT US ABOUT MALE MEIOSIS

Meiosis is the specialized cell process whereby an organism undergoes two nuclear divisions in preparation for gamete formation. In humans, this results in a reduction of chromosomes from the diploid (46) to the haploid (23) number, with each gamete containing one copy of each chromosome. In this fashion, genetic diversity is created because each daughter cell contains a different combination of alleles.

Several processes are unique to meiocytes. However, none are more profound than those that occur during prophase of MI, when homologous chromosomes are required to first find their partners, then become intimately associated with one another and finally exchange genetic

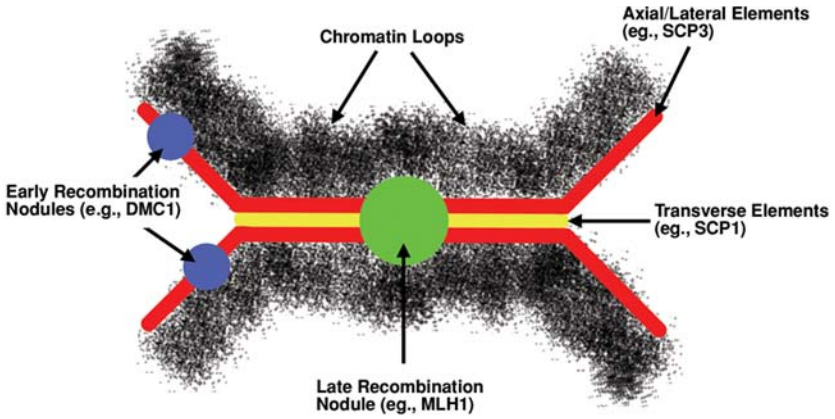

Fig. 1. The synaptonemal complex (SC) and associated recombination machinery proteins. The SC is a tripartite structure, consisting of two axial/lateral elements and the transverse filament in the central region. In leptotene, DNA from the two sister chromatids of each homolog becomes bound to the forming axial elements; by zygotene, the axial elements are completely formed. In zygotene, the transverse filament of the central region begins to bring the axial elements of each homolog into close register. By pachytene, the synaptic process is complete, with the chromosomal DNA bound to the fully-formed, mature SC; the axial elements are now referred to as lateral elements. Recombination occurs in the context of the SC, with "early" recombination nodules presumably representing sites of double strand breaks and "late" nodules a subset of those breaks that are processed as exchanges. Following pachytene, the SC dissolves, with the chiasmata (crossovers) acting to hold the homologs together until the metaphase/anaphase I transition.

Several of the components of the SC and the recombination machinery are now known, including SCP1 (a component of the transverse filament), SCP2, and SCP3 (components of the axial/lateral elements); DMC1 and RAD51 (components of early recombination nodules); and MLH1 (a component of late nodules). These can be detected cytologically using the appropriate antibodies; subsequently, specific chromosomes/chromosome regions can be identified with fluorescence *in situ* hybridization probes.

material, all in preparation for chromosome segregation at the first meiotic division. This series of events requires coordinated activity of a number of different protein families, two of which have been extensively studied in humans using immunocytogenetic methodology: synaptonemal complex proteins, responsible for linking pairs of homologous chromosomes, and recombination proteins, responsible for promoting exchanges (crossovers) between homologous chromosomes (Fig. 1).

2.1. Synaptonemal Complex Proteins

The synaptonemal complex (SC) is the prophase-specific supramolecular proteinaceous structure that forms between, and holds together,

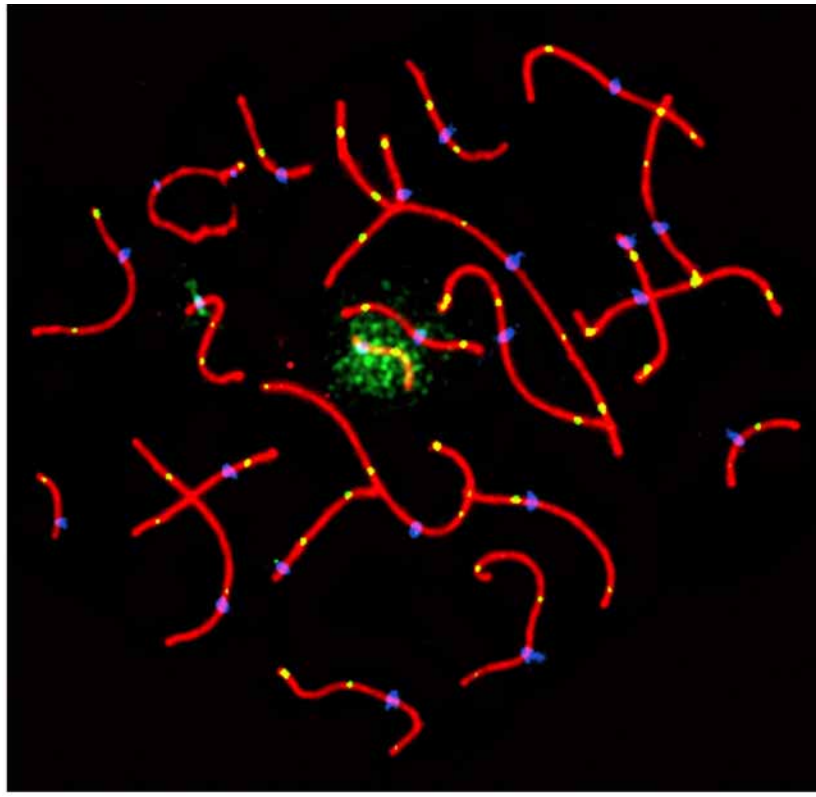

Fig. 2. Representative human pachytene stage spermatocyte, showing 23 fully formed synaptonemal complexes (SCs). SCs are detected by SCP3 (in red), centromeres by CREST (in blue), and sites of crossovers by MLH1 (in yellow). Two fluorescence *in situ* hybridization probes have been used to identify individual chromosomes: a paint probe for chromosome 22 (dispersed green signal) and a probe to the centromeric region of chromosome 18 (punctate green signal).

homologs (Fig. 1). Three component proteins of the SC have been identified: SCP1, SCP2, and SCP3. Of these, SCP3 has been the most useful in immunostaining studies because it localizes to the first-formed components of the SC (i.e., the axial elements; *see* Fig. 1) and thus can be used to monitor assembly and disassembly of the SC during prophase *(5–8)*. These and other studies have shown that, in the human male, short linear SCP3 fragments are first detectable in leptotene, coalescing to form full-length axial elements in zygotene, and by pachytene, the 46 axial elements have been "zippered up" by the transverse filament (i.e., by SCP1) to form 23 fully mature SCs (Fig. 2).

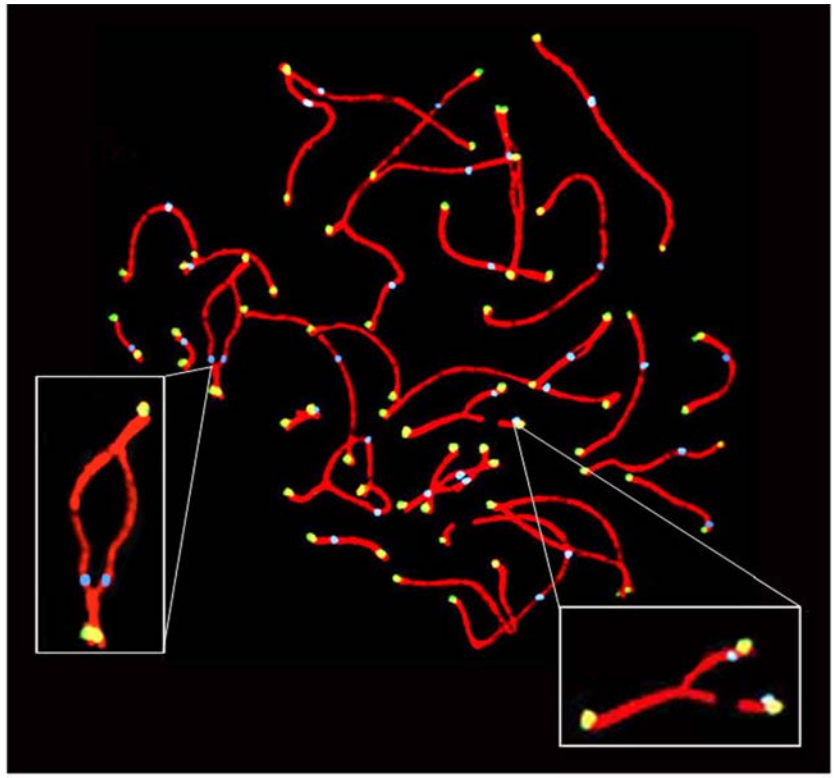

Fig. 3. Representative zygotene stage spermatocyte showing (left inset) a partially synapsed non-acrocentric chromosome with distally located regions of synapsis and an asynapsed interstitial region and (right inset) a partially synapsed acrocentric chromosome, with a region of synapsis extending from the distal region of the long arm toward the centromere, but with the proximal long arm and all of the short arm asynapsed.

Although these and other analyses have provided a broad outline of SC formation, few specifics have been available on the mechanics of human SC assembly. However, immunostaining studies of human males have finally begun to shed light on this process. Specifically, Brown et al. *(5)* analyzed formation of the SC in leptotene and zygotene spermatocytes from a series of control males (i.e., individuals with obstructive azoospermia [OA] in whom meiosis appeared unimpaired) and reported several general "rules" that apply to synapsis in males. Most importantly, the number of synaptic initiation sites appears to be tightly regulated in humans. On non-acrocentric chromosomes, there are invariably two sites per chromosome, one located near the short-arm telomere and one near the long-arm telomere; thus, there is

one initiation site per chromosome arm (Fig. 3). In contrast, acrocentric chromosomes have only one site, located distally on the long arm (Fig. 3). For both types of chromosomes, synapsis then proceeds toward the centromere.

Brown et al. *(5)* found little evidence that specific p- or q-arm sequences affect progression of synapsis. However, surprisingly, the centromere appears to have an inhibitory effect. For example, when one arm of a non-acrocentric chromosome is "zippered up" before the other, the centromere appears to act as a "stop sign," preventing further movement from that arm. This inhibitory role is in contrast to observations from some model organisms, in which the centromere promotes homologous pairing *(9)*. Further, these results provide evidence for a basic difference between synapsis and meiotic recombination in the human (i.e., there is now considerable evidence that chiasma interference is unaffected by the centromere, although it impedes the spread of synapsis). Thus it appears that, at least in the pericentromeric region, synaptic progression and transmission of recombination pathway signals are controlled differently.

In other studies, Brown et al. *(5)* demonstrated that synapsis is initiated in subtelomeric regions, not at the telomere proper. Specifically, they used pantelomeric probes to localize the telomeric repeat sequences (TTAGGG) in leptotene cells. In the vast majority of cases, they found that synaptic initiation sites were located proximal to the telomeric signals. However, it is not yet clear whether there are specific sequences that seed synapsis, nor is it known whether the synaptic initiation sites are located in regions housing subtelomeric repeats or in unique sequences.

Finally, Brown et al. *(5)* conducted studies comparing the number and location of synaptic initiation with the number and location of crossovers. The two processes share several features: for both, there appears to be a single "obligatory" event on p- and q-arms of non-acrocentric chromosomes and on q-arms of acrocentric chromsomes; both are excluded from the p-arms of acrocentric chromosomes and both are preferentially distally located on chromosome arms *(5,7,10)*. Thus, an obvious question is whether any, most, or all synaptic initiation sites are subsequently translated into crossovers. Although the answer to this question is not yet certain, the initial evidence suggests that the answer is no. That is, the number of crossovers (~45–50 per cell) exceeds that of synaptic initiation sites (~40 per cell), crossovers appear to be more proximally positioned than are initiation sites and, as discussed earlier, the centromere affects the two processes differently. Nevertheless, it may still be that some subset of initiation sites are associated with the

formation of crossovers. Detailed mapping of synaptic initiation sites and crossovers on individual chromosomes should allow us to directly test this possibility.

2.2. Recombination Proteins

Cytological identification of crossovers provides two important advantages over traditional, genetic linkage-based analysis of meiotic recombination: first, the ability to simultaneously visualize exchanges on each chromosome in a meiocyte and second, the ability to detect all exchanges in the cell (i.e., because only two of the four products are meiosis are recovered in gametes, linkage analysis can only detect one-half of all exchanges). However, until recently, the only available cytological methodology involved analysis of diakinesis/metaphase I preparations, an approach limited by the paucity of cells at the appropriate stage and by technical difficulties associated with generating good-quality images. Thus, relatively little information was generated with this approach.

With the advent of immunostaining technology, there was considerable optimism that crossover-associated proteins might be identified in pachytene cells, providing an alternative approach to diakinesis/metaphase I preparations. This optimism was realized about 10 yr ago, when analyses of mouse meiocytes suggested that the DNA mismatch repair protein MLH1 might colocalize with sites of crossover in pachytene cells *(11)*. Subsequent studies demonstrated that this was indeed the case both for mice and humans (*see*, e.g., Fig. 3; refs. *6*, *8*, *11*, and *12*). For example, human analyses of a series of control males *(7)* demonstrated that: the number of MLH1 foci per pachytene spermatocyte fit expectations from genetic linkage analyses, the foci were distally positioned, consistent with available data from linkage studies, and consistent with basic meiotic principles, the foci displayed positive interference (i.e., the presence of one focus reduced the likelihood that a second focus would be positioned nearby). These observations have now been confirmed by several groups *(6,8,13–19)* making it clear that MLH1 can serve as a marker for crossovers in studies of pachytene spermatocytes.

This observation has provided an important new tool to address questions that were previously intractable. For example, it has allowed us to directly examine possible interindividual variation, and to ask whether factors such as age might affect recombination levels. The answers to each of these two questions have been somewhat surprising. First, given the well-known association between disturbances in meiotic recombination and nondisjunction at MI *(20)*, it might have been

expected that recombination levels would be homogeneous among males. In fact, several studies have now demonstrated that apparently normal males may average as few as 45 exchanges per cell, whereas others may average as many as 55 per cell (i.e., an approx 20% difference). Whether or not this results in differences in the levels of aneuploid sperm between low and high "recombinators" is not yet known. Similarly, because the level of meiotic nondisjunction increases modestly with paternal age *(21)*, an age-related reduction in recombination might have been expected. In fact, the available data from MLH1 studies provides little evidence for such an effect *(6,12,22)*.

This approach has also allowed us to characterize the distribution of exchanges on individual chromosomes. These analyses indicate that, except for the short arms of acrocentric chromosomes, each chromosome arm contains at least one MLH1 focus (crossover). The human male complement contains 17 non-acrocentric autosomes, 5 acrocentric autosomes, and the sex chromosome pair, implying a minimum of 40 exchanges per cell. The fact that, normally, between 45 and 50 exchanges are identified in pachytene spermatocytes indicates that cells typically contain 40 "mandatory" and between 5 and 10 "optional" exchanges.

This approach also provides an important tool to identify the underlying factors that control the number and location of exchanges in meiocytes. Initial immunocytogenetic studies have led to the identification of both chromosome-specific and genome-wide influences. For example, Sun et al. *(23)* recently demonstrated a *cis* effect, as they found that small synaptic defects (e.g., "splits" or "gaps") on individual SCs reduced the overall level of recombination on those chromosomes. Lynn et al. *(7)* identified an important genome-wide effect, finding that the overall level of recombination in a cell was proportional to the length of the SCs; that is, the SC appears to "measure" genetic length, with cells containing longer SCs having more MLH1 foci than cells with shorter SCs. This suggests that the SC may be responsible for setting the number of exchanges per cell, although it is also possible that it is simply reacting to upstream effectors. By analyzing the number and location of earlier-acting recombination proteins (e.g., SPO11, DMC1, RAD51, MSH4, MSH5), it should be possible to distinguish between these alternatives. Indeed, in an initial study of the temporal progression of meiosis in human males, Martin et al. *(24)* have used just such an approach in examining several different recombination-associated proteins.

Finally, this approach has allowed us to examine the temporal relationship between synapsis and recombination. For many years it was assumed that the SC is indispensable for recombination (i.e., that recombination occurs only in the context of the mature, tripartite SC).

However, studies of model organisms have provided conflicting results: in some (e.g., yeast and mouse) the recombination pathway is initiated before the establishment of the mature SC, whereas in others (e.g., *Drosophila*) synapsis follows recombination (for review, *see* ref. *12*). Initial observations from the human male indicate that humans follow the yeast paradigm, because recombination proteins such as RAD51 are present in leptotene, long before the mature SC is evident *(5)*.

3. IMMUNOCYTOGENETICS AND THE ETIOLOGY OF MALE INFERTILITY

In the preceding discussion, the observations were based on analyses of meiosis in "control" males (i.e., individuals attending infertility clinics because of OA). However, several groups have now applied immunocytogenetic methodology to study individuals with nonobstructive azoospermia (NOA). In general, these investigations have addressed two major questions: What portion, if any, of cases of NOA are attributable to meiotic arrest in germ cell development? In cases of NOA in which cells proceed through meiosis, is there an increase in errors of synapsis and/or recombination?

Preliminary data relating to these questions are provided in Table 1, which summarizes four recent immunocytogenetic studies of MI in NOA individuals *(6,14,25)*. Most importantly, the studies suggest that abnormalities leading to meiotic arrest are an important contributor to NOA of unexplained origin. Combining the data from the four studies, it appears that approx 10% of all NOA is associated with meiotic arrest phenotypes. Further, if cases without any obvious germ cells (i.e., case with only Sertoli cells evident) are excluded, the portion of cases resulting from meiotic arrest increases to nearly 20% of NOA. Thus, in instances in which testicular biopsies are obtained for diagnostic purposes, immunostaining may actually be as informative as other standard diagnostic assays (e.g., karyotyping).

Virtually no information is yet available on the underlying causes of these meiotic arrest phenoypes, but preliminary data from our laboratory suggest that there are likely to be multiple reasons. Specifically, in an initial analysis of three cases (Table 2; ref. *19*), we found subtle differences among individuals in the "end-stage" germ cells that were present in the seminiferous tubules. Specifically, although spermatocytes in all three cases appeared to progress normally to zygotene, one had fully formed axial elements but there was no evidence for synapsis, one had partially synapsed SCs but no evidence for MLH1 localization, and one had partially synapsed SCs in the presence of MLH1 foci (a representative

Table 1
Summary of Results of Immunocytogenetic Studies of Individuals with Nonobstructive Azoospermia

Reference	*Total no. of patients*	*No. of patients with Sertoli cells only*	*No. of patients with meiotic arrest*	*No. of patients with post-pachytene cells*	*In patients with post-pachytene cells*	
					Decreased no. of MLH1 foci?	*Increase in synaptic defects?*
25[a]	18[a]	8 (44%)	1 (6%)	9 (50%)	Yes	Yes
6	40	21 (53%)	4 (10%)	15 (37%)	Yes	Yes
14[b]	12	0 (0%)	0 (0%)	12 (100%)	No	No
19	26	9 (35%)	3 (12%)	14 (54%)	No	No

[a]Eight of these 18 individuals are also represented in the data set of ref. *6*.

[b]Study population included one normospermic individual and seven individuals with either asthenoteratospermia or asthenospermia in addition to four individuals with nonobstructive azoospermia.

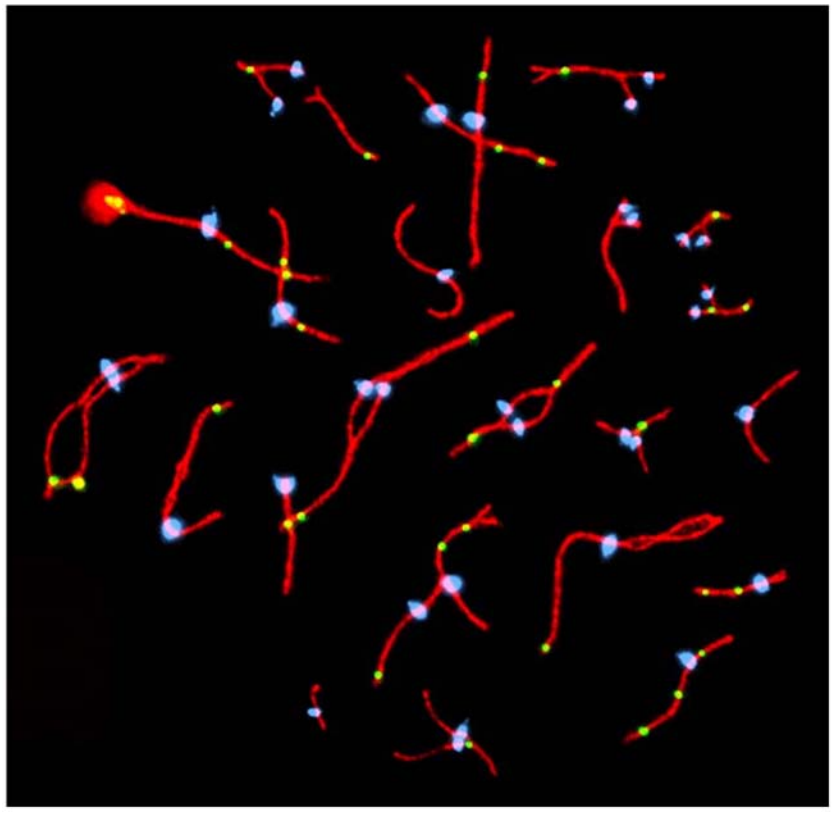

Fig. 4. Representative "end-stage" germ cell from an individual with a meiotic arrest phenotype. Note the presence of MLH1 foci (in yellow), despite the presence of multiple partially synapsed synaptonemal complexes. Synaptonemal complexes are detected by SCP3 (in red) and centromeric regions by CREST (in blue).

"end-stage" cell from this case is provided in Fig. 4). Clearly, in future analyses cases of meiotic arrest, it will be important to carefully examine the phenotypes of these "end-stage" cells, because this will be a crucial first step in the identification of the responsible molecular lesions.

The second question—whether there are disturbances in synapsis and/or recombination in individuals with NOA—also has important clinical ramifications. That is, abnormalities in either process will likely increase the rate of aneuploid sperm; thus, it is important to know whether there is an increase in such abnormalities in some, many, or all individuals with NOA in whom testicular sperm are evident. Unfortunately, the present data set is equivocal (Table 1). Two of the four studies reported increases in synaptic defects and decreases in recombination, whereas the other two failed to find an effect. Clearly, additional analyses of a more extensive series of cases will be needed to address this question.

Table 2
Variation in Meiotic Arrest Phenotypes in Three Individuals With Nonobstructive Azoospermia

Phenotype	*Patient 1*	*Patient 2*	*Patient 3*
Presence of early markers of recombination	√	√	√
Presence of full-length axial elements	√	√	√
Presence of partially synapsed SCs	—	√	√
Presence of MLH1 foci	—	—	√
Presence of fully synapsed SCs	—	—	—

4. CONCLUSION

From the preceding discussion, it is obvious that immunocytogenetic analysis of human spermatogenesis is in its infancy. Nevertheless, the initial results have already have been instructive. In studies of normal males, they have provided us with a general set of "rules" for synapsis of homologs; they have allowed us examine the distribution of crossovers in individual spermatocytes and the extent of variation in recombination among individuals; and they have provided an approach to examine factors that may influence either synapsis or recombination. Further, in cases of abnormal spermatogenesis, immunocytogenetic methodology has provided a new approach to the study of unexplained infertility. The initial results on this front are also encouraging, suggesting that immunocytogenetics may uncover the reason for infertility in as many as 10% of all cases of NOA. Clearly, there are limitations to immunocytogenetic analysis of spermatogenesis: most importantly, the approach requires acquisition of testicular tissue, material that is not easily obtained. Nevertheless, the initial results suggest that even small data sets will produce large advances in our understanding of both normal and abnormal human spermatogenesis.

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8 The Clinical Relevance of Sperm Aneuploidy

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Summary

The relevance and predictive capabilities of sperm aneuploidy frequencies have been assessed in normal men, men with a constitutional chromosomal abnormality, and infertile men with a normal karyotype. Sperm aneuploidy assessment by fluorescence *in situ* hybridization analysis (FISH) appears to be valuable and reliable. A number of studies have demonstrated that increased frequencies of aneuploidy in human sperm are mirrored by similar frequencies in embryos, fetuses, and newborns. It has been suggested that sperm aneuploidy should be employed as a routine screen before intracytoplasmic sperm injection treatment. This may be most valuable for the groups at highest risk, namely patients with Klinefelter syndrome; structural chromosomal abnormalities; macrocephalic, multinucleated, and multiflagellate sperm; and men with nonobstructive azoospermia. FISH analysis of sperm in these men should aid in counseling and decision making by the couples.

Key Words: Aneuploidy; meiosis; recombination; FISH; translocation.

1. INTRODUCTION

The analysis of sperm aneuploidy has been possible for decades, but many people wonder about the relevance of sperm aneuploidy frequencies and how predictive they are of aneuploidy in a conceptus or child. This chapter discusses the relevance of sperm aneuploidy in normal men, men with constitutional chromosomal abnormalities, and infertile men, and how it relates to risks of chromosomally abnormal children.

2. NORMAL MEN

The study of chromosome abnormalities in human sperm first became possible in 1978 with the advent of the human sperm–hamster oocyte fusion system *(1)*. Laboratories in Canada *(1,2)*, the United States *(3)*, Japan *(4)*, and Spain *(5)* provided strikingly consistent estimates of 1–2%

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aneuploid sperm in normal control donors. We were interested in using this technique to study the distribution of chromosome abnormalities to provide clues about the etiology of nondisjunction. All chromosomes were clearly susceptible to nondisjunction, but we determined that the G group chromosomes (chromosomes 21 and 22) and the sex chromosomes had a significantly elevated frequency of aneuploidy compared with other chromosomes *(6)*. The increased susceptibility of these chromosomes is probably related to the fact that they generally only have one crossover and a lack of recombination is clearly linked to nondisjunction in humans *(7,8)*. Human sperm karyotyping, using the hamster system, provides detailed information about each individual chromosome, permitting analysis of both numerical and structural abnormalities. However, there are significant disadvantages to this approach: sperm must be capable of fertilizing hamster oocytes; the technique is very difficult, time-consuming, and expensive; and the data yield is small. In fact, only 12 laboratories worldwide have had success with this technique despite many efforts.

Fluorescence *in situ* hybridization (FISH) analysis with chromosome-specific DNA probes was developed in the 1990s, providing a faster, cheaper, easier alternative for detecting aneuploidy in human sperm *(9–11)*. Also, sperm hampered by abnormalities in motility or other aspects of fertilization can be assessed using FISH analysis *(12–14)*. This is a simple technique that has been embraced by many laboratories, but it must be remembered that it is indirect: fluorescent signals, rather than chromosomes, are counted (*see* Fig. 1). We see considerable variability in aneuploidy frequencies for individual chromosomes among different studies. For example, for chromosome 21, the lowest disomy (two copies of chromosome 21) frequency has been reported as 0.05% *(15)* and the highest as 0.95% *(16)*. It is unlikely that these differences arise only from interindividual variation in disomy. Rather, different approaches in the experimental design, different probes, and scoring criteria used are regarded as being responsible (for a review, *see* ref. *17*). This makes it essential for each study to have controls analyzed in the same laboratory.

In a composite analysis of the distribution of disomy frequencies in studies on normal men, the mean disomy frequency for autosomes was 0.15 and 0.26% for sex chromosomes *(18)*. Our laboratory has determined that most autosomes have a similar frequency of nondisjunction, but chromosome 21 and the sex chromosomes have a significantly increased frequency *(19,20)*. These results corroborate our earlier studies in human sperm karyotypes *(6)*. This has also been confirmed by other groups *(21,22)*.

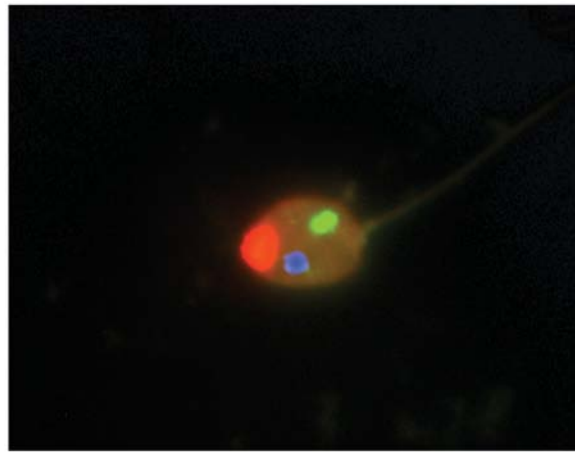


Fig. 1. Abnormal 24,XY sperm, with one red Yq12 signal and one centromere-specific green X signal. The presence of the single blue satellite III chromosome 1 signal, an internal control, indicates that this sperm is disomic rather than diploid.

3. MEN WITH CONSTITUTIONAL CHROMOSOME ABNORMALITIES

Constitutional chromosome abnormalities are relatively common in humans. These can be numerical chromosome abnormalities, such as men with an extra chromosome or structural abnormalities, such as translocations. Many of these abnormalities are associated with infertility and an increased risk of pregnancy loss. The frequency of constitutional chromosomal abnormalities in infertile men varies between 2 and 14%, depending on the severity of the infertility and the nature of the pathology *(23)*. Constitutional abnormalities have been studied using the hamster oocytes system, which provides precision in the human sperm karyotypes *(24,25)*. In the past decade, FISH analysis has been utilized with centromeric and telomeric probes to determine chromosome segregations and take advantage of the larger sample sizes *(26,27)*.

4. SEX CHROMOSOMAL ABNORMALITIES

Men with a 47,XYY karyotype generally produce normal children but there has been no systematic study of children born to those men. Theoretically, 50% of the sperm cells should be abnormal. In a study of 75 sperm karyotypes from a 47,XYY male, we found no sperm disomic for a sex chromosome *(28)*. Our results supported the hypothesis that the extra sex chromosome is eliminated during spermatogenesis.

FISH analysis on the same male with 10,000 sperm studied demonstrated a small but significant increase for XY disomy to 0.6% *(29)*. Similar FISH studies by other laboratories have demonstrated increased frequencies of sperm aneuploidy for the sex chromosomes ranging from 0.3 to 15% (refs. *30* and *31*; *see* ref. *32* for a review). However, when only the stringent three-color FISH studies are assessed, the frequency of 24,YY or 24,XY sperm was 1% or lower *(32)*. Thus, analysis of sperm chromosomal aneuploidy has largely been reassuring for these men.

Men with Klinefelter syndrome (47,XXY) or mosaic variants (e.g., 47,XXY/46,XY) generally have severe oligozoospermia or azoospermia and sperm can sometimes be obtained by a testicular biopsy. FISH analysis has demonstrated that the frequency of aneuploidy for the sex chromosomes varies from 1.5 to 7% *(33, 34)* in sperm from Klinefelter mosaics and 2 to 25% *(35, 36)* in the sperm of men who appear to have a nonmosaic 47,XXY karyotype (for a review, *see* ref. *23*). Chromosomally normal offspring as well as conceptions with a 47,XXY karyotype have been reported in Klinefelter syndrome males who have fathered a pregnancy through intracytoplasmic sperm injection (ICSI; ref. *23*). From both mouse *(37)* and human studies *(38)*, it appears likely that the extra sex chromosome is generally eliminated during meiosis (as in 47,XYY) but the abnormal testicular environment still leads to an increased frequency of aneuploidy sperm and embryos. Staessen et al. *(39)* have reported on ICSI and preimplantation genetic diagnosis (PGD) performed in 32 cycles of 20 couples with spermatozoa from nonmosaic Klinefelter patients. They found a significantly higher frequency of both sex chromosomal (13.2%) and autosomal (15.6%) abnormalities compared with a control group adjusted for age (3.1 and 5.2%). The three- to fourfold increase in chromosomal abnormalities in embryos is consistent with some of the sperm chromosome studies in these men, but there seems to be considerable variability in the frequency of sperm chromosome abnormalities in men with Klinefelter syndrome.

5. TRANSLOCATIONS

Robertsonian translocation carriers have a fusion of the long arms of two acrocentric chromosomes resulting in a balanced state with 45 chromosomes. Pairing of the chromosomes at meiosis can lead to chromosomally balanced and unbalanced gametes. Sperm karyotyping studies have demonstrated that 3 to 27% of the spermatozoa are unbalanced *(40)*. FISH studies in 23 Robertsonian translocation heterozygotes

have shown similar frequencies of imbalance varying from 7 to 36% *(41,42)*.

Reciprocal translocations occur when there are exchanges of chromosome material between any chromosomes. During meiosis, four chromosomes must pair in reciprocal translocation heterozygotes and the resulting segregations have a higher frequency of unbalanced chromosomes than Robertsonian translocations. Sperm karyotyping studies of more than 30 reciprocal translocation heterozygotes have shown that 19–77% of spermatozoa are unbalanced *(24)*. FISH analyses of chromosome segregations in more than 30 carriers have reported frequencies of unbalanced chromosomes ranging from 37% *(43)* to 79% *(27)*. In one study, four male family members of a kindred segregating a chromosome 15;17 translocation were studied by FISH analysis *(44)*. The segregation patterns were very similar in all four men, with approx 50% of sperm chromosomally unbalanced. Also, Morel et al. *(43)* found similar frequencies of imbalance of 37 and 43% in two brothers heterozygous for a chromosome 7;8 translocation. These studies demonstrate that the risk of meiotic imbalance is primarily determined by the characteristics of the chromosomes involved and the breakpoint positions. They also demonstrate the reproducibility of the method. Because the frequency of chromosome abnormality is very high, some men carrying reciprocal translocations have undergone PGD to implant only chromosomally normal or balanced embryos. Studies comparing the frequency of chromosome abnormalities in sperm and embryos from reciprocal translocation carriers show a very close agreement in the abnormality frequencies *(45)*.

6. INVERSIONS

Inversion occurs when there are two chromosome breaks in the same chromosome and the segment heals in an inverted manner. During meiosis, the inverted chromosome may pair with a normal chromosome by an inversion loop. If an uneven number of crossovers occur in this loop, half of the gametes will be chromosomally unbalanced. There have been seven inversion heterozygotes studied by the sperm karyotyping technique with frequencies of chromosome imbalance varying from 0 to 31% *(46,47)*. There have been six studies of inversion heterozygotes by FISH analysis with 1 to 54% unbalanced sperm *(48,49)*. Thus, there is a considerable variation in the frequency of abnormal sperm produced by inversions and sperm aneuploidy testing is valuable to determine which inversion carriers have a significant risk.

7. INFERTILE MEN WITH A 46,XY KARYOTYPE

It is clear from the studies discussed earlier that men with a constitutional chromosomal abnormality have an increased risk for sperm chromosomal anomalies. However, it has also been determined that infertile men with a normal somatic karyotype produce sperm with an increased frequency of chromosome abnormalities. There have been more than 30 FISH studies of sperm chromosome abnormalities in 46,XY infertile men and the great majority have demonstrated a significantly increased frequency of aneuploidy for the autosomes and particularly for the sex chromosomes (for a review, *see* ref. *23*). Most studies have reported the increase of sperm chromosome abnormalities in infertile men to be about three times higher than control donors *(50–52)*. Reports based on prenatal diagnosis *(53)* of ICSI pregnancies and newborns *(54)* have indicated the risk of *de-novo* chromosome abnormalities to be approx 2 to 3%, which is threefold higher than normal pregnancies. Thus, the increased frequency of chromosome abnormalities in ICSI pregnancies and newborns mirrors the increased frequency observed in sperm of the infertile ICSI patients. Furthermore, studies have indicated that these chromosome abnormalities are of paternal origin *(55)*, underscoring the fact that chromosomally abnormal sperm in ICSI patients become chromosomally abnormal fetuses and children. We have found that this increased frequency of sperm chromosome abnormalities in 46,XY infertile men is observed for all types of abnormal semen profiles: oligozoospermia *(56)*, teratozoospermia *(57)*, aesthenozoospermia *(13)*, and azoospermia *(58)*. Many of these men have approximately three times the risk observed in control donors, although a number of studies suggest that the risk is higher for azoospermia with sperm retrieved from a testicular biopsy *(59,60)*. One important exception to this rule is men with a high percentage of macrocephalic, multinucleated, and multiflagellate sperm. A number of studies have reported very high frequencies of aneuploidy and polyploidy sperm in these men (50–100%; refs. *61–64*).

8. SPERM SELECTION BASED ON CHROMOSOMAL CONTENT

To many people, it seems intuitive that sperm selection must exist. After all, with millions of sperm to choose from, surely the best (i.e., most chromosomally normal) should have an advantage in developing into a mature sperm and fertilizing an oocyte. However, there is very little

evidence that this is the case. In fact, the evidence against sperm selection is quite compelling. Most striking is the evidence from constitutional chromosomal abnormalities, such as translocations. Reciprocal translocations lead to a very high frequency of chromosome imbalance, with an average of 50% unbalanced sperm *(24,65)*. These can often be very abnormal after adjacent 2 or 3:1 segregation with the equivalent of an extra chromosome. The fact that we find such a high frequency of chromosome abnormalities in sperm argues against selection during spermatogenesis. Also, the theoretical expectations of the various segregations are generally observed *(43,66)*; for example, an equal frequency of balanced and normal chromosomes are seen in sperm after alternate segregation and similarly an equal frequency of the two outcomes expected from adjacent 1 segregation are observed, although some clearly have more chromosomal imbalance. Oliver-Bonet et al. studied human translocations at different spermatogenic stages (metaphase I, II, and mature sperm) and found no evidence of selection based on chromosomal content *(67)*. Furthermore, PGD studies have demonstrated the same frequency of aneuploidy in early embryos as in sperm, demonstrating that fertilization is not a barrier to chromosomally unbalanced sperm *(45)*. The selection occurs after this stage because the chromosome imbalance interferes with the normal embryological development so that by the time of the prenatal diagnosis at approx 16 wk gestation, only 11% of fetuses from male translocation carriers are chromosomally unbalanced *(68)*. Most studies in mice *(69,70)* and hamsters *(71)* have also shown no evidence for sperm selection.

9. RETROSPECTIVE STUDIES RELATING ANEUPLOIDY IN SPERM AND OFFSPRING

A number of studies have analyzed populations of paternally derived aneuploid children and then studied the fathers to determine if sperm aneuploidy was significantly elevated. Blanco et al. studied a population with a high prevalence of Down syndrome *(21)*. They determined that the two cases of paternally derived trisomy 21 had fathers with significantly increased disomy 21 sperm. Further studies showed that these men had generalized tendencies to meiotic nondisjunction, because other chromosomes had elevated aneuploidy frequencies *(72)*. In a similar study, however, Hixon et al. did not find a significantly elevated frequency of disomy 21 sperm in cases of Down syndrome of paternal origin *(73)*. Martinez-Pasarell et al. *(74,75)* found a significantly increased frequency of sex chromosomal aneuploidy in sperm from fathers of paternally derived Turner syndrome patients and Tang

et al. *(76)* found an exceedingly high frequency of sex chromosomal nullisomy (19.6%) and XY disomy (18.6%) in the infertile father of a 45,X abortus, shown to be caused by a lack of paternal X chromosome. Eskenazi et al. *(77)* studied children with Klinefelter syndrome and found that paternally derived cases had higher frequencies of XY sperm than maternally derived cases. Thus, the majority of studies show an elevated frequency of chromosomally abnormal sperm in fathers of paternally derived aneuploid offspring.

10. PROSPECTIVE STUDIES RELATING ANEUPLOIDY IN SPERM AND OFFSPRING

In the first publication of an increased frequency of sperm chromosome abnormalities in infertile men with a normal karyotype, we reported on one male who had a frequency of 24,XY sperm that was ninefold higher than controls *(46)*. This man subsequently had ICSI and fathered a pregnancy that resulted in a 47,XXY fetus *(78)*. Nagvenkar et al. *(79)* studied men with severe oligozoospermia and normal fertility and related the sperm aneuploidy frequency to the outcome after ICSI. They found a significantly higher frequency of sex chromosomal disomy in the severe oligozoospermia group and this correlated with a lower pregnancy and live birth rate. Petit et al. *(80)* studied sperm aneuploidy in three groups of men with different ICSI outcomes: group A had at least four ICSI treatments without a pregnancy, group B had a pregnancy after one to three ICSI attempts, and group C consisted of a fertile control group. FISH analysis for chromosomes 8, 9, 13, 18, 21, X and Y demonstrated a higher aneuploidy frequency in group A compared with group B, and group B compared with group C. They suggested that sperm aneuploidy could be used as a predictive test before ICSI to improve genetic counseling for patients.

Gianaroli et al. *(81)* studied sperm aneuploidy and correlated it to results in blastomeres after PGD in couples in which the female partner was younger than 36 yr old. They found a higher incidence of monosomies and trisomies in embryos from microepididymal sperm aspiration and testicular sperm extraction sperm and aneuploidy for the sex chromosomes increased proportionally to the severity of the male-factor condition. These authors suggested that it is important to include sperm FISH analysis in preliminary tests given to infertile couples, especially in the case of repeated in vitro fertilization failures. These preliminary studies suggest a correlation between sperm aneuploidy frequencies and ICSI outcome.

10.1. Should Sperm Aneuploidy Testing Be Recommended for Infertile Men With a 46,XY Karyotype?

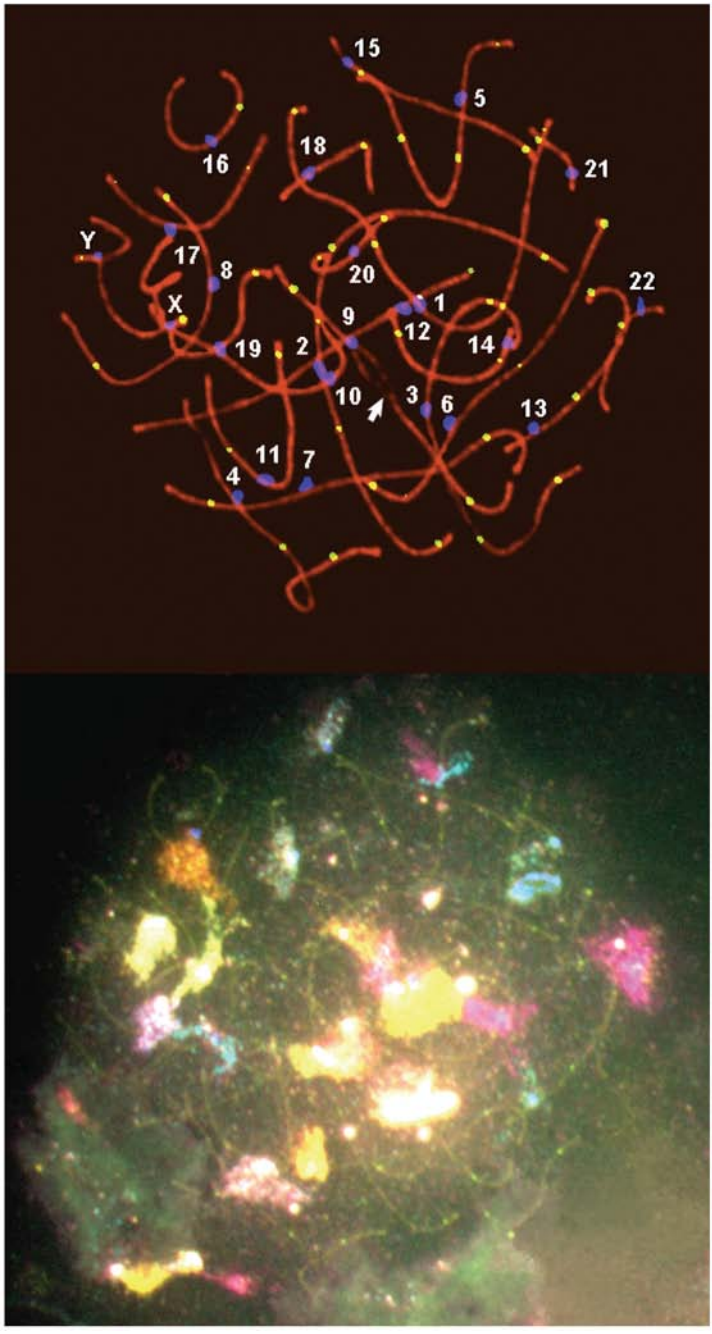
After a decade of analysis, it is clear that infertile men with a normal somatic karyotype have an increased risk of sperm aneuploidy. The magnitude of this risk has varied from 2- to 10-fold that of control donors. However, most studies have shown an increase of approximately threefold *(46–48)*, which mirrors the risk observed in newborns after ICSI. Is this enough of a risk to consider advocating sperm aneuploidy testing for all male-factor infertility patients? It is certainly important that all patients be informed of the risk but it is unlikely that patients would be dissuaded from treatment as a result. Sperm aneuploidy testing might be worthwhile if it uncovered some men with a very high frequency of sperm aneuploidy. However, these men appear to be relatively rare. In my laboratory, there were 6 of 66 infertile patients (with a normal karyotype) who had an XY disomy frequency that was at least fivefold greater than controls and 2 of 66 men with frequencies more than 10-fold higher (unpublished results). Thus, the majority of men have moderately increased risk. Although a number of studies have advocated routine sperm aneuploidy testing in infertile men with a normal karyotype, it is unlikely that this would uncover many men with a substantially increased risk of aneuploid children.

11. IS TESTING RECOMMENDED FOR MEN WITH NONOBSTRUCTIVE AZOOSPERMIA?

We have demonstrated that men with nonobstructive azoospermia have severe meiotic abnormalities including chromosome pairing and synapsis defects and a significant decrease in the frequency of recombination *(82–84)*. Because chromosome pairing and recombination is intimately linked with the normal segregation of chromosomes *(7,8)*, these defects may be responsible for the increased frequency of aneuploidy observed in testicular sperm from these men *(55–57)*. It is difficult to study sperm aneuploidy in men with nonobstructive azoospermia because, generally, such a small number of sperm are retrieved. However, when it is possible, it is certainly worthwhile. The new immunocytogenetic techniques, which allow analysis of chromosome synapsis and recombination, are also very valuable (*see* Fig. 2); presently, they are only available on a research basis in a few laboratories worldwide.

12. CONCLUSION

FISH analysis of sperm aneuploidy is clearly a valuable and reliable technique. A number of studies have shown that increased frequencies of aneuploidy in human sperm are mirrored by similar increases in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early embryos, fetuses, newborns, and children. As a result, there have been suggestions that sperm aneuploidy should be employed routinely for all infertile men before ICSI treatment. Because most infertile patients are likely to have only a moderate increase in sperm aneuploidy, it may be effective to target those groups at highest risk:

1. Patients with Klinefelter syndrome.
2. Patients with structural chromosomal abnormalities, such as translocations and inversions.
3. Men with a high proportion of macrocephalic, multinucleated, and multiflagellate sperm.
4. Men with nonobstructive azoospermia, when enough sperm are retrieved from the testicular biopsy.

Patients in these four groups have quite variable frequencies of sperm aneuploidy and thus FISH analysis of sperm could aid in counseling and decision making. The couple might then be reassured by a low risk or, in the event of a high risk, decide against ICSI treatment or proceed with ICSI combined with PGD.

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Fig. 2. *(Opposite page)* (Upper) Pachytene spermatocyte from a nonobstructive azoospermic patient, with synaptonemal complexes (SCs) shown in red, centromeres in blue, and MLH1 (recombination) foci in yellow. Note that SC 9 has an unpaired chromosome region (arrow). (Lower) Subsequent centromeric multicolor fluorescence in situ hybridization analysis allows identification of individual chromosomes so that MLH1 foci can be analyzed for each SC.

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9 DNA Repair Genes and Genomic Instability in Severe Male Factor Infertility

Francesca K. E. Gordon, BS and Dolores J. Lamb, PhD

Summary

The maintenance of genomic integrity is of key importance for gametogenesis. Nevertheless, the processes of DNA replication, mitosis, and meiosis are surprisingly error-prone and subject to damage. Accordingly, a series of DNA repair mechanisms have evolved that recognize and repair DNA damage and DNA replication errors to maintain the fidelity of the DNA sequence. Gradually translating findings from targeted gene deletion and mutant mouse models to human male infertility, we have learned that the processes of mitosis and meiosis require proper functioning of the entire DNA repair mechanism in the cell for normal fertility to be present in the male. This chapter focuses on our current understanding of the processes required for the maintenance of DNA integrity during spermatogensis.

Key Words: DNA repair; meiosis; synaptonemal complex; SPO 11; spermatogenesis.

1. INTRODUCTION

Despite the relatively common incidence of male infertility, relatively little is known about the etiology. From a therapeutic perspective, spermatogenic defects are among the most severe of these impairments, presenting as severe oligozoospermia (<1 million sperm per milliliter of ejaculate) and nonobstructive azoospermia (ejaculate with no sperm in the absence of genital tract obstruction).

Nonobstructive azoospermia may result from genetic and environmental factors. Exposure to toxins, legal or illegal drugs, and trauma to the testes can reduce or ablate sperm count and/or viability, either temporarily or permanently *(1–3)*. Numerical and structural sex chromosomal

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anomalies are associated with male infertility. Men with Klinefelter's syndrome (47,XXY), the most common cause of nonobstructive azoospermia, are azoospermic as a result of hypogonadism and chromosomal disjunction during meiosis *(4)*. Individuals with more subtle defects, like Y-chromosome microdeletions, may not present with phenotypic abnormalities, aside from spermatogenic defects that vary in severity with the location of the microdeletion *(5,6)*. Gross autosomal rearrangements, such as reciprocal translocations or unbalanced translocations, are associated with azoospermia as a result of chromosomal disjunction during meiosis I *(7,8)*.

A diverse array of genetic disorders cause infertility in the male *(9)*. Targeted gene deletion in mouse models defined key pathways required for normal male fertility, although translation of these findings to the clinic is slow. Fertility genes affecting the somatic cells of the testis include agents such as growth factors and their receptors, genes involved in hormone biosynthesis, metabolism and action, cell adhesion and tight junctions, and signal transduction pathways. Spermatogonia also require proper growth factor and receptor function, as well as expression of genes involved in the regulation of stem cells, mitosis, and apoptosis. Spermatocytes have entered the meiotic pathway and, not surprisingly, expression of proteins required for chromosome pairing and synapsis, homologous recombination, genomic integrity, and DNA replication and repair are necessary. Other genes affect spermatid differentiation include those required for cell remodeling, chromatin packaging, nuclear condensation, and spermiation. This chapter focuses on the contribution of proteins required for mitosis and meiosis during spermatogenesis, in particular those involved in homologous recombination and DNA repair, and the consequence(s) of mutations in these genes for the successful completion of this process and fertility.

An understanding of spermatogenesis is necessary to appreciate the importance of DNA repair pathway proteins in male germ cell development. Spermatogenesis requires primordial germ cell proliferation and later, the presence of spermatogonial stem cells in the testis, as well as the highly proliferative type A spermatogonia, a few of which differentiate to become type B spermatogonial—the cells destined to enter the meiotic pathway. Meiosis occurs during the spermatocyte stage and involves two divisions that ultimately produce a haploid spermatid that in turn undergoes extensive differentiation and morphology changes to become a mature spermatozoon. Thus, genes controlling genetic fidelity are of importance in both mitosis and meiosis during spermatogenesis.

Meiosis I differs from meiosis II and mitosis because it involves a reduction in the number of chromosomes per cell and an exchange of

genetic material between homologous chromosomes or homologous recombination. This provides the basis for the continual mixing of the gene pool and evolution of the species (Fig. 1).

This exchange of genetic material occurs during prophase I and requires proper functioning of the mismatch repair proteins as demonstrated by work in humans, mice, and *Saccharomyces cerevisiae* *(10–13)*. Prophase I is further subdivided into leptotene, zygotene, pachytene, diplotene, and diakinesis (Fig. 2A). Chromosomes thicken and homologs begin to synapse during leptotene. Recombination is also initiated at this stage by formation of double-stranded breaks (DSBs; refs. *14* and *15*). Pairing of homologous chromosomes to facilitate recombination is stabilized by synaptonemal complex (SC) formation, which is complete during zygotene *(16–18)*.

A host of factors expressed during this phase of prophase I coordinates processing of DSBs into single-end invasions (SEIs) and then into holliday junctions (HJs; Fig. 1; refs. *16–21*). Resolution of HJs occurs during the pachytene stage of spermatogenesis *(11,17,19,22–25)*, completing exchange of genetic material between homologs. The SC then dissolves during diplotene, causing the chromosomes to separate where crossover has not occurred *(15)*. Further condensation occurs during diakinesis, making the tetrads clearly visible. As in mitotic metaphase, chromosomes line up on the metaphase plate. However, sister chromatids attach to the same pole rather than different poles as they do in mitotic metaphase. One copy of each chromosome moves to opposite poles of the primary spermatocyte during anaphase I. Like the events during mitosis, the nuclear envelope reforms and gradually generates two separate cells during telophase I. The only difference is that during meiosis the two daughter cells are haploid instead of diploid.

Gene expression is required for each step of this process and the following discussion provides a brief overview of some of the key factors important for progression through meiotic prophase I.

2. DEFECTIVE MITOSIS BECAUSE OF DNA REPAIR DEFECTS AND GERM CELL LOSS LEADING TO MALE INFERTILITY

2.1. Defective Recombination and Male Infertility

Studies in a number of model organisms define a series of meiotic checkpoints that arrest spermatogenesis when defective recombination is present, leading to male infertility. Men with maturation arrest pathology on testis biopsy show histology suggestive of this type of

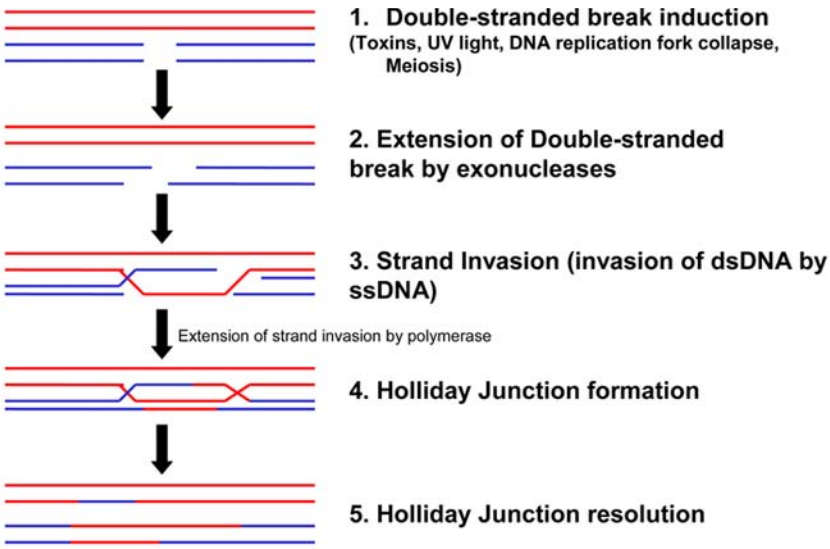

Fig. 1. A generalized model of homologous recombination. A double-stranded break is induced by UV light, irradiation, free-radicals, toxins or factors expressed in the early stages of Meiosis I. This promotes exchange of genetic material between homologous chromosomes (1). Exonucleases together with other factors create regions of single-stranded extension of the break on one homolog. Proteins which bind to this piece of single-stranded DNA catalyze its invasion of the double-stranded homolog generating an intermediate single-end invasion structure (2). These single-stranded regions invade the intact double-stranded homologous chromosome (3). DNA Polymerase extends the site of this invasion. Mismatch repair proteins and associated factors bind to the single-end invasion structure and promotes both conversion of this structure into a Holliday Junction (4) and resolution of the Holliday Junction (5). Resolution of the Holliday junction into two separate double-stranded homologs completes the process of homologous recombination.

defect. This led Reijo-Pera, Martin, and colleagues to examine the frequency and location of recombination events in men with maturation arrest compared with those with histologically normal spermatogenesis *(26,27)*. Nearly half of the infertile patients examined showed measurable defects in recombination during spermatogenesis, suggesting that men with arrest at the zygotene stage of prophase in meiosis I have a phenotype similar to that seen in mice with mutations in recombination genes, as described later. For these men, genetic checkpoints stop spermatogenesis when faulty meiotic recombination is present and eliminate the defective cells through apoptosis *(27)*. Accordingly, it is important to translate the findings in the animal models to human infertility to define this etiology, because patients with nonobstructive azoospermia are candidates for treatment with an assisted reproductive technology.

2.1.1. Initiation of Events Required for Meiotic Recombination: SPO11 and Related Proteins

SPO11 is a topoisomerase variant required for the initiation of homologous recombination. Human and mouse SPO11 are about 80% homologous, but are quite different from other eukaryotic homologs. In both yeast and mammals, DSB initiation during leptotene requires SPO11, as demonstrated by recombination and synapsis defects in both organisms when expression of this gene is ablated *(15)*. In mice, disruption of *Spo11* results in defective meiosis and in both males and females *(28,29)*. DSB breaks do not form. Homologous recombination synapsis defects are present in the *Spo11–/–* mice resulting in apoptosis during early spermatocyte prophase. Interestingly, disruption of *Atm (30,31)*, *Dmc1 (32,33)*, *Mei1 (34,35)*, and *Morc (36)* result in an altered SPO11 localization and/or expression suggesting that SPO11 may have additional roles in synapsis. Not surprisingly, targeted deletion of these genes in other mouse models result in similar spermatogenic deficiencies. Likewise, the *Mei1* mutant mouse shows similarities to the *Spo11*-deficient mice, suggesting that it may work together with Spo11 in DSB formation.

SPO11 is then displaced from the DSB site by Mre11, which is abundantly expressed during early meiotic prophase *(37)*. Mutation of *Mre11* blocks meiotic recombination. This protein works in a multiprotein complex including RAD50, p200, p400, and NBS1, all of which are required for meiotic recombination. Other proteins (reviewed in ref. *15*) in complex with MRE11 generate 3′ overhangs from DSBs, providing substrates for factors like Rad51 involved in SEI formation *(13,15)*. Rad51 functions in recombination and DNA repair of DSBs that occur in meiosis, although the *Rad51* deletion is a preimplantation lethal mutation indicating a key role early in development.

2.1.2. SC Proteins

When the homologous chromosomes must pair and recombine, the chromosomes link through a supramolecular proteinacious structure (comprised of a complex aggregate of SYCP1, 2, and 3 proteins) known as the SC. SC formation facilitates recombination by stabilizing interaction between homologs and through interaction with proteins involved in recombination, such as Msh4-Msh5 and Rad 51 *(13,16,20)*.

Experimental models have used *S. cerevisiase* where SC formation is better understood than in mammals. These organisms are easier to genetically manipulate. In both yeast and mammals, this structure is comprised of lateral elements, central elements, and transverse elements that link the first two (Fig. 2; ref. *18*). The Zmm-family

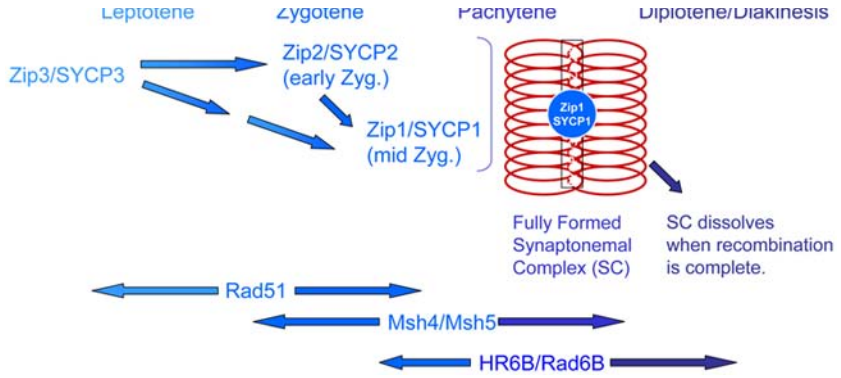

Fig. 2. Overview of synaptonemal complex formation in propase I. The synaptonmenal complex forms during Leptotene and Zygotene and both stabilizes pairing of homologous chromosomes and interacts with proteins such as Rad51, Msh4-Msh5, and HR6B/Rad6 to facilitate recombination. Zip3/SYCP3 expression begins during Leptotene. It promotes association of Zip2/SYCP2 with chromosomes during early Zygotene which drives Zip1/SYCP1 polymerization along the length of each pair of homologs during mid-Zygotene. SYCP3 is first expressed and associates with condensing chromatids during Leptotene. It directs interaction of SYCP2 with the lateral element of the homologous pair during early Zygotene. Both SYCP2 and SYCP3 on the lateral element of each homologous pair then mediate association of SYCP1 with the central element. The synaptonemal complex persists through Pachytene and dissolves in Diplotene/Diakinesis when recombination is complete and spermatocyte development progresses to Metaphase I.

proteins, Zip1, Zip2, and Zip3, are the main SC structural proteins in yeast. Zip3 directs association of Zip2 with chromosomes, promoting Zip1 polymerization along the length of the homolog and presumably at the center where it makes up the central element *(16,20)*. Zmm-family proteins interact physically and genetically with Mre11, Msh4, and Msh5, which are important for recombination, suggesting an additional role for these structural proteins in progression through prophase I *(20)*.

Mammalian core SC proteins SCP1, 2, and 3 have minimal structural homology but considerable secondary structure and functional homology with Zmm-family proteins. The lateral elements of the SC are formed by SCP2 and SCP3 and the central and transverse elements are composed of SCP1 polymers *(18)*. Spermatocytes in the *Sycp1*-null mice are arrested at pachytene, with a few cells reaching diplotene stage of meiosis. *Scp2* has not been deleted in mice, but it expressed during the meiotic prophase in the rat *(38)*. Targeted deletion of *Sycp3* results in male sterility because of apoptosis during meiotic prophase as a result synapsis failure *(39,40)*. The azoospermic phenotype of men

with mutant SYCP3, the human homolog of SCP3, because of failed synapsis during prophase I indicates that mammalian SCP proteins facilitate chromosomal synapsis and recombination in a similar way to Zmm-family proteins in yeast *(41)*.

FKBP6 is another protein that localizes to the regions of chromosome synapsis and deletion of this gene results in the absence of normal pachytene spermatocytes and evidence of abnormal chromosome pairing and misalignments between homologous chromosomes *(42)*. Deletion of exon 8 of *Fkbp6* is the basis of the aspermia mutation observed in rats *(42)*.

2.1.3. RAD51

Rad51 expression is initiated during leptotene and peaks during mid- to late-zygotene (Fig. 3; ref. *17*). Given this window of expression, it is not surprising that Rad51 interacts with factors important in synapsis and SEI formation. A physical interaction between Zip3 and Rad51 has been observed despite little co-localization of these factors *(16)*. Because SC formation ends in late zygotene, it is possible that little co-localization between Rad51 and Zip3 is observed because of differences of peak expression times of these factors. Alternatively, Rad51 and Zip3 co-localization may only be observed at sites of recombination. SEI formation occurs during zygotene, which coincides with peak expression of Rad51. Other studies demonstrate that Rad51 forms a filament on single-stranded DNA and catalyzes strand exchange with homologous double-stranded DNA *(20,21)*. The Msh4-Msh5 (Fig. 5) heterodimer may stabilize this process, consistent with similarities in spatial and temporal expression patterns between these factors *(13,17)*.

2.1.4. MSH4-MSH5 HETERODIMER

The meiosis-specific expression of the homologs of the *Escherichia coli* MutHLS system is of particular interest. Mammalian *Msh4* and *Msh5* are expressed exclusively in germ cells and have a high homology to these same factors in yeast at core functional regions: the DNA-binding helix-loop-helix motif and the adenosine triphosphatase region *(10,43–45)*. MSH4 is meiosis-specific but is not involved in mismatch correction. The role of this protein is to ensure reciprocal recombination and segregation at meiosis I. MLH3 is also present in spermatocytes and co-immunoprecipitates with MSH4.

Msh4 or *Msh5* knockout mice exhibit chromosomal pairing and form DSBs but cannot complete synapsis resulting in a failure of primary spermatocytes at the zygotene/pachytene checkpoint *(10,13)*. These results

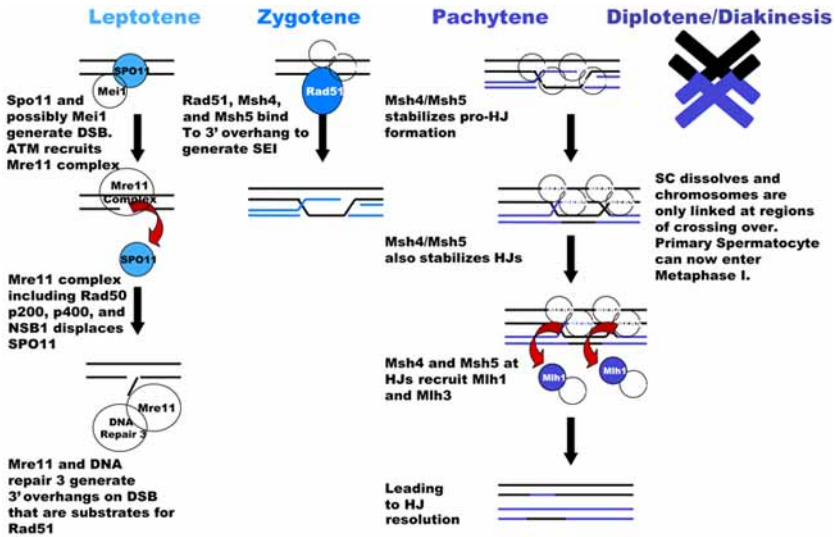

Fig. 3. Overview of homologous recombination during prophase I of meiosis I. Double-stranded breaks are induced by SPO11 and possibly Mei1. ATM is recruited to the double-stranded break where it recruits the Mre11 complex, displacing SPO11. The Mre11 complex generates the 3 single-stranded overhang that is the substrate for Rad51 which catalyzes formation of the single-end invasion intermediate along with Msh4 and Msh5. The Msh4-Msh5 heterodimer then mediates conversion of the single-end invasion intermediate to a proto-Holliday junction and then to a Holliday junction. It then recruits the Mlh1-Mlh3 heterodimer to the Holliday junction to mediate resolution of this structure. The Synatonemal complex then dissolves during Diplotene/Diakinesis leaving only the sites of crossover linked on homologous chromosomes. Once Holliday junctions are resolved and the synaptonemal complexes degrade, the germ cell is ready to transition to Metaphase I.

are consistent with other studies that define the function of Msh4-Msh5 in detail.

Msh4-Msh5 co-localize in vivo with Rad51 in mammals *(17)* and Rad51 and Zmm-family proteins in yeast *(10,16)*, indicating that Msh4-Msh5 is vital for the completion of synapsis that must take place before the transition into pachytene. Expression of Msh4 peaks during mid- to late-zygotene along with Rad51, suggesting that the Msh4-Msh5 heterocomplex acts in concert with Rad51 to promote SEI formation *(17)*. Msh4-Msh5 also seems to promote HJ formation through stabilization of an intermediate proto-HJ (pHJ) structure *(13,19)*. The Msh4-Msh5 heterocomplex binds with high affinity to a pHJ, triggering the exchange of adenosine 5′-diphosphate for adenosine 5′-triphosphate at the adenosine 5′-triphosphate-binding site, causing a sliding

clamp to bind to the pHJ. This binding event stabilizes the pHJ and facilitates extension of the 3′-end by DNA polymerase, generating two adjacent HJs, which promotes the loading of more clamps onto the structure *(19)*. Msh4-Msh5 heterodimers recruit Mlh1-Mlh3 heterodimers to resolve the HJ and complete recombination *(17,19)*.

2.1.5. MLH3 AND MLH1

Mlh3 encodes a DNA repair protein that interacts with MLH1. Together they play a role in the maintenance of genomic integrity. Studies suggest a functional redundancy of this protein with Pms1 and Pms2. Targeted deletion of *Mlh3* in mice results in an arrest at metaphase with apoptosis *(46)*. Primary spermatocytes of *Mlh3* knockout mice fail at metaphase because of misaggregation of chromosomes. Mlh1–/– germ cells, in contrast, fail at the end of prophase I, suggesting that Mlh3 binds to recombination sites and subsequently recruits Mlh1 *(11)*. The presence of an Mlh1-binding site on Mlh3 *(46)* and later work showing a delay in co-localization of Mlh1 and Mlh3 at Msh4-postive foci during pachytene confirms these findings (Fig. 3; refs. *17* and *22*). Mlh1 and Mlh3 localize to recombination nodules during pachytene and diplotene, where they facilitate recombination and chaismata separation. During the progression from diplotene to metaphase 1, the number of Mlh1-Mlh3 foci decreased to zero, suggesting that these proteins are involved in resolution of chiasmata and prophase I to metaphase I transition *(22)*.

Recent data indicating that Mlh1-Mlh3 heterodimers resolve HJs during mid- to late-pachytene is bolstered by findings from earlier studies and from results in humans *(11,12,22,24)*. Genetic analysis in yeast and co-immunoprecipitation with mammalian proteins demonstrate both a physical and genetic interaction between Msh4 and Mlh3 that is further substantiated by co-localization experiments in mammalian germ cells *(17,24)*.

Loss of *MLH1* expression in human males results in a phenotype of azoospermia similar to that observed in mice *(11,47)*. Another study found that men with nonobstructive azoospermia had a smaller number of Mlh1-positive foci and less primary spermatids at the pachytene stage of meiosis. There were also more gaps (discontinuities) and splits (unpaired chromosomes) in men with nonobstructive azoospermia than in normal controls or men with obstructive azoospermia *(12)*, suggesting that reduced Mlh1 expression adversely affects recombination and may promote germ cell failure. Progression of germ cells through meiosis I was also absent in individuals with a truncation in SYCP3, the human homolog of SCP3. The C-terminal region of

SYCP3 was shortened in these individuals, preventing association with other SYCP3 proteins and presumably ablating SC formation. Mismatch repair mutations are observed in men with nonobstructive azoospermia that match results obtained from mouse studies, indicating that these mutations could account for some previously inexplicable cases of azoospermia.

3. OTHER DNA REPAIR-RELATED PROTEINS AND MALE INFERTILITY

3.1. Retinoblastoma

Mutational inactivation of Rb1 causes alteration or ablation of Rb protein expression leading to development of retinoblastoma (RB), a highly malignant pediatric ocular cancer. Loss of functional Rb leads to tumorogenesis via loss of cell cycle control *(48,49)* or impairment of the DNA damage repair pathway *(50,51)*. O6-methylguanine-DNA methyltransferase (MGMT) is involved in the repair of DSBs induced by alkylating agents in vitro *(52)* and is important for the recognition of these lesions by mismatch repair proteins in vivo *(53,54)*. The MGMT promoter is hyper-methylated in some patients with RB, leading to reduced or absent MGMT expression and consequent reduction in DNA damage repair *(50)*.

Not surprisingly, patients with RB also display similar Mlh1 deficiencies, also resulting from Mlh1 promoter hyper-methylation *(51)*. Inactivation of Mlh1 in RB patients can lead to microsatellite instability as well as reduction in DNA damage repair, both of which promote cancer progression as demonstrated by findings of Mlh1 and Msh2 and other DNA damage repair pathway proteins such as Mlh3 in hereditary nonpolyposis colon cancer lineages *(51,55–57)*. Results from these studies demonstrate that the Rb protein interacts with mismatch repair pathway proteins during DNA damage repair in somatic cells.

3.2. The Ubiquitin–Proteasome Pathway

The ubiquitin–proteasome pathway is important for protein degradation *(58)* and other cellular processes including chromatin remodeling *(59,60)*. Ubiquitination of a protein substrate requires the action of three enzymes: a ubiquitin-activating enzyme (E1) that converts ubiquitin into a form in which it can bind, a ubiquitin-conjugating enzyme (E2) that brings the ubiquitin molecule to its target, and finally a ubiquitin ligase (E3) that catalyzes binding of ubiquitin with the substrate *(61)*. The testis is one of the regions of maximal ubiquitin–proteasome pathway activity in mammals *(62)* where it functions in chromatin condensation

during meiotic prophase I and in postmeiotic chromatin condensation during spermiation *(63,64)*.

3.2.1. HRad6B Ubiquitin-Conjugating Enzyme (HR6B)

Immature male mice deficient in the Hr6b ubiquitin-conjugating enzyme experience germ cell failure in the first wave of spermatogenesis coincident with meiosis I. Pachytene spermatocytes show less condensed chromatin leading to an overall reduction in SC width and premature breakdown of these complexes at telomeres *(63)*. These results indicate that chromatin condensation during synapsis is important for proper SC formation and maintenance.

Homologous recombination was increased in *hr6b–/–* primary spermatocytes as indicated by an increased number of Mlh1-positive foci. This increase in recombination can be explained by increased accessibility of SPO11 to chromatin to initiate DSBs as a result of looser chromatin structure *(63)*.

HR6B, also known as Rad6B, is also involved in DNA damage repair in somatic cells (Fig. 4). Rad6B is recruited to chromatin upon DNA damage by exogenous (UV light, toxin exposure) or endogenous (free radical) agents by the RING finger protein Rad18 *(65,66)*. Rad6 associates with DNA where it mediates both error-free and error-prone postreplication repair, which is a different mechanism of DNA damage repair than mismatch repair *(65)*. Both Rad6 and Rad18 mutants in yeast can be suppressed by Srs2, which is involved in DNA damage repair through homologous recombination. It is possible that this suppressor mutation is effective because it allows DNA damage repair by homologous recombination to compensate for damaged post-replication repair *(67)*. Mammalian *Rad18–/–* ES cells also have increased sister chromatid exchange indicative of increased homologous recombination because of loss of Rad18 expression. Sister chromatid exchange increases still further in Rad18 knockout embryonic stem cells when they are exposed to UV light or toxins, suggesting that homologous recombination can compensate in part for an impaired postreplication repair pathway *(66)*.

3.2.2. The Fanconi Anemia Protein Complex Genes and Defective Germ Cell Proliferation

Fanconi anemia genes play an important role in male and female fertility. This is of importance because Fanconi anemia is a DNA damage repair disease. These proteins function in the response pathway that involves the BRCA1 and BRCA2 breast cancer susceptibility genes. Patients with Fanconi anemia are sensitive to DNA cross-linking agents. One of the most important steps in the pathway involves the

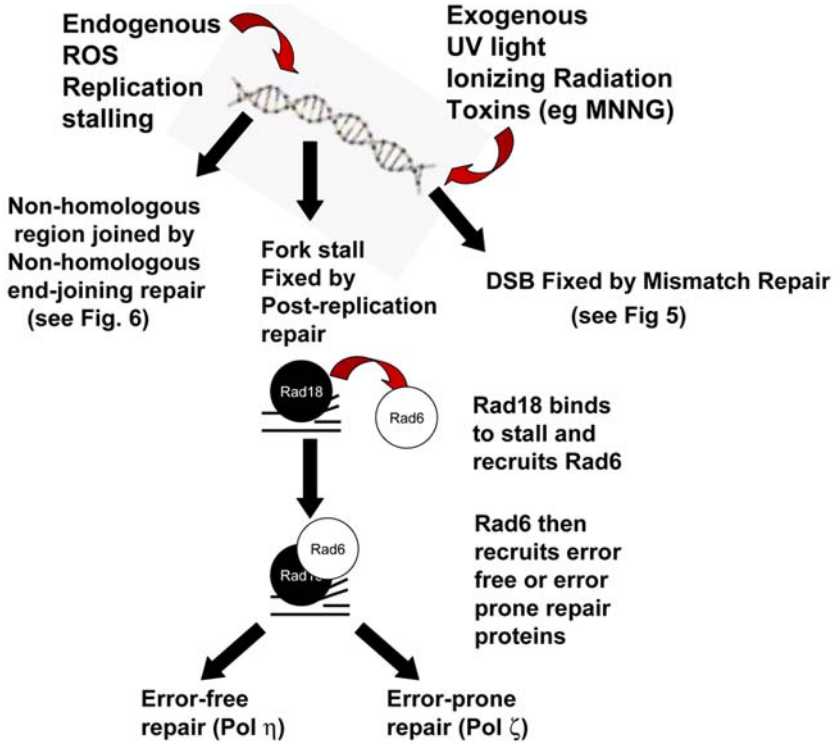

Fig. 4. Mechanisms of DNA damage repair in somatic cells. Endogenous stresses (free radicals) or exogenous agents (UV light, Ionizing radiation, toxins) cause DNA damage that the cell must either repair or undergo apoptosis to preserve integrity of its genome. Cells can repair this damage by double-stranded break repair (*see* Fig. 5), nonhomlogous end joining (*see* Fig. 6), or post-replication repair. Post-replication repair fixes lesions in DNA that result in stalled replication forks. Rad18 binds to the stalled replication fork and recruits Rad6. Rad6 then recruits either DNA polymerase η to promote error-free repair or DNA polymerase ζ that promotes error-prone repair.

mono-ubiquitination of FANCD2 that changes the subcellular localization of this protein to distinct foci in the nucleus. Targeted deletion of the Franca genes results in germ cell deficiencies resulting from defective proliferation of the germ cells *(68–74)*.

Nadler and Braun showed that loss of the Fanconi anemia complementation group C locus (Fancc) results in germ cell loss that occurs at the time of the mitotic proliferation of the primordial germ cells *(72)*. Similarly, *Pog* gene defects, which underlie the germ cell deficient (*gcd)* mutant mouse phenotype, are consistent with an association with Fanconi anemia *(70,75)*. *Pog* or *PHF9* encodes an E3 ubiquitin ligase associated with Fanconi anemia. It is thought

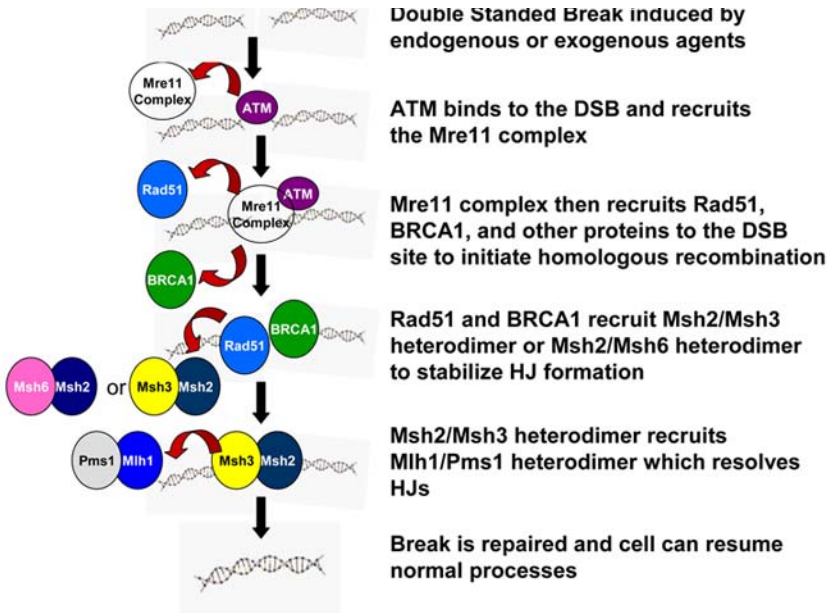

Fig. 5. Endogenous or exogenous agents can cause double-stranded break repair in somatic cells. Induction of a double-stranded break leads to activation of ATM and its movement to the site of DNA damage where it recruits the Mre11 complex. The Mre11 complex then recruits Rad51, BRCA1, and other factors to the double-stranded break to initiate homologous recombination. Rad51 and BRCA1 in turn recruit either an Msh2-Msh6 or an Msh2-Msh3 heterodimer to the site of recombination where it helps stabilize Holliday junction formation and to recruit Mlh1-Pms1. Thus, Msh2-Msh3 (or Msh2-Msh6) catalyzes the resolution of this structure. These factors then resolve the Holliday junction and repair the break allowing the cell cycle to progress.

that this is a component of the Fanconi anemia protein core complex, possibly a catalytic subunit, required for FANCD2 monoubiquitination *(70)*.

3.3. Ataxia Telangiectasia/ATR

Men with ataxia telangiectasia (ATM) have gonadal atrophy and azoospermia because of failure of primary spermatocytes at the leptotene/zygotene transition *(76)*. This phenotype is identical to the mouse phenotype *(30)*. ATM is expressed in germ cells from B-type spermatagonia through pachytene *(77)*. ATM self-activates by autophosphorylation in the presence of DSBs, whether they are induced by SPO11 in meiosis or by exogenous factors in somatic cells *(78,79)* (Fig. 5). Because ATM is a kinase, it then phosphorylates Histone H2AX, which then becomes a substrate for the Mre11 complex during

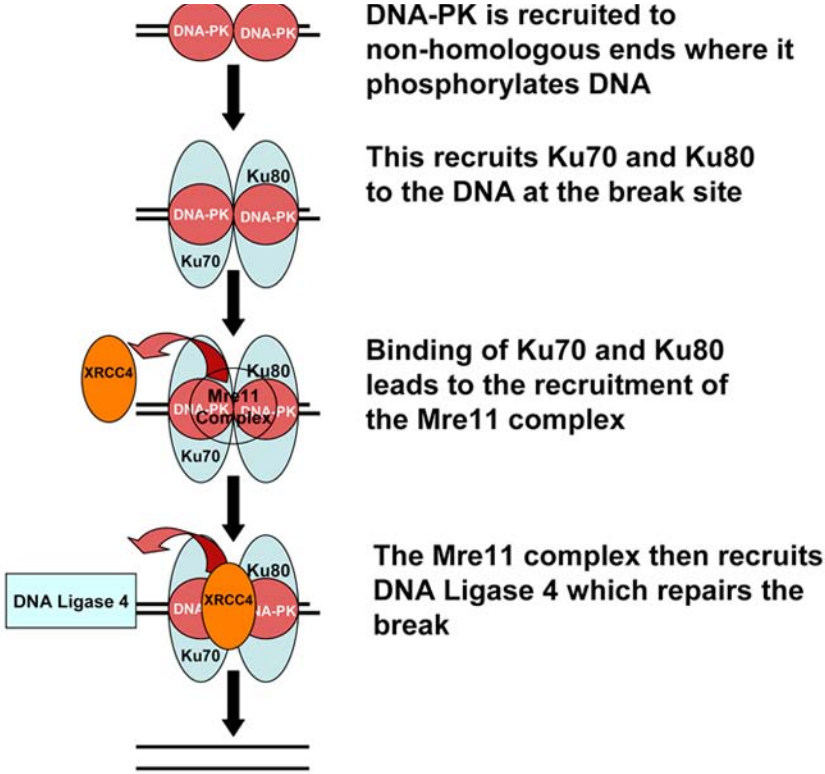

Fig. 6. Nonhomologous end joining repair. A break in DNA can occur where the ends are not complimentary. In this case, DNA phosphokinase (DNA-PK) binds to and phosphorylates histones at the non-homologous break site. Binding of DNA-PK recruits Ku70 and Ku80 which leads to the recruitment of the Mre11 complex. Association of the Mre11 complex with Ku70 and Ku80 recruits XRCC4 which recruits DNA ligase 4 to the break site.

meiosis I or BRCA 1 and 2 during somatic DNA damage repair *(80,81)* (Fig. 6).

4. CONCLUSION

This chapter highlights the many genes involved in meiosis required for DNA repair, recombination, and replication. Research in this area is advancing rapidly, yet despite significant gaps in our knowledge, it is clear that defects in genes required for homologous recombination and DNA repair are integral to the maintenance of genomic integrity, as well as to the process of spermatogenesis. It is likely that a significant percentage of men with nonobstructive azoospermia harbor genetic defects in some of these functionally important genes. Mutations in

genes required to maintain the fidelity of the genome might have implications for these patients in later life. In addition, the current practice of using the assisted reproductive technologies to achieve pregnancy for the severe male-factor couples with nonobstructive azoospermia may require additional evaluation of safety and efficacy for the offspring of this select patient population.

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III The Y Chromosome, Development, Spermatogenesis, and Sperm Maturation

10 Germ Cell-Specific Genes and Posttranscriptional Regulation in the Testis

Mark S. Fox, PhD,
and Renee A. Reijo Pera, PhD

Summary

Ten to 15% of couples are infertile. One of the most common causes of infertility is defective spermatogenesis characterized by the production of few or no sperm (oligospermia and azoospermia, respectively). However, little is known of the molecular causes of spermatogenic failure. Current assessment of infertility is based on sperm counts and testicular biopsies with little molecular analysis, apart from Y chromosome analysis in some cases. Thus, we sought to examine whether microarray technology might enable us to profile gene expression in infertile men and define subclasses of spermatogenic failure with a biological basis. We performed microarray analysis on a small group of infertile men and then focused further on analysis of a subset of genes that encode RNA-binding proteins in the *DAZ* gene family and/or interact with proteins encoded by this family. In this chapter, we review these experiments and also discuss the target RNAs of these RNA-binding proteins in more detail.

Key Words: Microarray; Sertoli cell only; azoospermia; oligospermia; germ cell; DAZ; DAZL; PUM1; PUM2.

1. INTRODUCTION

The birth of a healthy child begins with the fusion of functional gametes, the egg and sperm, resulting in the propagation of a functional embryo. The development of male gametes through spermatogenesis is characterized by mitotic replication of the spermatogonial stem cell population, meiotic differentiation, and spermiogenesis.

In 10–15% of couples, fertility is severely compromised *(1)*. One of the most common causes of infertility is poor sperm production, oligozoospermia, or azoospermia (the production of few or no sperm,

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respectively). Further, the testicular histology associated with severe oligozoospermic or azoospermic men can be categorized as nonobstructive azoospermia, Sertoli cell-only (SCO) syndrome, maturation arrest (pre- or postmeiotic arrest), and hypospermatogenesis. Recently, there has been much interest in using modern technologies to further define the causes of infertility, including microarray analysis *(2)*. Indeed, such analysis may both identify new candidate genes and confirm the role of many genes in spermatogenesis, including genes such as transition protein 1, and protamines 1 and 2; proteins involved in the sequential replacement of histones by transition protein and finally by protamines during nuclear compaction of the sperm nucleus; deleted azoopsermia (DAZ) and deleted azoospermia-like (DAZL; refs. *3–18*). Nonetheless, studies from model organisms suggest that perhaps several thousand genes may be required for germ cell development, thus the vast majority of genes that might be mutated or deleted in men with spermatogenic defects remain to be identified.

2. GENE EXPRESSION PROFILES OF HUMAN SPERMATOGENIC GERM CELLS

Recent technical and analytical advances have made it practical to examine and quantify expression of thousands of genes in parallel using microarrays from tissue samples with as few as 1000 cells *(2)*. We tested whether these advances would allow us to compare gene expression in testis biopsies from infertile men. We began by comparing the transcriptional profiles of biopsy samples from three patients diagnosed with SCO syndrome to those with hypospermatogenesis (Fig. 1). The samples were hybridized to a microarray containing probes for 21,618 cDNAs. Initially, a hierarchical clustering algorithm identified distinct groups comprised of genes expressed primarily in Sertoli cells or germ cells within testicular tissue and those genes ubiquitously expressed between tissue types (Fig. 2; ref. *2*). We examined a list of the 689 genes that had the greatest differential in expression to identify candidate genes whose expression are specific to germ cells within testicular tissue *(3)*. We then used several methods of gene selection to create a more limited set of genes for further exploration. Moreover, when we searched gene and clone reports for information regarding expression and function in humans and/or model organisms, we found that 239 genes had previously been shown to be either significantly or primarily expressed in the testis. This list of significant genes was then further reduced to 177 genes by removal of false-positives through a number of algorithms *(3)*.

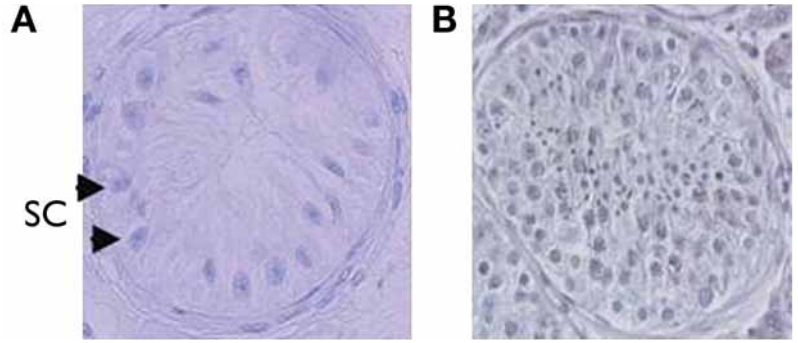

Fig. 1. Testiclar biopsies from **(A)** a patient diagnosed as Sertoli cell only with somatic cells, Sertoli cells, and interstitial cells but no germ cells and **(B)** a patient with normal spermatogenesis with seminferous tubules containing all stages of spermatogenesis.

If the individual microarray data points are an accurate reflection of germ cell transcription, then it would be expected that clones demonstrating a large variance in expression between SCO and normal samples would include genes previously known to have testis/germ cell function, as suggested previously. Examples of such genes included those that have been shown to be expressed during specific stages of spermatogenesis and sperm function, such as primordial germ cell development and migration (*DAZ*, *TSPY*), spermatogonial proliferation and survival (*DAZL*, *zona pellucida binding protein*, *basonuclin*, *T-STAR*; refs. *4–9*), various stages of meiosis (*cdc25c*, *TBP-like*, *serine/threonine kinase 13 [aurora/IPL1-like]*; refs. *10–12*), and spermiogenesis (*protamine 2*, *mitochondrial capsule selenoprotein*, *acrosomal vesicle protein SP-10*, *AKAP-associated sperm protein*, *testis-specific protein TPX-1*, *calmegin*; refs. *13–17*). Another group of genes has more limited data that also suggested a role in spermatogenesis including *testis-specific bromodomain*, *testis-specific ankyrin motif protein*, and *testis-specific expressed transmembrane 4 protein*, as well as others *(18–26)*. Finally, perhaps the most interesting subset of genes identified was that which also included genes highly enriched or specific to female germ cells/ovary. Twenty genes were identified in this category, including six genes that encode hypothetical proteins, four encoded by expressed sequence tags, a gene involved in calcium modulation, another involved in apoptosis, and several that bind DNA or RNA or encode membrane proteins *(4,6,8,9,27–32)*.

To further establish a correlation between the microarray data and gene expression during spermatogenesis, nine genes spanning all stages of the spermatogenic differentiation and a control gene were

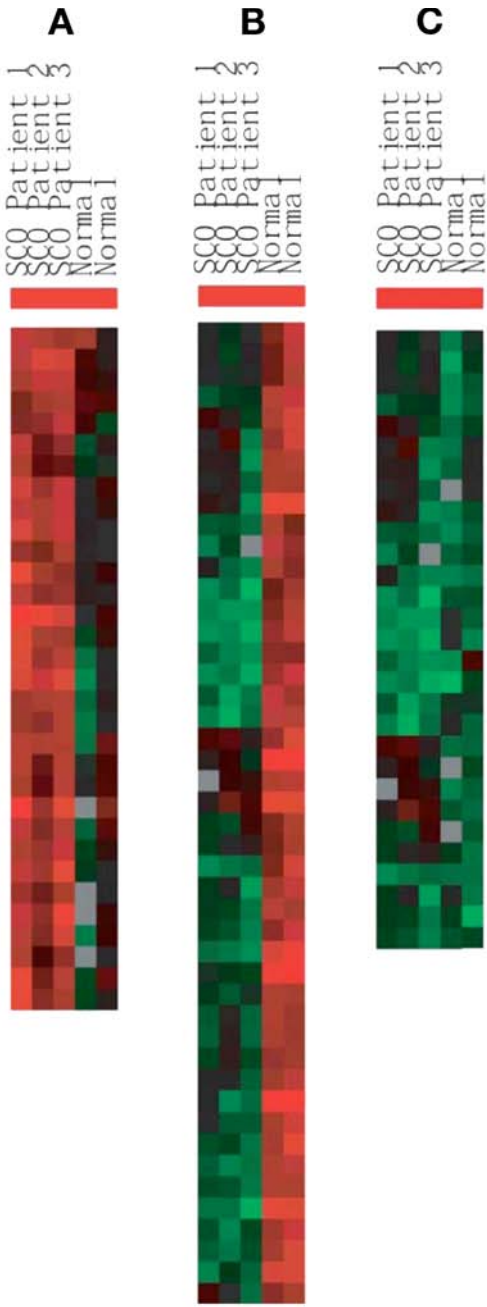

Fig. 2. Diagnosis of spermatogenic defects via molecular analysis. **(A)** Expression pattern of gene upregulated in biopsies containing Sertoli cells, **(B)** those upregulated in normal testis, and **(C)** those genes expressed ubiquitously expressed between tissue types.

selected for conformational experimentation. Eight of the nine genes were selected for their known association with spermatogenesis as described in the literature. *DAZ (4)* and *TSP (5)* genes are expressed in primordial germs cells and expression of these genes has been observed until the secondary spermatocyte stage of spermatogenesis. Expression of *zona pellucida-binding protein (7)* has been detected as early as primary spermatocyte continuing until maturation of the sperm. Replacement of histones by protamines begins with the expression of the transition proteins, specifically *transition protein 1 (33)*, whose expression can be detected after the first meiotic division. Both *synaptojanin (34)* and *protamine 2 (13)* genes are transcribed postmeiotically in the round spermatid stage of spermatogenesis. Three other genes were also selected for further analysis: the *neural polypyrimidine tract-binding protein (35)*, *testis-specific bromodomain (18)*, and *testis-specific ankyrin motif-containing protein (19)*. Expression of these genes during spermatogenesis has as yet not been specifically detailed. The *glucuronidase* β gene was used as a positive control because expression is found in somatic and germ cells.

cDNAs were initially synthesized from RNA from normal testis, biopsy samples from SCO patients and subjected to polymerase chain reaction (PCR) amplification with respective gene-specific primers. This strategy allowed for a rapid preliminary evaluation of expression on a number of specific genes on a panel of tissue samples. In the three patients with SCO syndrome, as predicted by biopsy and microarray analysis, we found specific PCR products were only detect for the control *GUS*, except for the unexpected detection of *transition protein 1* in SCO patient 1 (Fig. 3). Reverse transcriptase-PCR enabled the detection of extremely few germ cells, suggesting that SCO patient 1 may have a very few number of spermatogenic cells that were not detected by the original histological method.

3. PRESENCE OR ABSENCE OF GERM CELLS: VALIDATION

Expression analysis using DNA microarrays has provided an overview of the genes that comprise a set of instructions for a complex developmental process, germ cell development. This list can now be searched for genes of interest in future experimental efforts (such as mouse genetic studies) or for genes that may be implicated in human male infertility. In particular, it is hoped that future studies may correlate gene expression with testicular biopsies in a large population of infertile men. Then, by combining expression analysis and clinical data, analysis might be extended toward the goal of predicting outcomes

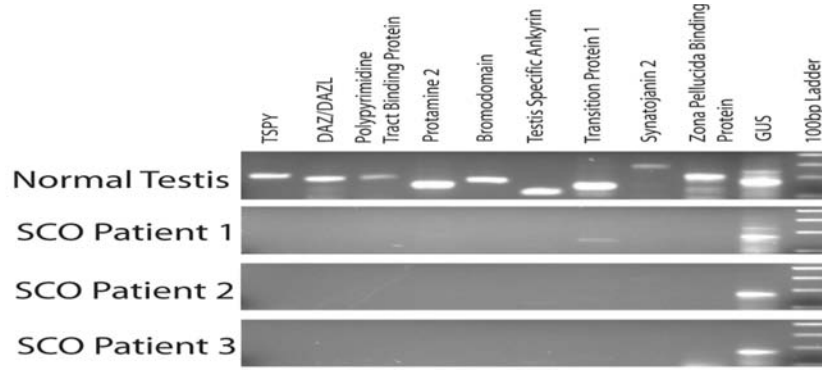

Fig. 3. Gene expression validation. Reverse transcriptase-PCR products of *TSPY*, *DAZ/DAZL*, *polypyrimidine tract-binding protein gene*, *protamine 2*, *bromodomain*, *ankyrin motif protein*, *transition protein 1*, *synaptojanin 2*, *zona pelucida-binding protein*, and *GUS* in normal adult testicular tissue and testicular tissue from Sertoli cell-only patients.

of assisted reproductive techniques, such as intracytoplasmic sperm injection.

4. IDENTIFICATION OF GERM CELLS-ENRICHED RNA-BINDING PROTEINS

Analysis of global gene expression in the normal testis identified many genes that are implicated in spermatogenesis with reported functions in cell envelope and membranes, cellular processes, metabolism, nucleotides, proteases and proteins modifications, replication and repair, transcription and regulation, transport, and in the regulation of RNA. Eight transcripts were identified that encoded proteins that could bind RNA (Table 1), two ribonuclease proteins, two members of the DEAD-box family, a polypyrimidine tract-binding protein, and two members of the *DAZ* gene family, DAZ and DAZL proteins.

The essential role of RNA–protein interactions for normal germ cell development is highlighted by the severity of the defects that result when this system is perturbed. Chromosomal deletions of the Y chromosome that encompass a cluster of *DAZ* genes that encode proteins with ribonucleoprotein motifs cause oligospermia and azoospermia (the production of few or no sperm, respectively) in men *(36–39)*. The *DAZ* genes arose via transposition of a germ cell-specific autosomal gene, *DAZL*, during primate evolution *(40–43)*. *DAZ* and *DAZL* are 90% identical; homologs are found in diverse organisms and are required for germ cell allocation or maintenance early in development

Table 1
RNA-Binding Proteins Enriched for in Male Adult Germ Cells

Name	*Accession number*
Ribonuclease, RNase A family, 11	AA609760
Three prime histone mRNA exonuclease 1	AA682626
Deleted in azoospermia 4	AA133797
Deleted in azoospermia-like	AA774538
KH domain-containing, RNA-binding, signal transduction-associated 3	AA456299
DEAH (Asp-Glu-Ala-His) box polypeptide 36	AA430052
DEAD (Asp-Glu-Ala-Asp) box polypeptide 20	AA460305
Polypyrimidine tract-binding protein 2	NM 021190

and/or meiosis *(44–47)*. To further define the function of the DAZ and DAZL proteins, additional studies have focused on localization of DAZ/DAZL and on defining proteins that interact with DAZ/DAZL *(4,48,49)*. *DAZ* and *DAZL* are expressed prenatally in gonocytes or primordial germ cells *(4,48)*. In the adult, the DAZ protein is restricted to multiple stages of male germ cells, whereas DAZL is expressed in adult germ cells of both sexes *(4,48)*. These results suggest that proteins encoded by the *DAZ* family may function at multiple points in human germ cell development. They may act during meiosis and during the establishment of stem cell populations.

5. IDENTIFICATION OF DAZ/DAZL INTERACTING PROTEINS AND RNAS

Within germline stem cells it has been established that multiple proteins containing RNA-binding motifs interact with DAZ and DAZL proteins (Fig. 4; refs. *49–53*). One of these proteins is the human homolog of *Drosophila* Pumilio, called PUMILIO-2 (PUM2). Human PUM2 shares 80% identity with *Drosophila* Pumilio over more than 280 amino acids that define the RNA-binding domain *(49,54)*. The homology of human PUM2 to *Drosophila* Pumilio is of particular interest given the well defined role of *Drosophila* Pumilio as a translational repressor that is required for both anterior–posterior patterning and germ cell development in the fly embryo and adult *(55–60)*. In addition, data from the evolutionarily distant roundworm, *Caenorhabditis elegans*, indicates that two members of the Pumilio family, fem-binding factor-1 and -2, act together to regulate germline stem cell maintenance by interacting with the 3′-untranslated region (UTR) of *gld-1* messenger

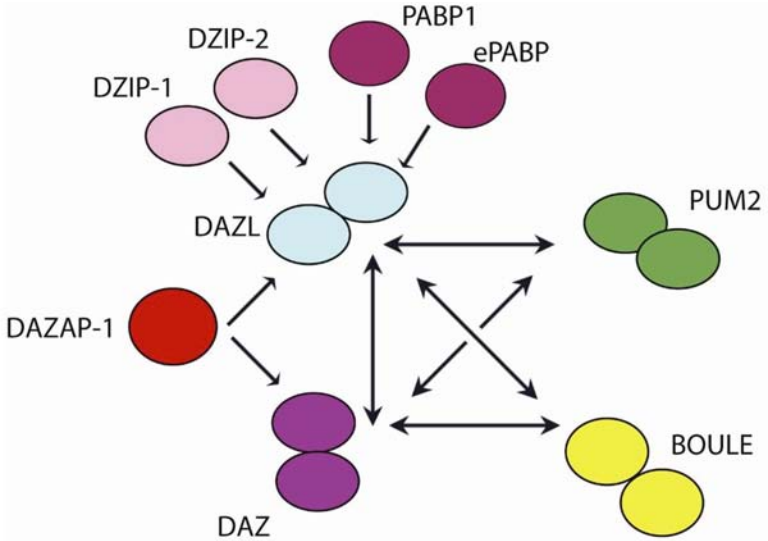

Fig. 4. Diagram illustrating protein–protein interactions with DAZ/DAZL *(49,50,52,53)*.

RNAs (mRNAs; ref. *61*). A third Pumilio family member in *C. elegans*, PUF-8, also acts during germ cell development to regulate completion of meiosis *(62)*. Thus, the interaction of the human DAZ, DAZL, and PUM2 proteins allows us to hypothesize that DAZ/DAZL and PUM2 proteins interact to regulate RNA ligands necessary for early development of the germ cell lineage: allocation, maintenance, and differentiation of primordial germ cells and germline stem cells.

In model organisms, Pumilio binds to two different mRNAs, the *hunchback* mRNA (specifically, the nanos regulatory element [NRE] sequences) during embryonic abdominal patterning in *Drosophila* and *cyclin B* mRNA during embryonic germ cell migration in *Xenopus (56,63)*. Similarly, the mouse Dazl may bind to the 3′-UTRs of several mRNAs, including *Cdc25A* and *Tpx-1 (64)*. However, it is unlikely that these genes are the major targets of Dazl protein given the observations that Cdc25A is not essential for germ cell development and Tpx-1 is only expressed in the testis, whereas Dazl is essential for both male and female germ cell development *(65,66)*. Thus, we sought to identity mammalian target mRNAs that are regulated by these RNA-binding proteins by taking advantage of the observation that the PUM2 and DAZ/DAZL proteins can form a stable complex and colocalize in the germ cell lineage *(49,51)*. We immobilized PUM2 or DAZL fusion proteins to beads to co-immuoprecipitate human testis mRNAs. Messenger

ribonucleoprotein complexes bound by each fusion protein were extracted and mRNAs were subjected to amplification via reverse transcriptase-PCR and the products were cloned. To enhance the specificity of the products obtained, serial copurifications were performed for each protein and resulting mRNAs were reverse-transcribed, radiolabeled, and used to screen colonies identified in the first screen. mRNAs specifically bound by both the DAZL and PUM2 fusions were identified as positives in at least two rounds of screening. This resulted in the identification of potential candidate target mRNAs of which three transcripts overlapped with those of Jiao et al. *(64)*, with both groups pulling down transcripts for *transition protein 1*, *XAGE*, and *GAGE*.

6. IDENTIFICATION OF PUM2 RESPONSE ELEMENT

Given the high conservation between *Drosophila* and human Pumilio homologs, we sought additional information regarding PUM2 RNA binding by examining its interaction with the *Drosophila* NRE. Using the methodology described in Moore et al. *(49)*, we cloned the minimal NRE-binding site into a plasmid, which allows the expression of a hybrid RNA molecule, and mutagenized 11 nt, 5 nt within Box A and 6 nt within Box B, sequences described by Zamore et al. *(67)*. The interaction between the RNA targets and PUM2 was assayed for via the three-hybrid system. This allowed us to define a minimal sequence that is required by PUM2 to bind to the NRE as "GNNNNN NNNNNNUGUA," as shown in Fig. 5. In previous studies *(67)*, mutations within a region defined as "Box A" of the NRE had no effect on binding of human PUM1 to the NRE sequence; likewise, mutation of the first two nucleotides of Box A and a second region, Box B, had no effect on *Drosophila* Pumilio binding. In contrast, when we compared binding of PUM2 to the NRE within Box A, PUM2 required only the first nucleotide. Similarly to PUM1 and *Drosophila* Pumilio, however, the sequence UGUA in Box B is essential for binding, confirming the assumption that the three proteins have different sequence similarities in vivo.

We next analyzed the sequences of the clones identified in the original screen to identify mRNAs that contained the 17-nt binding sequence. Twenty elements were identified in 14 transcripts (Tables 2 and 3), which were then screened for binding with PUM2. Two of the identified mRNAs for *Hypothetical protein FLJ10498* (SDA1 domain containing 1) and *Bullous Pemphigoid Antigen 1 (BPAG1)* recruited PUM2, as shown in Fig. 6. No interaction by either member of the DAZL gene family or by the pACT plasmid expressing only the GAL4 activation

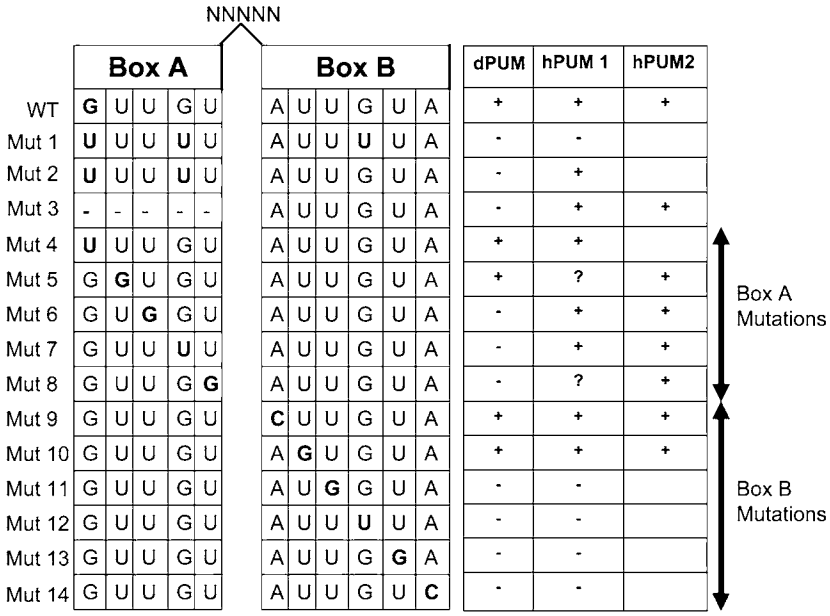

Fig. 5. Pumilio and homologs bind to nanos regulatory elements (NREs). *Drosophila* Pumilio (dPUM), human PUM1 (hPUM1), and PUM2 (hPum2) proteins specifically recognize the NRE sequence. Several mutant messenger RNAs with single-nucleotide changes abolish binding of Pumilio proteins to the NRE sequences.

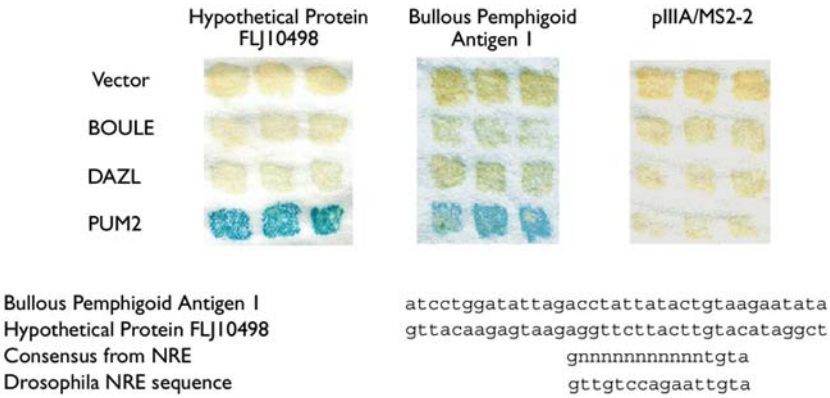

Fig. 6. RNA–PUM2 interactions in yeast. Of the 21 potential PUM2 *cis*-elements screened, only two were positive for binding in a yeast three-hybrid assay. These sequences are located in the 3′-untranslated regions of transcripts for *hypothetical protein FLJ10498* (SDAD1) and *bullous pemphigold antigen 1*. RNA-binding proteins DAZL and BOULE did not recognize either sequence. No interactions were detected with the vector alone or PUM2 in the absence of the nanos regulatory element-related sequence.

Table 2
Potential Sequences Recognized by PUM2 Within the 3′-Untranslated Region of Each Gene

Name	*Accession number*
Hypothetical protein FLJ12910	BC011348
Triosephosphate isomerase 1 (TPI1)	NM 000365
Fatty acid desaturase 1 (FADS1)	NP 037534
Hamartin (tuberous sclerosis)	NM 000368
F-box-only protein 21 (FBXO21)	NM 015002
HSPB-associated protein 1 (HSPBAP1)	NM 024610
Stromal membrane-associated protein 1 (SMAP1)	NM 021940

Table 3
Potential Sequences Recognized by PUM2 Within the 3′-UTR of Each Gene

Name	*Accession number*
Integrin alpha 6 precursor (VLA-6) (CD49f)	NM 000210
Trytophenyl tRNA synthetase (WARS)	NM 004184
Diablo homolog	NM 019887
SDA1 domain-containing 1 (SDAD1)	BC063797
Hypothetical protein LOC57821	AK223354
Bullous pemphigoid antigen 1 (BPAG1)	NM 183380
Enhancer of rudimentary (*Drosophila)* homolog	NM004450

domain was detected, thus confirming that the RNA–protein interaction was specific to PUM2. FLJ10498 (SDAD1) is a protein of 86.6 kDa located on chromosome 4 that was first identified as a transcript in teratocarcinomas, whereas BPAG1 is a 230/240 kDa protein located on chromosome 6 that serves as an autoantigen in the blistering disease bullous pephigiod *(68–70)*.

7. IDENTIFICATION OF DAZL AND PUM2 RESPONSE ELEMENT

To define whether DAZL in conjunction with PUM2 could bind to the *SDAD1* transcript, overlapping 90-bp fragments were constructed and analyzed for their ability to support binding of DAZL and PUM2. RNA–PUM2 interactions were detected for fragments 0–90, 128–218, 530–620, and 666–756, whereas DAZL was found to only bind two regions of the 3′-UTR, next to the stop codon in fragment 0–90 and in fragment 666–756, a sequence that also binds PUM2 (Fig. 7). A series

Fig. 7. Binding of proteins to the 3′-untranslated region (UTR) of the SDAD1 messenger RNA (mRNA). The 3′-UTR of the *SDAD1* transcript was subcloned into a vector and screened for protein binding via the yeast three-hybrid system. The fragments are numbered from 0 (corresponding to the stop codon of the predicted open reading frame of *SDAD1*) to 837 (the start of the polyadenylated tail of the transcript). Blue color indicates interaction between the RNA sequence and fusion proteins in the yeast three-hybrid assay. Multiple RNA-binding proteins bind the *SDAD1* mRNA.

of 5′ and 3′ deletion constructs were generated using annealed oligonucleotides and analyzed for the ability of these constructs to recruit binding of PUM2 and DAZL. In this way we were able to map two distinct PUM2-binding elements whose binding constraints were confirmed by gel shift assays competition experiments: PBE1–UNUUANUGUA (spacing between UNUUA and UGUA elements can differ up to three bases) and PBE2–UAUANNUAGU). Furthermore, comparison of sequences that DAZL binds in our analysis with those obtained by Jiao et al. yields a consensus sequence (UAUGUAGUUAUUAAAAAUUUUUAAAUCA; ref. *64*). In addition, we identified sequences in the SDAD1 3′-UTR that may also be bound by conserved RNA-binding proteins that are known to interact with DAZ and DAZL (Fig. 4). Analysis of BOULE binding indicated that this protein can bind three regions of the 3′-UTR, a fragment near the stop codon (0–90), fragment 666–756, and fragment 734–824. DAZ-2 binds to fragments 0–90, 530–620, 666–756, and 734-824, whereas DAZAP-1 recognizes fragments

Table 4
Consensus Pumilio Regulatory Elements in the 3′-Untranslated Region of Genes Whose Expression Is Specific to Germ Cells Within Testicular Tissue

Name	*Accession number*
Zona pellucida-binding protein (ZPBP)	NM 007009
Homo sapiens acrosomal vesicle protein 1 (ACRV1)	NM 001612
GDNF family receptor alpha 2 (GFRA2)	NM 001495
Guanine nucleotide-binding protein (G protein), alpha-activating activity polypeptide, olfactory type (GNAL)	NM 182978
cDNA DKFZp434G1726	AL162052
Chromosome 13 open reading frame 23 (C13orf23)	NM 170719
MLF1-interacting protein (MLF1IP)	NM 024629
Chromosome 6 open reading frame 64 (C6orf64)	NM 018322
Protein-O-mannosyltransferase 1 (POMT1)	NM 007171
Casein kinase 2, alpha prime polypeptide (CSNK2A2)	NM 001896
Glycerol kinase 2 (GK2)	NM 033214
Aurora kinase C (AURKC)	NM 003160
PDZ-binding kinase (PBK)	NM 018492
Serine/threonine/tyrosine interacting protein (STYX)	NM 145251
BUB1 budding uninhibited by benzimidazoles 1 homolog	NM 004336
Nucleoporin 155kDa (NUP155)	NM 004298
DEAH (Asp-Glu-Ala-His) box polypeptide 36 (DHX36)	NM 020865
Bromodomain, testis-specific (BRDT)	NM 001726
cAMP responsive element modulator (CREM)	NM 001881
Suppressor of hairy wing homolog 2 (SUHW2)	NM 080764
ADP-ribosylation factor 1 (ARF1)	NM 001658
DnaJ (Hsp40) homolog, subfamily A	NM 005147
Lysozyme-like 6 (LYZL6)	NM 020426
Hypothetical LOC197387	BC038761.1
Solute carrier family 36 (proton/amino acid symporter)	NM 181774
Spermatogenesis-associated 4 (SPATA4)	NM 144644
Ribonuclease, RNase A family, 11	NM 145250

332–422, 462–552, 530–620, and 666–756 (Fig. 7). We were unable to detect any interactions between the 3′-UTR of the *SDAD1* gene and DZIP-1 or DZIP-2. A schematic of RNA–protein interactions suggest the potential for coordinate regulation of RNAs and the presence of a complex of proteins and RNAs that are definitive of germ cells.

8. PUM2 REGULATORY ELEMENTS WITHIN GERM CELL-ENRICHED TRANSCRIPTS

Sequence analysis of the clones identified by the co-immunoprecipitation screen demonstrated that five transcripts possess a putative PUM2 RNA-binding site (hPBE1; ref. *71*). Using the yeast three assay, we identified RNA–protein interactions for four out of the five transcripts, suggesting that this PUM2-binding sequence had been characterized in sufficient detail to be used in database searches *(71)*. Previously, we proposed that major targets of DAZL, that are essential for germ cell development, have not yet been identified. However, the constraints of the DAZL-binding element have not been fully elucidated to allow for an effective search of the germ cell profile. Another possibility would be to search the germ cell transcription profile using a *cis*-element of a protein known to interact with DAZL, in this case PUM2. A search through the 3′-UTRs of the 177 human genes either significantly or previously expressed in germ cells identified 27 possible targets of PUM2 (Table 4). Future analysis of these transcripts will identify those genes that are also bound by DAZL *(3)*.

9. CONCLUSION

The male germline has the unique ability to pass genetic information to the next generation via a highly organized differentiation process called spermatogenesis. In this chapter, we describe 177 potential transcripts that can be explored as prognostic markers for spermatogenic failure. Moreover, we propose that gene expression profiling may also be a useful tool to explore the relationship between gene expression and in vitro fertilization outcome. In addition to its clinical potential, gene profiling may also assist in design of experiments to uncover genetic requirements in spermatogenesis. This may be achieved by comparing gene profiles from patients suffering from different syndromes or from data mining, as in the case of discovering genes important in germ cell development that are posttranscriptionally regulated.

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11 The Genetics of Cryptorchidism

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Summary

Cryptorchidism is one of the most frequent congenital abnormalities, with a recorded frequency of 3–4% among newborn males. Before sex determination, both female and male embryonic gonads are located in the same high intra-abdominal position. The developing testes migrate through a multiphase process of testicular descent (TD), first into a low abdominal position and then into the developing scrotum. A critical role in TD belongs to the gubernacular ligaments. Analysis of mouse mutants revealed a number of genes involved in this process. Insulin-like factor 3 (Insl3) controls the first abdominal phase of TD through its receptor, leucine-rich-containing repeats G protein-coupled receptor (Lgr8). The inguinoscrotal stage of TD is believed to be controlled by the hypothalamic–pituitary–gonadal axis, and specifically by androgens. Additionally, the targeted ablation of several transcription factors, such as Hoxa10, Hoxa11, and Desrt, causes cryptorchidism in mice, suggesting an involvement of multiple signaling pathways in TD. In this chapter, we review the mutation analysis and allele association studies for the candidate genes in human patients with cryptorchidism.

Key Words: Cryptorchidism; testicular descent; INSL3; LGR8; Steroid hormones.

1. CLINICAL CONSEQUENCES OF CRYPTORCHIDISM

Cryptorchidism, which will be used as a synonym to the undescended testes in this chapter, is one of the most common maladies in newborn males. The reported frequency of cryptorchidism is as high as 3–4%; however, among premature infants the rate cryptorchidism at birth is about 30% *(1,2)*. At 1 yr of age and later, when surgical intervention is recommended, the incidence of cryptorchidism in the general population is 0.8–1% *(2)*. In the last 30–40 yr, the frequency of cryptorchidism at 1 yr of age appears to have increased in some countries by 60% *(3,4)*, however, it is not clear whether such an

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increase reflects better recording methods, diagnostics, or the actual events *(2)*. The effect of the environmental endocrine disruptors affecting embryonic development and the role of hereditary factors in susceptibility to teratogens should be considered *(2,5)*. The most common form of cryptorchidism in humans is inguinoscrotal and retractable testes, which can affect one or both gonads. Although familial cases of cryptorchidism have been reported, most human occurrences are sporadic.

Testis exposure to the relatively high body temperature was shown to lead to germ cell degeneration. Consequently, patients with intraabdominal or intracanalicular testes manifest rapid decline in germ cell population *(6,7)*. Men with a history of testicular maldescent account for 5–10% of infertile couples *(2)*. Germ cell degeneration and dysplasia are thought to be the causes of an increased risk of testicular cancer in individuals with a history of cryptorchidism. About 10% of all patients with testis tumors have a history of cryptorchidism. On the other hand, men with testicular tumors have more than a 30-fold increase in the rate of bilateral cryptorchidism and a 15-fold higher incidence of unilateral cryptorchidism in their history *(2,8,9)*. However, it remains unclear whether cryptorchidism, infertility, and testicular cancer share a common cause, such as an intrinsic developmental/genetic defect, or if the latter two abnormalities are the result of the anomalous testis position *(6,7)*.

Testis descent follows the stage of testis determination, and is a part of the male sexual differentiation pathway. It is clear that the etiology of cryptorchidism is multifactorial, because several anatomical, neurological, and endocrine anomalies in human patients are associated with undescended testes *(7)*. Early surgical intervention (orchiopexy) is recognized as the most reliable treatment of cryptorchidism. With the use of recent advances in treatment, including the use of advanced reproductive technology methods, such as testicular sperm extraction and intracytoplasmic sperm injection, cryptorchidism-associated infertility can be effectively treated *(10)*. One should consider, however, that such treatment might facilitate the propagation of putative inherent abnormalities in the progeny of the affected fathers.

2. MAJOR DEVELOPMENTAL STEPS IN TESTICULAR DESCENT

The two-stage model of testicular descent (TD) in mammals separates two major phases, transabdominal and inguinoscrotal descent (Fig. 1; ref. *7*). It should be pointed out, however, that up to five anatomically recognized stages of TD were defined based on the

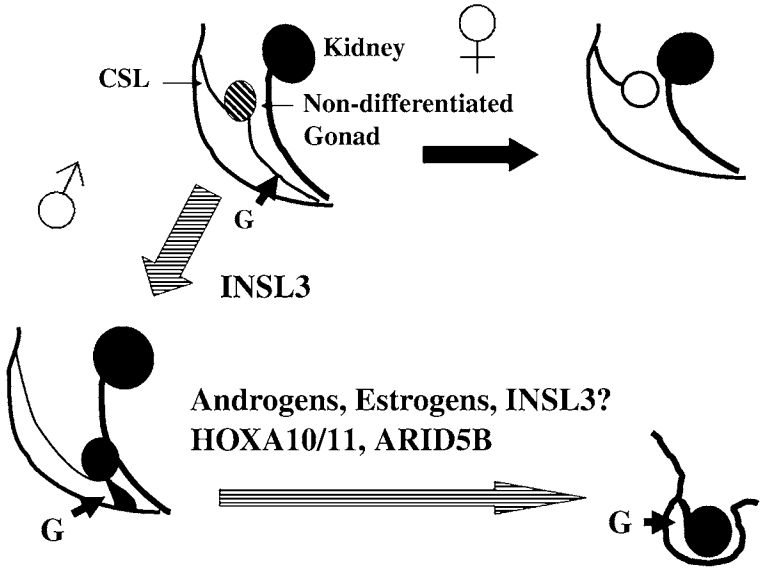

Fig. 1. The nondifferentiated gonad is located near the attached kidney with two mesenteric ligaments, cranial suspensory ligament (CSL) and the gubernaculum (G). Abdominal position of the ovary in females is provided by the persistence of CSL in the absence of gubernacular differentiation. INSL3-induced differentiation of gubernacula directs the first, transabdominal phase of testicular descent. The second, inguinoscrotal descent is controlled by androgens, estrogens, and transcription factors HOXA10, HOXA11, ARID5B, and possibly INSL3.

detailed histological analysis of human embryos *(11)*. The cranial suspensory and gubernacular ligaments are believed to play a major role in TD *(7)*. During the transabdominal phase, which occurs between weeks 10 and 23 of gestation in human embryos (in mouse between 15.5 and 17.5 d postcoitum), the testes move from their original position to the inguinal region, and by birth to the bladder neck. The high position of the ovary, attached to the abdominal wall through cranial ligament, seems to follow a default pathway. The process of transabdominal descent occurs in parallel with a shortening of the gubernacular ligament cord, outgrowth of the gubernacular bulb, and differentiation and eversion of the cremaster muscle. The human gubernaculum deposits a significant amount of the extracellular matrix rich in hyaluronic acid to form a cone-like structure at the caudal end of the male gonad *(7,12)*. In the human fetus, the second phase occurs before birth at weeks 24–34 of gestation, whereas in mice it is completed within 20 d after birth. This stage is characterized by the caudal extension of the gubernaculum, its involution and protrusion into the scrotal sac,

development of the processus vaginalis, dilation of the inguinal canal by the gubernacular bulb, and some intraabdominal pressure to force the testis through the canal. In mice, TD is mediated by the contractions of the cremasteric muscle *(7)*. Human gubernacular histology evolves from a hydrated structure with a loose extracellular matrix and poorly differentiated fibroblasts into an essentially fibrous structure rich in collagen and elastic fibers. In rodents, however, the adult gubernaculum is differentiated into the cremaster muscle component of the spermatic cord *(13)*. Such differences in development, anatomy, and histology of this and other male organs should be taken into consideration when using model organisms *(14)*.

3. TRANSABDOMINAL TESTICULAR DESCENT AND INSULIN-LIKE 3 FACTOR

Insulin-like 3 factor (INSL3) is a secretary peptide hormone, structurally similar to the insulin and relaxin peptides *(15,16)*. The expression of INSL3 is limited to testicular fetal and adult Leydig cells in males. There is a differentiation-dependent increase in the level of INSL3 in the blood, and the expression of the gene coincides with the time of TD. It was shown that the INSL3 promoter is strongly regulated by steroidogenic factor 1 (SF1), a transcription factor and an important player in male differentiation *(17,18)*. The involvement of SF1 in *INSL3* gene expression provides the direct link between the sex determination pathway and TD mechanism.

In mice, the mutation of Insl3 results in high intraabdominal cryptorchidism and male sterility *(19,20)*. The mutant gubernaculum fails to differentiate, directly implying a regulatory role for this hormone in TD. Notably, surgical correction of cryptorchidism in young males fully restores fertility, indicating that spermatogenic arrest is secondary to cryptorchidism in these mutants. Remarkably, the transgenic overexpression of Insl3 in female mice causes male-like differentiation of gubernacula and descent of the ovaries *(21)*. Thus, INSL3 alone in the absence of androgens is capable of inducing first stage of TD. Insl3-transgenic females also develop inguinal hernia. The latter fact indicates that INSL3 might also play some role in the second stage of TD, specifically in the development of the processus vaginalis.

Through positional cloning of the crsp mouse mutant, our group isolated the GREAT receptor, later renamed as leucine-rich-containing repeats G protein-coupled receptor (LGR8; ref. *22*). Specific targeting of this gene results in the same phenotype as in Insl3-mutant mice *(23)*. Based on the similarity of the phenotypes and biochemical characteristics

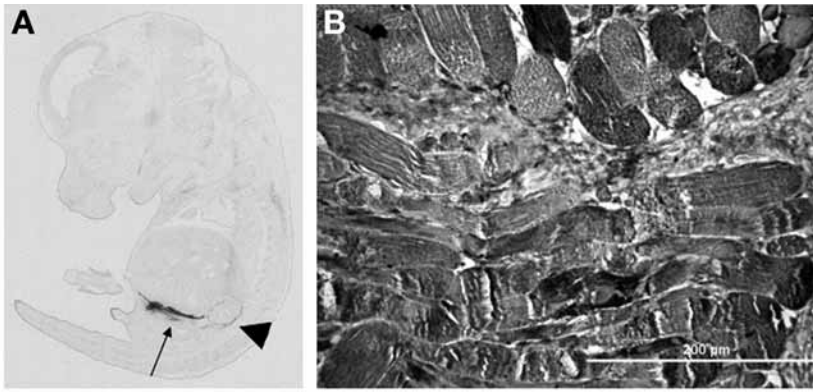

Fig. 2. Expression of mouse Lgr8 receptor in embryonic and adult gubernacula. **(A)** *In situ* hybridization of *Lgr8*-specific RNA probe to gubernacular ligaments (arrow). Testis is marked with an arrowhead. Hybridization was performed by GenePaint *(52)*. **(B)** Immunohistochemical localization of Lgr8 protein in the muscle cells of adult mouse gubernaculum.

of the receptor, we have suggested that INSL3 is in fact a cognate receptor for LGR8 *(22)*. This was confirmed both in experiments in vitro and in vivo *(24,25)*. It was shown that the INSL3 stimulation of cells transfected with LGR8 leads to an increase in the intracellular cyclic adenosince monophosphate concentration. The ligand binds to the receptor with a Kd in the low nanomolar range *(24)*. To address the question of whether Lgr8 is the only receptor for Insl3 in vivo, we have produced transgenic mice overexpressing Insl3 and deficient for Lgr8 *(25)*. Analysis of the gonadal position in such females revealed that Insl3 did not stimulate male-like differentiation of gubernaculae in the absence of the Lgr8 receptor. Transgenic females overexpressing Insl3 with the Lgr8 deletion, had a wild-type phenotype with ovaries in the normal, high abdominal position. Males of the same genotype developed cryptorchidism. Thus, the deletion of the *Lgr8* gene completely abrogated the abnormal phenotype associated with the overexpression of Insl3, providing a direct proof that Lgr8 is the only receptor for Insl3 *(25)*. During embryonic development, Lgr8 is expressed exclusively in the gubernaculum, thus confirming its direct involvement in gubernacular differentiation (Fig. 2). In adult mouse gubernacula Lgr8 is expressed mainly in striated muscles, indicating a role for Insl3 in myogenesis (Fig. 2). No specific targets of the INSL3 signaling are currently known. It was shown, however, that the treatment of the organ culture of the embryonic gubernaculum caused cell proliferation in vitro, suggesting a role of INSL3 in cell growth or survival *(26,27)*.

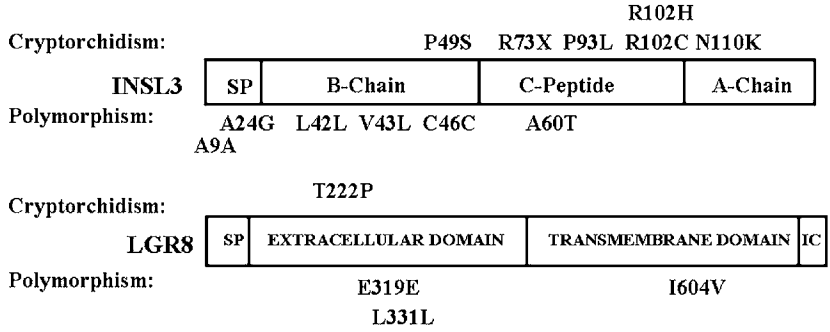

Fig. 3. Mutations of the human INSL3 and LGR8 proteins detected in human. The mutations shown above the protein were identified only in patients with testicular maldescent; the mutations shown below the protein were identified both in patients and control samples. SP, signal peptide; IC, intracellular domain.

Based on the specific involvement of the *INSL3/LGR8* pathway in TD in rodents, extensive mutation screening of these genes was undertaken in human cryptorchid patients at several laboratories *(15,16)*. The analysis of both sporadic and familial cases indeed revealed several mutant alleles. Most of them represented common polymorphisms, found both in controls and in affected patients. Some of them, however, were detected exclusively in the latter group. Surprisingly, all the patients with the *INSL3/LGR8* mutations were heterozygous for the wild-type allele (Fig. 3), implying that the mutation-dependent mechanism of testicular maldescent may be related to gene haploinsufficiency. In support of this conclusion, it was shown recently that in Leydig cells the *Insl3* gene is expressed constitutively *(28)*, and thus any decrease in its expression or an expression of INSL3 receptor in gubernaculae might be responsible for testicular maldescent.

As with other members of insulin-relaxin family of peptides, INSL3 is translated as a preprohormone and contains signal peptide, B-chain, C-peptide, and A-chain. Upon hormone maturation, signal peptide and C-peptide are excised, while the A and B chains are assembled with two interchain and one intrachain disulfide bonds. The majority of the INSL3 mutations led to a single amino acid substitution (missense mutations; Fig. 3; refs. *15* and *16*). The only exception is the R73X mutation, resulting in the termination of translation and most probably rendering a physiologically inactive hormone. The R73X mutation was found in one patient with unilateral cryptorchidism. However, the mutation does not seem to be explicitly associated with the abnormal phenotype, because at least one male relative with the same mutation did not have cryptorchidism at the time of examination

(29). The other cryptorchidism-specific mutations include R102H and R102C substitutions in C-peptide, described in patients with bi- or unilateral cryptorchidism, and P93L, found in two patients with unilateral cryptorchidism *(30)*. A mutation in the A-chain of INSL3 (N110K) was identified in a patient with the right testis located in the inguinal canal. N110 represents a highly conservative residue, therefore a suggestion was made that N110K substitution would be deleterious for the function of INSL3 *(31)*. Another study reported a B-chain mutation (P49S) in a patient with bilateral intraabdominal testes and under-masculinized genitalia. This patient had a 46XY karyotype, female external genitalia, but no uterus *(32)*. Functional analysis of the recombinant INSL3 peptides, containing all aforementioned substitutions, revealed that only P49S mutation affects the ability of INSL3 to activate its cognate receptor *(25)*. The region of INSL3 B-chain surrounding P49 is involved in the interaction with the receptor *(33)*, which might explain the compromised properties of the mutant peptide. Further functional, population, and if possible, hereditary analysis of other INSL3 alleles are needed to ascertain the role of these mutations in the etiology of cryptorchidism.

The INSL3 receptor, LGR8, is a G protein-coupled receptor with a large extracellular N-terminus, intracellular C-terminus, and a central part composed of seven transmembrane domains. The extracellular domain of LGR8 includes 10 leucine-rich repeats, believed to form highly organized structure participating in the ligand binding. T222P represents the only LGR8 mutation found so far, specific for the cryptorchid phenotype (Fig. 3; refs. *23* and *34*). The mutation was described in five patients with uni- and bilateral cryptorchidism, although some of the patients experienced spontaneous TD at puberty. Again, all patients contained this mutation in the heterozygous condition, which may explain the variability in the severity of the disease. At the amino acid level, the mutation affects one of the leucine-rich repeats and, according to functional analysis, renders the protein unable to be expressed on the cell surface membrane *(23,35)*.

Out of 730 patients analyzed to date for INSL3 variations, unique cryptorchidism-specific mutations were found in eight cases. Out of 184 patients analyzed for LGR8 variations, the cryptorchidism-specific mutation T222P was found in five cases. Thus, even if one assumes that all these mutations are deleterious for INSL3/LGR8 signaling and may lead to the testicular maldescent, this would explain only a small portion of all of the disease cases. An analysis of patients with a family history of undescended testes failed to reveal any of the INSL3/LGR8 mutations *(35)*. The fact that individuals with similar deleterious mutations are presented with different clinical phenotypes suggests that the

other genetic and/or endocrine factors might affect the severity of INSL3-related deficiency.

4. THE ROLE OF STEROID HORMONES IN INGUINOSCROTAL DESCENT

A critical role in the second phase of TD was attributed to the hormonal status of the developing embryo *(7)*. It has been shown that androgen antagonists can produce cryptorchidism. This is consistent with the phenotype of testicular feminized (*Tfm*) mice and human patients lacking androgen receptors. In both cases, intraabdominal testes are situated at the level of the bladder neck with no eversion of the scrotal sac *(36,37)*. It is well known that testosterone regulates development of derivatives of the Wolffian duct, such as the epididymis, vas deferens, and seminal vesicles, as well as the development of the external male sex organs. During TD, testosterone fails to cause full differentiation of the gubernacula in *Tfm* and in *Gnrh* (*hpg*) or *Gnrh* receptor-mutant mice. Males of these two mutant genotypes, as well as mice with mutations of the LH receptor *(38)*, show low intraabdominal cryptorchidism. Additionally, androgens are involved in proper development of the processus vaginalis and differentiation of the inguinal canal.

It was proposed that in addition to the direct effect on gubernacular differentiation, androgens might act indirectly through the genitofemoral nerve (GFN) releasing its principal neurotransmitter calcitonin gene-related peptide. Disruption of the GFN in pathological cases or experimental animals leads to cryptorchidism. In the latter situation, it can be at least partially corrected by calcitonin gene-related peptide *(39)*. It has been suggested that in addition to the direct androgen action on the gubernaculum, the GFN mediates androgen effects on early postnatal gubernacular DNA synthesis and growth, especially in differentiating muscle cells of the gubernacular bulb and cremaster muscles.

The link between cryptorchidism and hormone deficiencies is also evident in human congenital disorders that cause hypogonadism or androgen resistance, such as functional prepubertal castrate syndrome, Noonan's syndrome, Klinefelter's syndrome, Reifenstein's syndrome, and hypogonadotropic eunuchoidism. All these conditions share a common theme of inadequate androgen function. At the same time, the significance of the hypothalamic–pituitary–gonadal (HPG) axis on the transabdominal stage of TD and its effect on gubernaculum differentiation is less clear.

The relation between INSL3 and the hormones of the HPG axis is not clear. The hpg and Gnrhr–/– mice apparently express Insl3 hormone, although the mutant males are cryptorchid *(40)*. Androgens synergize the effect of INSL3 on gubernaculum growth in vitro. On the other hand, as indicated previously, in tfm animals, the testes descend to the low abdominal position; the cranial suspensory ligaments remain in these mutants. Recently, we have shown that the overexpression of transgenic Insl3 on the Gnrhr-deficient background is sufficient for gubernacular differentiation and transabdominal descent of the gonads, both in male and female mice. However, the androgens are apparently required for the second stage of TD *(40)*. From the clinical standpoint, cryptorchidism in humans in most cases is not associated with abnormalities of androgen signaling. No specific mutations in the genes affecting androgen signaling were reported in patients with isolated cryptorchidism, although, as indicated previously, cryptorchidism is a common feature of different syndromes affecting hormonal balance in the developing embryos.

5. ENVIRONMENTAL FACTORS AND GENETICS SUSCEPTIBILITY

The modulation of hormonal homeostasis during development is another potential cause of testicular maldescent. It has been shown that the uterine exposure to environmental endocrine disruptors (EEDs) has deleterious effects on male reproductive tract development *(2,5,6)*. Experiments with laboratory animals clearly show that male progeny of pregnant females treated with various EEDs develop with the cryptorchid phenotype. TD was significantly inhibited by estradiol or the nonsteroidal estrogenic substance diethylstilbestrol *(2,5)*. The estrogen effect might be mediated through suppression of fetal Leydig cell development *(41)*, with a resulting decrease of androgen and INSL3 production. Alternatively, estrogens can directly target development of the cranial gonadal ligament and the gubernaculum, which both express estrogen receptors *(42)*. The failure of gonadotropin and testosterone injections to reverse estrogen-induced cryptorchidism in fetal mice *(43)* suggests that the second scenario may be true. It has been reported that the incidence of cryptorchidism in human populations seems to be on the rise, at least in industrial countries *(2)*. In farming communities, an increase risk of cryptorchidism was associated with exposure to EEDs *(6)*. From a genetic standpoint, the question arises whether there are specific alleles of the genes involved in TD that may have an increased sensitivity to EEDs.

Recently, an association was reported between a homozygosity for a specific haplotype (AGATA) of the estrogen receptor 1 (ESR1) gene and cryptorchidism *(44)*. It was shown that the homozygosity for one of the single-nucleotide polymorphism-defined haplotype of ESR1 variant was found only among Japanese patients with undescended testes. Significantly, no deviations from the normal range of basal serum gonadotropin and testosterone were detected in the patients, and there were no mutations in the androgen receptor gene, 5α-reductase-2, INSL3, or other genes, as well as no karyotype or Y-chromosome abnormalities. Based on these results, the authors suggested that a specific ESR1 allele might be responsible for cryptorchidism. Surprisingly, no mutations were found in the exons of ESR1 allele associated with these haplotype. We have recently screened 44 patients with idiopathic cryptorchidism from the United States and Europe for the presence of the AGATA haplotype. Although we have detected five patients heterozygous for this haplotype, no homozygous patients were found in our samples, thus indicating that the ESR1 link with cryptorchidism might be population-specific.

6. TRANSCRIPTIONAL FACTORS INVOLVED IN TESTICULAR DESCENT

Genetic targeting of *Hoxa10* and *Hoxa11* homeobox genes resulted in uni- and bilateral low abdominal cryptorchidism *(45–47)*. Both the gubernacular cord and bulb are sites of strong *Hoxa10* expression at day 15.5 postcoitum and postnatally, indicating that the abnormality is directly affected this organ. It was also shown that the spinal nerves undergo transformation, which might affect the GFN innervation of gubernacular and cremaster muscles. Shortening of the gubernacular cord and outgrowth of the bulb failed to occur in mutant mice. Cremasteric myocytes are disorganized and reduced in number. In addition to cryptorchidism, the mutant mice display homeotic transformation of vertebrae and lumbar spinal nerves. In our experiments, an overexpression of transgenic Insl3 in mice deficient for the *Hoxa10* gene does not correct the cryptorchid phenotype in males. These results suggest that although Insl3 is sufficient to direct the first transabdominal phase of TD in the absence of HPG axis signaling or Hoxa10, their presence is important for the inguinoscrotal phase of TD.

Mice deficient for Desrt, also known as AT-rich interactive domain 5B (Arid5b), manifest growth retardation, defects in male reproductive organs, and a cryptorchid phenotype *(48)*. A recent study showed an

induction of smooth muscle marker genes, including smooth muscle α-actin and smooth muscle 22α, and retarded cellular proliferation in cells overexpressing Arid5b *(49)*. These data implicate Arid5b as a novel regulator of smooth muscle cell differentiation and proliferation. It should pointed out that although Hoxa10, Hoxa11, and Desrt mutant mice exhibit the cryptorchid phenotype, they also display an array of abnormalities not related to male development, complicating the assessment of their role in TD.

Mutation analysis of human *HOXA10* gene has been conducted in patients with cryptorchidism *(50,51)*. The first paper described several mutations and a 24 bp deletion in *HOXA10*, some of them found exclusively in affected patients *(50)*. However, no cryptorchidism-specific variants were detected in another population *(51)*. Thus, the significance of the detected mutations for the functional properties of the mutant proteins as well as their cause–effect role in cryptorchidism remains to be proven.

7. CONCLUSIONS

Progress in genome sequencing and analysis of transgenic mouse mutants has uncovered a number of genes involved in TD. The analysis of the *INSL3/LGR8* signaling revealed its function in the transabdominal phase of TD. Several mutant alleles of these two genes were associated with human cryptorchidism, and it was demonstrated that the function of some of the mutant ligands and receptors was compromised. However, direct evidence of the involvement of these genes in human disease is still missing. Similarly, some specific alleles of *HOXA10* and the homozygosity for the ESR1 haplotype were associated in some populations with cryptorchidism. It is clear, however, that mutations in these genes are unlikely to explain the majority of the abnormalities observed in human populations.

The question arises, “what is the basis for the failure to detect human mutations in the candidate genes identified from mouse mutant studies?” Several possible reasons can be pointed out. First, TD is a complex process, which occurs during a significant time period in embryonic development and involves a number of different phases. Each step may be controlled by a number of factors, and thus the resulted cryptorchid phenotype can be caused by a failure of multiple genetic and hormonal signaling pathways. Second, the cryptorchidism-causing mutations directly affect male fertility, and therefore are under constant negative selective pressure in the population. Third, the candidate genes identified based on the rodent studies may

play a lesser role in the etiology of the human disease as a result of the significant differences in the development of reproductive organs between these species. With completion of the genome sequencing of other mammalian species, such as dog and pig, new genetic animal models and phenotype-driven cloning of the cryptorchidism causative genes may provide new clues to the mechanisms of the testicular maldescent in human population.

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12 The Chromatoid Body and microRNA Pathways in Male Germ Cells

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Summary

The chromatoid body (CB) is a finely filamentous, lobulated perinuclear granule located in the cytoplasm of male germ cells. The role of the CB in the mouse has remained elusive, although it was proposed to be involved in RNA storing and metabolism. We have found that the CB is related to the RNA processing body of somatic cells and that it seems to operate as an intracellular nerve center of the microRNA (miRNA) pathway. Our findings underscore the importance of posttranscriptional gene regulation and of the miRNA pathway in the control of postmeiotic male germ cell differentiation.

Key Words: Transcription; translation; VASA; MIWI; argonaute; RISC; Dicer.

1. INTRODUCTION

The program of gene expression during spermatogenesis is based on unique rules. A very special feature concerns the process of chromatin remodeling, which involves various unconventional steps with respect to somatic cells. In addition, many genes use alternative promoters and splice isoforms specific for the male germline. Transcription in germ cells during spermatogenesis follows a highly specialized program corresponding to a series of differentiation steps occurring in spermatogonial cells, spermatocytes, and haploid spermatids *(1,2)*. In the testis, specific gene expression is in part achieved through transcription factor cyclic adenosine monophosphate response element modulator (CREM)

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and its cofactors, which demonstrate that a testis-specialized transcription machinery has evolved. This germ cell-specific program of activation and silencing of gene expression is essential for the coordination of postmeiotic events required for sperm development and function. One characteristic event is the arrest of transcription in spermiogenesis, which coincides with changes in the acquisition of the transcriptional machinery and chromatin compaction. Indeed, at the transition from round to elongating spermatids there is accumulation of a remarkable amount of transcripts whose translation is repressed for various days. Although this process constitutes an essential regulatory step, the molecular mechanisms involved in translational repression are not fully understood.

2. UNIQUE CHROMATIN REMODELING AND TRANSCRIPTIONAL REGULATION IN MALE GERM CELLS

Spermatogenesis follows a program finely regulated by the hypothalamic–pituitary axis that features the transformation of an undifferentiated diploid stem cell into highly differentiated haploid spermatozoa. Differentiation of germ cells into spermatozoa occurs in the seminiferous epithelium, and relies on a complex paracrine dialog with Sertoli cells. Testosterone secreted by Leydig cells under the influence of pituitary-secreted luteinizing hormone and follicle-stimulating hormone acting on Sertoli cells stimulates gene transcription and the secretion of peptides that promote germ cell differentiation. Biochemical stimulation of germ cells is thought to occur via the secretion of regulatory molecules from Sertoli cells, such as growth factors and proteases. Although evidence exists for the control of gene transcription from the hypothalamic–pituitary axis *(3)*, how these highly specialized endocrine regulations are involved in the control of chromatin remodeling is still unclear.

The postmeiotic developmental phase of spermiogenesis involves the differentiation of spermatids into spermatozoa. It constitutes a remarkable process as germ cells undergo an enormous morphogenetic transformation involving acrosome and flagellar formation, DNA compaction, and cytoplasmic ejection. Male germ cell-specific nuclear proteins, the transition proteins and protamines, sequentially replace histones to allow for DNA compaction and permit reshaping of the round spermatid nucleus. In addition to the histone-to-protamine transition process, a remarkable number of histone variants are present in germ cells, whose expression follows a highly dynamic program *(4)*. Many of the histone

variants are testis-specific, underscoring the importance of highly specialized epigenetic events in the structuring and regulation of chromatin.

A significant number of gene promoters display a highly restricted activity to male germ cells. This notion provides evidence for the presence in these cells of a unique balance of common regulators and germ cell-specific factors. Following meiosis, the beginning of spermiogenesis is characterized by a massive wave of transcriptional activity *(5)*, which results in the activation of a number of essential postmeiotic genes in early haploid cells. To insure this efficient and timely transcription, various general transcription factors, such as TATA binding protein (TBP) and Transcription Factor IIB (TFIIB), are differentially regulated in germ cells *(6)*. In addition, some testis-specific examples of transcriptional regulatory complexes have been reported. One of these includes CREM and its co-activator, activator of CREM in testis (ACT; ref. *7*). The targeted ablation of both *crem* and *act* genes by homologous recombination in the mouse germline results in impaired spermatogenesis *(8,9)*. The activity of the CREM–ACT complex is modulated by a special regulatory system: a testis-specific kinesin, KIF17b, is responsible for the nuclear export of ACT, determining the interruption of CREM-dependent postmeiotic gene transcription *(10)*.

One aspect of gene regulation that is not fully explored in male germ cells concerns the regulation of RNA function, stability, and translation by the microRNA (miRNA) pathway. Because of the previously mentioned translational repression of postmeiotic transcripts, it is reasonable to assume that miRNAs are indeed likely to play an essential role during spermatogenesis. Interestingly, MIWI, a member of the PIWI subfamily of Argonaute proteins, associates with ribohomopolymers and specifically with transcripts of CREM-target genes, suggesting a functional connection of MIWI to RNA processing *(11)*.

Argonaute proteins are widely expressed in many tissues. In contrast, all members of the PIWI family are expressed mainly in testis *(12,13)*, although their role in the RNA interference (RNAi) pathway is still obscure. It is tempting to speculate that MIWI may operate as a male germ cell-specific miRNA pathway component involved in functional messenger RNA (mRNA) regulation in testis.

3. THE CHROMATOID BODY

The remarkable history of the chromatoid body (CB) began more than 100 yr ago, when it was first described by Benda *(14)*. For many years, its presence remained unappreciated and then its origin was debated, as being nuclear *(15)*, nucleolar *(16)*, or a derivation from intermitochondrial dense

material during the late pachytene stage of the prophase of the first meiotic division *(17,18)*. The CB appears to be a very conserved feature of germ cells throughout the animal kingdom *(19)*. These contain cytoplasmic cloud-like accumulations of material-denominated nuage. In *Drosophila*, the polar bodies present in the oocytes are also identified as nuage. In mammalian spermatogenic cells, the equivalent of nuage corresponds to the CB. In early spermatids of the rat, it has a diameter of 1–1.5 μm and a finely filamentous lobular structure (Fig. 1). Typically, it is found associated with a multitude of vesicles. The CB is first seen in mid- and late-pachytene spermatocytes as an intermitochondrial dense material. During early spermiogenesis, the CB is seen near the Golgi complex and frequently connected by material continuities through nuclear pore complexes with intranuclear particles *(20)*.

The varying localization of the CB during successive stages of spermatogenesis is one of its primary features. It appears at the nuclear envelope in association with nuclear pore complexes during early spermiogenesis and associates with the annulus later in spermiogenesis *(17,21)*. In living cells, the CB moves around the Golgi complex and has frequent contacts with it. The CB also moves perpendicularly to the nuclear envelope and even through cytoplasmic bridges to the neighbor spermatids *(20)*.

The presence of RNA *(22)* and ribonucleoprotein *(23)* in the CB as well as in all nuage material in spermatogenic cells *(24)* was first suggested by histochemical studies, whereas the presence of DNA has been excluded *(25)*. There are indications that the CB may function as a source of mRNA and/or of its partially processed precursors during the late stages of spermiogenesis, when the spermatid nucleus becomes gradually inactive *(25,26)*. In support of this hypothesis, it was found that TP2 mRNA and mRNA-binding proteins p48/52 localize in the CB *(27,28)*.

Interestingly, a major CB component is the DEAD-box RNA helicase, VASA. This protein is thought to act as an RNA chaperone and is a general marker of all germ cells. The mouse VASA homolog, MVH, was recently used as a marker of sperm formation from embryonic stem cells *(29)*. The features described previously and the presence of MVH indicate that the CB could constitute a structure underlying the mechanisms of posttranscriptional processing and storage of several mRNA species in germ cells.

4. THE miRNA PATHWAY

RNAi and miRNA pathways are evolutionarily conserved control mechanisms that use RNA molecules to inhibit gene expression at the

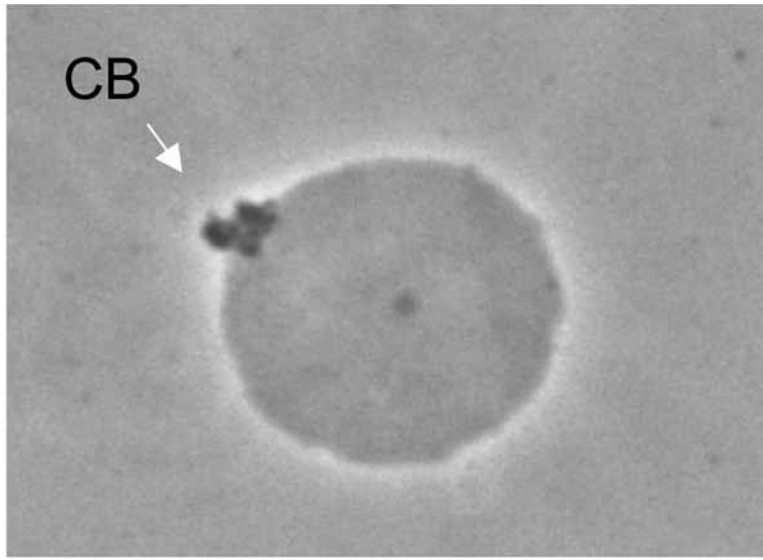

Fig. 1. The chromatoid body (CB) appears as a cloud-like, dense structure in the cytoplasm of male germ cells. Its perinuclear localization is evident in this phase contrast image. For a comprehensive review article on the CB, *see* ref. *20*.

level of mRNA degradation, translational repression, or chromatin modification and silencing *(30–32)*. RNAi has been shown to be present throughout spermatogenesis in mice *(33)*, but its function and cellular control during germ cell development remain uncertain.

Two classes of 21- to 25-nucleotide small RNAs, small-interfering RNAs (siRNAs) and miRNAs, act as sequence-specific regulators of gene expression *(31)*. siRNAs mediate degradation of mRNAs, having sequences fully complementary to their sequence, whereas miRNAs are proposed to regulate gene expression by inhibiting protein synthesis through imperfect basepairing to the 3′-untranslated region of target mRNAs *(30,31)*. Both siRNA and miRNA precursors are processed to mature small RNAs in the cytoplasm of cells by the large endonuclease Dicer *(30,34)*. Mature miRNAs and siRNAs are assembled into miRNA- and siRNA-induced silencing complexes (miRISC and siRISC, respectively), which subsequently act on their targets by translational repression or mRNA cleavage (Fig. 2). Essential components of RISC complexes are the members of the Argonaute family of proteins *(13)*. These proteins share the so-called PAZ and PIWI domains and are classified into two subfamilies depending on sequence similarity to either *Arabidopsis argonaute1* or *Drosophila piwi (12,13)*. In mammals, argonaute1 subfamily members, Ago1 to Ago4, have been

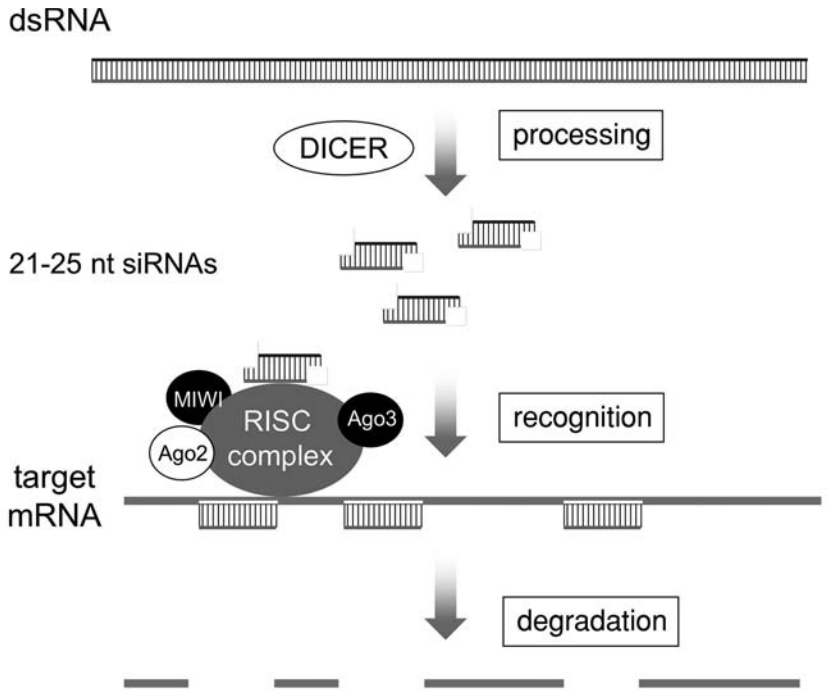

Fig. 2. Schematic representation of the silencing pathway of gene expression induced by small RNAs. The RNA interference machinery converts the sequence-specific information of long double-stranded RNAs (dsRNAs) into small, 21- to 25-nt long dsRNAs (small interfering RNAs, microRNAs), which assemble into an effector complex, the RNA-induced silencing complex. For the role of Dicer and the Argonaute proteins, *see* Subheading 4, and refs. *30–34*.

shown to be involved in the RNAi/miRNA pathway *(35,36)*. All four members of the PIWI subfamily are mainly expressed in testis *(12)*, and two of them, MIWI and MILI, are crucial for progression through spermatogenesis in mouse *(11,37)*.

5. FUNCTIONAL LINK OF THE RISC PATHWAY WITH THE CB

The highly restricted localizaton of MVH in the CB has been used as specific marker for this structure *(38)*. We analyzed the formation of the CB along the differentiation of postmeiotic germ cells and found that MVH is expressed throughout the development of round spermatids. Strikingly, this pattern correlates with MIWI distribution, which indeed colocalizes with MVH. In addition, other members of the Argonaute family of proteins, including Ago2 and Ago3, were found in

the CB *(39)*. As it has been recently shown that in somatic cells Ago proteins localize in cytoplasmic processing bodies (P-bodies; refs. *40* and *41*), these findings suggested a possible functional similarity between P-bodies and the CB. This similarity included also other important components, such as the decapping enzyme component Dcp1a *(42)*, the 5′ to 3′ exonuclease Xrn1, and the RNA-binding protein GW182, all present in P-bodies and found in the CB. Thus, the P-bodies of somatic cells and the CB of male germ cells share a number of significant similarities.

The coordinate presence of various components of the RISC pathway in the CB was strongly suggestive of miRNAs production in this site. Indeed, *in situ* hybridizations using probes specific for various miRNAs known to be expressed in testis demonstrated their localization with Ago proteins in CBs. The analysis revealed high concentration of miR-21, miR-122a, and let-7a, which all colocalized with MVH in CB *(39)*. Interestingly, the use of *in situ* hybridization using oligo dT probes for mRNA molecules, the natural targets for miRNA-mediated regulation, demonstrated accumulation of mRNAs in CBs. Thus, the CB seems to concentrate important elements of the RISC complex and of the miRNA pathway.

The role of the protein Dicer in the miRNA pathway is central (Fig. 2). Dicer processes miRNA precursor molecules folded into double-stranded RNA-like hairpins to mature miRNAs. These are subsequently transferred to the miRISC complex where the effector phase of the process takes place *(30,34)*. A direct interaction between Dicer and Ago proteins may be required for the transfer of miRNAs to the effector complex *(34)*. Importantly, Dicer is present in both meiotic spermatocytes and postmeiotic round spermatids, but not in elongated spermatids. We have found that the Dicer protein in testis is enzymatically active and that it concentrates in CBs, coordinately with the other elements of the miRNA machinery *(39)*.

One additional finding that could be highly relevant for understanding the role played by the CB is the physical interaction of Dicer with MVH *(39)*. Although the role of this DEAD-box RNA helicase with respect to the enzymatic activity of the Dicer protein still needs to be established, it would appear that MVH constitutes a male germ cell-specific component of the miRNA pathway.

6. CONCLUSIONS

The functional role and the molecular nature of the CB in germ cells has been elusive and debated for many years *(20)*. Our results indicate

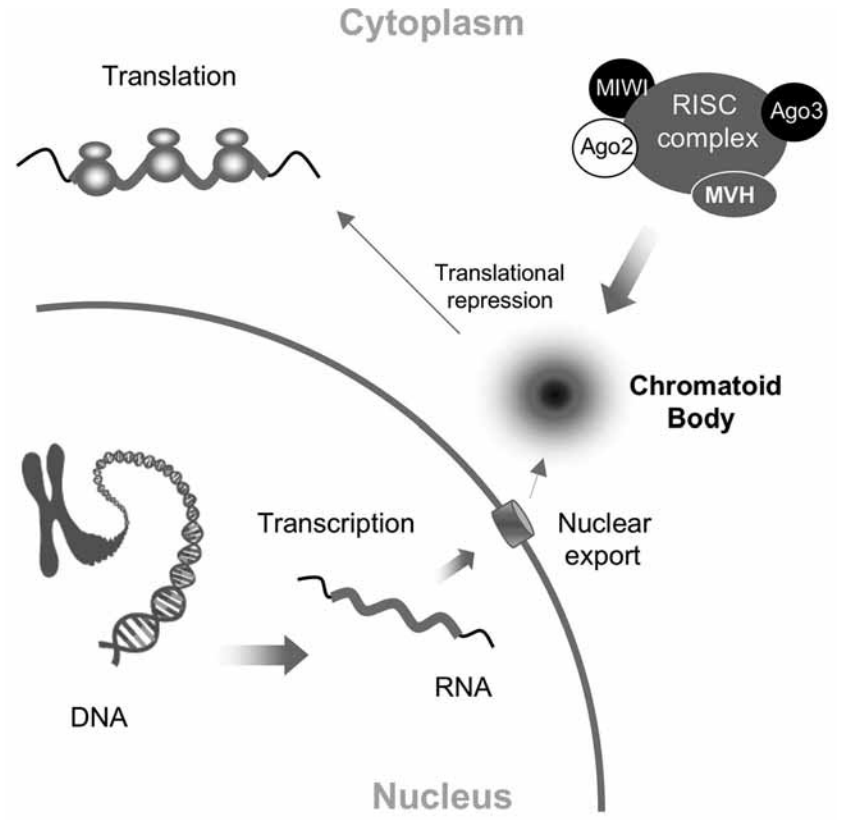

Fig. 3. Model of chromatoid body (CB) function in postmeiotic male germ cells. After transcription, haploid gene transcripts are assembled in the ribonucleoprotein particles containing RNA-binding proteins and transported through nuclear pore complexes into the cytoplasm where they are loaded to the CB. Argonaute family members, including MIWI, Ago2, and Ago3, and the RNA-induced silencing complex are also located in the CB. CBs contain RNA-binding and -processing proteins, such as the adenosine triphosphate-dependent RNA helicase of the DEAD-box protein family, mammalian VASA homolog, and the components of miRNA pathway that associate with mRNAs and direct them to storage or degradation.

that the CB would function as an RNA storing and processing structure. Specifically, the presence of Dicer/Argonaute and miRNAs reveals that the CB occupies a privileged position in posttranscriptional control of gene expression through the small RNAs pathway (Fig. 3).

The expression and localization of components of the siRNA and miRNA pathways during spermatogenesis has not been fully explored. RNAi has been shown to be active during the whole spermatogenesis program *(33)*, and many miRNAs whose expression is enriched in testis have been identified *(43,44)*. Our findings importantly expand our

knowledge and link these pathways to a cytoplasmic, structural organelle: the CB *(39)*. Interestingly, the CB is endowed of the remarkable property of moving very actively and three dimensionally in the cytoplasm of round spermatids. During these movements, CBs make frequent contacts with the nuclear envelope. Continuity in electron-dense material between the nucleus and the CB through nuclear pore complexes has been observed *(20)*. One hypothesis suggests that rapidly moving CBs collect mRNA leaving the nucleus. Interestingly, miRNA precursors processed in the nucleus are exported to the cytoplasm through nuclear pore complexes *(45)*. Based on our findings, we suggest a model in which pre-miRNAs transported to the cytoplasm are loaded through nuclear pores to the CB. Thus, the CB functions as a subcellular concentration site for components of the miRNA pathway, centralizing the miRNA posttranscriptional control system in the cytoplasm of haploid male germ cells.

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13 Sperm Maturation in the Epididymis

Role of Segment-Specific Microenvironments

Gail A. Cornwall, PhD
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Summary

Spermatozoa undergo a maturation process and acquire motility and fertility as they migrate from the proximal to the distal end of the long convoluted tubule known as the epididymis. Regions of the epididymis are subdivided into discrete segments defined structurally and functionally by connective tissue septa. Each segment is a unique microenvironment that together allows maturation to occur. This chapter focuses on how these distinct microenvironments are created, including the involvement of segment-specific expression of secretory proteins and cellular proteins allowing each segment to respond uniquely to external stimuli and novel mechanisms the epididymis uses to deliver and remove proteins from the epididymal lumen.

Key Words: Epididymis; sperm maturation; CRES; fertilization; lumacrine.

1. WHY STUDY THE EPIDIDYMIS?

Although it is well known that the development of germ cells occurs in the testis in a process known as spematogenesis, it is often overlooked that testicular sperm are nonfunctional gametes and lack the ability to naturally fertilize an egg. It is only after sperm migrate through the epididymis and undergo a maturation process that they acquire progressive motility and the ability to fertilize. Although significant progress has been made over the past years, we still have yet to identify the critical molecular and biochemical pathways that allow maturation to occur.

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The necessity of understanding the normal processes of epididymal sperm maturation is emphasized by the fact that up to 40% of infertile men exhibit idiopathic infertility, which in many cases can reflect maturational disorders. Assisted reproductive technologies, such as intracytoplasmic sperm injection, are effective treatments for ididopathic infertility, because one only needs access to spermatozoa regardless of functional maturity. However, intracytoplasmic sperm injection seems to have become the end to all treatments, such that the basic investigation of epididymal function has, to some degree, fallen by the wayside. This is indeed a frightening observation because common sense would dictate that most couples trying to conceive would prefer the least invasive method possible to circumvent their fertility problem and, if the processes of sperm maturation were defined, approaches to maturing sperm in vitro could be developed.

The importance of epididymal study is also underscored by the continued lack of development of a male contraceptive. Although considerable emphasis has been placed on developing male contraceptives, the focus has primarily been on using a hormonal approach (a combination of testosterone and progestin) to disrupt spermatogenesis. Although this approach should work in theory, currently there are profound limitations, including the requirement of repeated injections, long periods of time to achieve an effect as well as to reverse the contraceptive effect, and the possibility of undesirable side effects including changes in libido. Unless these disadvantages can be improved, the worldwide acceptance of a hormone-based male contraceptive comes into question. Indeed, although oral hormonal contraceptives for women have been in place for decades and are considered to be relatively safe, the worldwide acceptance rate is only 20%, whereas in the United States the acceptance is only 10% *(1)*. Thus, alternatives are needed for a male contraceptive. Targeting specific molecules in the epididymis would be a more attractive approach than disrupting spermatogenesis because the contraceptive effects would be rapid and more readily reversible and side effects associated with alterations in hormones would be avoided.

With these priorities in mind, epididymal biologists have identified key areas of epididymal research that need further emphasis, including studying spermatozoa and associated maturational changes during epididymal transit, the specifics of sperm–protein interactions as well as sperm interactions with nonprotein molecules in the epididymal lumen, studies of the cytoplasmic droplet and its role in maturation, the development of better in vitro systems to facilitate study of sperm maturation, understanding the mechanisms of sperm storage and maintenance of sperm activity, examination of interstitial–epithelial cell interactions,

as well as identifying functions for the vast number of epididymal secretory proteins that are part of the epididymal luminal microenvironment *(2)*. From studies thus far it is becoming apparent that sperm maturation is not only the secretion and binding of a select group of proteins to the sperm surface, but rather involves an incredibly sophisticated and finely tuned relationship between the epididymal epithelium and spermatozoa. Further studies in the many areas described previously are needed to provide a sound knowledge of the epididymis from which new therapies and contraceptive targets can be developed.

The goal of this chapter is to summarize in brief the recent progress in the field of epididymal biology with particular focus on regionalized gene expression and study of epididymal secretory proteins and their putative functions in sperm maturation, novel mechanisms for the delivery of proteins to spermatozoa during epididymal transit, as well as unique mechanisms for the functional control and removal of secretory proteins from the epididymal lumen. It is hoped that the reader will become as intrigued as the authors are by the incredible complexities of this organ including the distinctive mechanisms the epididymis uses to achieve its primary goal, namely the maturation of spermatozoa and thus perpetuation of the species.

2. SPERM MATURATION IN THE EPIDIDYMIS: SEGMENT-SPECIFIC MICROENVIRONMENTS

In brief, spermatozoa exiting the testis and entering the single, long, convoluted tubule known as the epididymis are nonfunctional in that they lack progressive motility and the ability to fertilize an egg. As sperm migrate from the proximal to the distal epididymis, they undergo a maturation process and acquire motility and fertility. It is generally accepted that epididymal sperm are, for the most part, synthetically inactive and thus maturation requires the interaction of sperm with proteins that are synthesized and secreted by the epididymal epithelium. The epididymis is grossly divided into three regions: the caput (head), corpus (body), and cauda (tail). The most proximal caput region, in some species, is also known as the initial segment. Each epididymal region carries out distinctive functions, with the caput and corpus carrying out early and late sperm maturational events, respectively, while the cauda region primarily serves as a storage site for functionally mature spermatozoa.

Although the primary cell type along the epididymal tubule remains the same from the proximal to the distal epididymis, of considerable interest to epididymal biologists is the highly regionalized gene expression

within these epithelial cells. Neighboring cells can express quite different subsets of genes, which contribute to the ever-changing luminal environment that spermatozoa encounter as they move from the proximal to the distal epididymis and which ultimately allow sperm maturation to occur. Although previously it was thought that varying patterns of gene expression along the tubule were loosely associated with different epididymal regions, work by Turner et al. in 2003 demonstrated that the presence of connective tissue septa further subdivides the caput, corpus, and cauda epididymis into discrete intraregional segments and that region-specific gene expression may in fact be highly ordered and compartmentalized within these precise segments *(3)*. Indeed, examination of β-galactosidase activity by immersion of whole mouse epididymis in X-gal solution revealed a precise staining in the initial segment, no staining in the remainder of the caput, and intense staining in the corpus epididymis. Furthermore, the β-galactosidase activity was precisely contained with discrete segments delineated by the connective tissue septa with changes in expression occurring as the tubule passed through the septa *(3)*. By using size exclusion dyes and radiolabeled molecules, these authors further demonstrated that the connective tissue septa may also act as barriers, restricting the movement of molecules from the interstitial space of one segment to the next. This would allow segment-specific paracrine signaling to occur between stromal and epithelial cells, which could regulate the tightly controlled segment-specific expression of genes *(3)*.

Thus the epididymal tubule is a highly ordered and segmented organ, with each segment representing a unique physiological compartment. Each compartment possesses distinctive gene expression profiles within the epithelium that dictate segment-specific secretion of proteins into the luminal fluid, directly or indirectly affecting sperm maturation. Segment-specific expression of genes encoding signaling molecules, regulatory proteins, transporters, and receptors also contribute to the formation of unique microenvironments by allowing the epithelium to respond uniquely to different stimuli, such as hormones and other regulatory factors (Fig. 1). Identifying and determining the function of segment-specific proteins is of paramount importance for understanding epididymal sperm maturation.

2.1. Segment-Specific Gene Expression

Space limitations preclude a thorough discussion of the many genes that exhibit regionalized gene expression in the epididymis. Therefore, we mention only briefly groups of genes based on their putative functions. Although much of the earlier data was drawn from published

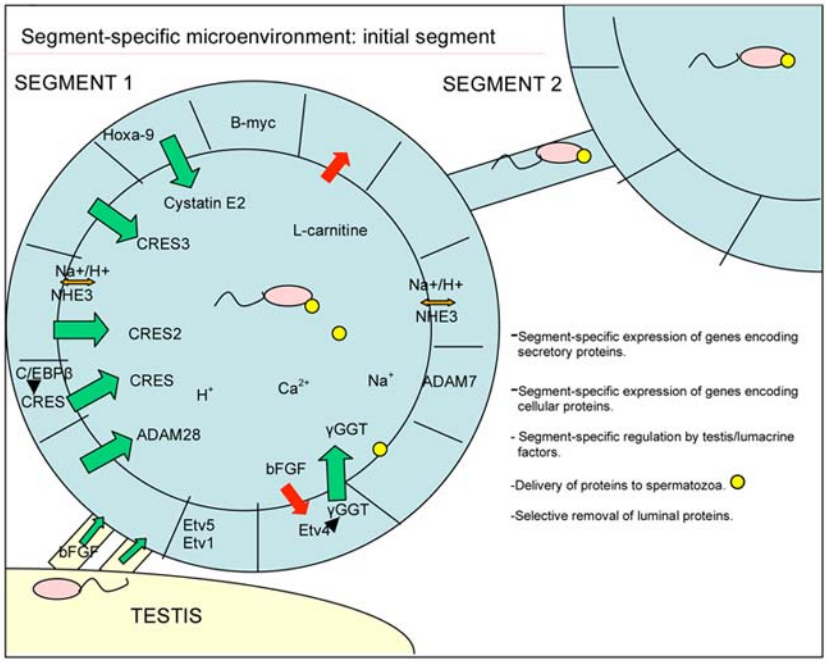

Fig. 1. Schematic representation of factors contributing to the formation of a segment-specific microenvironment in the epididymal initial segment of the mouse. Examples of secreted and cellular proteins are noted.

reports of expression levels determined by reverse transcriptase-polymerase chain reaction or Northern analysis of epididymal regions *(4)*, most recently the application of gene profiling technology to the epididymis has yielded volumes of information of segment-specific gene expression that is available to the public. In particular, our laboratory screened 15K expressed sequence tags from the National Institute of Aging that were derived from pre- and peri-implantation embryos, embryonic day 12.5 female gonad/mesonephros, and newborn ovary comparing the mouse initial segment with the remainder of the epididymis *(5)*. Because the gene chips used in these studies represented sequences derived from early-stage embryos, the microarray analysis was biased toward identifying new subsets of genes expressed in the adult epididymis. This information is available at http://www.ttuhsc.edu/cbb/faculty/cornwall/nelson/supplemental.xls. Subsequent to our studies, Johnston et al. examined the varying expression profiles of genes from all segments of the mouse epididymis using Affymetrix mouse gene chips *(6)*. This information is available in a searchable website at the Mammalian Reproductive Genetics database (http://mrg.

genetics.washington.edu). Because each study used different sources of sequences, a broad representation of sequences is presented. Following is a brief summary of genes and gene products exhibiting regionalized expression in the epididymis, including proteases and protease inhibitors, modifying enzymes, signaling molecules, and transcription factors.

During epididymal transit, sperm-associated proteins including ADAM2 (fertilinβ), ADAM3 (cyritestin), ADAM24 (testase), CE9, and others are proteolytically processed to their mature and presumably functionally active forms *(7–10)*. Although the identity of the proteases involved in the activation of these proteins is not known, furin-like proteases have been implicated for several of these processing events *(7)*. Recent studies by our laboratory show that several members of the prohormone convertase family of proprotein processing enzymes including furin, PC7, PC4, and PACE4 are expressed in the epididymis in a regionalized manner and several are present in epididymal fluid suggesting a possible role for these proteases in sperm maturational events (Cormier and Cornwall, unpublished data). Indeed, PC4 knockout male mice are infertile despite normal spermatogenesis and motility, suggesting that critical fertilization molecules may not be processed correctly *(11)*. Other proteases expressed in a segment-specific manner in the epididymis include several of the matrix metalloproteases, MMP2, MMP3, MMP9 *(12)*, ADAM28 *(13)*, and procathepsin L *(14)*.

Several protease inhibitors that show segment-specific expression have also been identified. Several members of the cystatin-related epididymal spermatogenic (CRES) subgroup of family 2 cystatins of cysteine protease inhibitors including CRES, CRES2, CRES3, and cystatin E2 are highly restricted to the initial segment region of the mouse epididymis *(15–18)*. Although the function of these secretory proteins in vivo is not known, in vitro CRES is an inhibitor of several members of the prohormone convertases *(19)*, suggesting CRES may regulate proprotein processing events in the epididymis. Other protease inhibitors expressed in the epididymis include Eppin, a member of the whey acidic protein type four–disulfide core gene family. In the human, Eppin associates with semenogelin on the surface of ejaculated spermatozoa and may provide antimicrobial activity for spermatozoa *(20)*. Studies in which primates were immunized with Eppin resulted in a contraceptive effect in 78% of the monkeys that was reversible in 71%, suggesting an important function for this protein in fertility and a possible role as a male contraceptive *(21)*.

Other gene and gene products expressed in a region-dependent manner in the epididymis include those encoding antioxidant enzymes such γ glutamyl transpeptidase, glutathione peroxidases, and superoxide dismutase *(22–25)*. Because the epididymal lumen is oxygen-rich, unsaturated fatty acids in sperm membranes may be susceptible to oxidative damage. The expression of these enzymes may protect spermatozoa from oxidative damage. Lysosomal enzymes such as β-hexosaminidase, α mannosidase, and β-galactosidase are secreted into the epididymal lumen, where they may affect sperm function either directly or indirectly by modifying carbohydrate moieties *(4,26,27)*.

A variety of signaling molecules are also expressed in a region-dependent manner in the epididymal epithelium. It is likely that these proteins respond to various external stimuli in the luminal environment, ultimately affecting epithelial cell function. For example, in the caput, several members of the retinoid signaling pathway are expressed such as epididymis-specific extracellular retinoic acid-binding protein and the related MEP17, mMUP4-L, and mEP19, cellular retinoic acid-binding protein and retinoic acid α receptor *(28)*. It is likely that these proteins participate in the delivery and trafficking of retinoids to and within the epididymal cells.

The bone morphogenetic proteins belong to the transforming growth factor-β superfamily of growth factors and function as signaling molecules. Bone morphogenetic protein (BMP) 7 and BMP8a are both expressed in the epididymis and the loss of *Bmp8a* gene function leads to epididymal degeneration that ultimately results in infertility *(29)*. Interestingly, although both BMPs are expressed in the initial segment, the degenerative effects observed in the *Bmp8a* knockout are observed in more distal epididymal regions, suggesting a possible paracrine role for BMP8a in the epididymis. Several peptides such as proopiomelanocortin, β-endorphin, proenkephalin, and neuropeptide Y are expressed in the epididymis and may also function in paracrine signaling pathways *(4)*.

The region-dependent expression of genes implies that there are region-dependent transcription factors. Perhaps the most well studied are the members of the *Etv4* subfamily including *Etv4*, *Etv5*, and *Etv1*. All family members are expressed in the initial segment, and their messenger RNAs (mRNAs) are profoundly reduced following the loss of signaling from the testis (*see* section 3.), suggesting these transcription factors may regulate a subset of genes dependent on testicular luminal fluid factors *(30)*. Other transcription factors expressed in the initial segment include the androgen receptor *(31)*, estrogen receptor α *(32)*, B-myc *(33)*, C/EBPβ *(34)*, and Pem *(35)*. Estrogen receptor α expression

is important for normal fluid resorption by the epithelium and specifically is necessary for expression of a critical transporter NA+/H+ exchanger 3 as evidenced by examination of the knockout mouse *(32,36)*. C/EBPβ function is necessary for transactivation of the CRES gene *(34)*. Although the epididymal gene targets for B-myc are not known, cell culture studies suggest that it is involved in the regulation of cell growth *(37)*.

Although the regional or segment-specific expression of genes has been well-documented in the epididymis, the biological roles their gene products carry out is for the most part unknown. However, recent generation of knockout mouse models have revealed the critical roles some of these epididymal expressed genes play. The loss of several genes that are involved in fluid transport or signaling in the initial segment region including that for apolipoprotein E receptor 2 *(38)*, estrogen receptor α*(Esr1) (32,36)*, HE6 *(Gpr64) (41)*, or the complete loss of the initial segment region resulting from the loss of the c-ros tyrosine kinase receptor *(Ros1) (42)* leads to an inability of sperm to regulate their cell volume, resulting in a characteristic hairpin loop of the sperm flagella (Table 1). Other epididymal expressed genes, including those for lysosomal proteins, such as β-hexosaminidase and cathepsin A (*ctsa*) or follicle-stimulating hormone receptor (*Fshr*), exhibit an altered epithelium and/or changes in lysosomal size (Table 1; refs. *49–51*).

3. TESTIS/LUMACRINE REGULATION OF THE INITIAL SEGMENT

Studies have clearly established that the epididymis is an androgen-dependent organ. Indeed, following castration, epididymal weight decreases to 25% of intact after 2 wk. Restoration of circulating testosterone reverses the cellular changes in the caput, corpus, and cauda epididymis but not in the initial segment *(59)*. Supra-physiological levels of androgens also do not reverse these changes in the initial segment. Interestingly, ligation of the efferent ducts, which connect the testis to the epididymis and are the passageway for sperm and luminal components to enter the initial segment from the testis, results in a profound regression of the initial segment region *(59)*. Because ligation of the efferent ducts does not affect circulating androgen levels, these studies suggest that the maintenance of initial segment morphology requires components in the luminal fluid from the testis (i.e., lumacrine regulation; ref. *60*). Furthermore, gene expression studies revealed a subset of initial segment expressed genes that are down-regulated following efferent duct ligation, including CRES subgroup members, and others, suggesting that luminal factors are not only needed

Table 1
Gene Knockouts That Exhibit Epididymal Phenotypes

Gene	*Function/expression/Phenotype*	*Reference*
Apolipoprotein E receptor-2 *(apoer2)*	Member of the low-density lipoprotein (LDL)receptor gene family, functions in endocytosis and signal transduction. Expressed in initial segment. Cauda sperm exhibit flagellar angulation and impaired motility. Decreased level of phospholipid hydroperoxide glutathione peroxidase protein observed in epididymal sperm from knockout suggests altered ability of sperm to regulate cell volume decrease resulting in hairpin morphology of . sperm. Increased levels of clusterin in epididymal luminal fluid of the knockout suggests apoER2 also functions as a clusterin receptor.	*38*
Apolipoprotein B *(Apob)*	Lipid absorption and triglyceride homeostasis. Expressed in testis and epididymis. Heterozygous males show reduced fertility. Sperm do not fertilize in vivo or in vitro but fertilize eggs if zona pellucida is removed. Sperm counts and motility decreased.	*39*
Estrogen receptor-α *(Esr1)*	Transcription factor. Primarily expressed in efferent ducts and initial segment. Disruption of fluid reabsorption in the efferent ducts. Dilation of rete testis and efferent ducts with subsequent fluid accumulation in seminiferous tubules, dilution of sperm, and resulting infertility. Decreased expression and activity of the Na^+/H^+ exchanger 3 transporter affecting Na^+ reabsorption and passive water transport.	*32*,*36*
Anion exchanger 2 *(Slc4a2)*	Na^+-independent anion transporter that mediates exchange of Cl^- and HCO^-_3 across cell membranes. Expressed primarily in initial segment, caput, with less expression in the cauda. Squamous metaplasia of the epithelium.	*40*

(Continued)

Table 1 ***(Continued)***

Gene	*Function/expression/Phenotype*	*Reference*
HE6 (Gpr64)	Orphan member of the LNB-7TM (B_2) subfamily of G protein-coupled receptors. Expressed in efferent ducts and initial segment. Heterozygous males showed a dysregulation of fluid reabsorption within efferent ducts leading to fluid accumulation in testis . Sperm exhibit flagellar angularity, decreased motility, and decreasing fertility with age. Sperm accumulate in efferent ducts.	41
c-ros tyrosine kinase receptor *(Ros1)*	Tyrosine kinase receptor. Expressed in initial segment. Failure of initial segment to develop. Male mice are infertile. Sperm exhibit flagellar angulation attributed to inability to regulate cell volume, some decreased motility. Increased luminal pH in cauda epididymidis.	42–44
Nuclear oxysterol receptor LXR α and β *(lxr)*	Transcription factor, activated by oxysterols, oxidized derivatives of cholesterol. LXR proteins highest in caput, less expression in cauda. In double knockout, regression of proximal caput epithelium, amorphous substance in lumen, cauda spermatozoa exhibited detached heads, flagellar angulation. Late onset of defects at 6 months of age.	45
Prosaposin *(psap)*	Secreted protein targeted to lysosomal compartment, lysosomal activator of hydrolases, trophic factor. Decreased epididymal weight, smaller tubular diameter, shorter undifferentiated epithelial cells.	46
Nuclear phospholipid hydroperoxide glutathione *(Gpx4)*	Selenoenzyme, member of the glutathione peroxidase family. Expressed in germ cells. Decreased thiol oxidation in cauda spermatozoa of the knockout.	47

(Continued)

Table 1 ***(Continued)***

Gene	*Function/expression/Phenotype*	*Reference*
Inositol polyphosphate 5-phosphatase *(Inpp5b)*	Signaling protein in IP3 pathway. Expressed in Sertoli cells, germ cells, epididymis. Sperm from knockout mice exhibit decreased fertility, reduced motility. Conditional germ cell knockouts are fertile suggesting phenotype is a result of defects in Sertoli cell and/or epididymal function. Reduced levels of fertilin beta protein processing in the knockout sperm.	*48*
FSH receptor *(Fshr)*	Follicle-stimulating hormone receptor involved in maintenance of spermatogenesis. Expressed in Sertoli cells. Decreased caput and corpus epithelium, decreased sperm motility.	*49*
Cathepsin A *(Ctsa)*	Lysosomal carboxypeptidase. Lysosomal abnormalities, vacuolated, expanded epithelium in caput and corpus. Abnormal halo cells.	*50*
β-hexo-saminidase A and B *(Hexa,b)*	Lysosomal enzyme. Increased number and size of lysosomes in initial segment and intermediate zone.	*51*
Mononuclear phagocytic growth factor stimulating colony factor *(Csf1)*	Decreased density of macrophages in testis, caput, and cauda epididymis. Macrophages do not exhibit normal localization in caput.	*52*
Somatic- and testis-specific angiotensin-converting enzyme *(Ace)*	Regulator of the renin-angiotensin system. Reduced fertility in male mice. Impaired sperm transport in oviducts and zona binding. Mice lack the somatic ACE but have the testis-specific ACE are fertile. Conflicting data on whether dipeptidase or glycosylphosphatidylinositol releasing activity of testicular ACE is critical for fertility.	*53–55*

(Continued)

Table 1 ***(Continued)***

Gene	*Function/expression/Phenotype*	*Reference*
Bone morphogenetic protein 8A *(Bmp8A)*	Degeneration of epididymal epithelium.	*29*
γ- Glutamyl transpeptidase *(Ggtp)*	Hypoplasia of the epididymis.	*56*
Hoxa-10, Hoxa-11	Transcription factors. Hoxa-10 knockout results in homeotic transformation of corpus to caput epididymidis and proximal vas deferens to cauda epididymidis, whereas hoxa-11 knockout knockout results in homeotic transformation of proximalvas deferens to cauda epididymis.	*57–58*

for the maintenance of initial segment morphology but for function as well *(15,16,60)*.

Although it is not known if one or many testis factors are required to maintain initial segment function, studies by Lan et al. suggest that basic fibroblast growth factor may be one such factor. Administration of fibroblast growth factor-2 but not epidermal growth factor to efferent duct-ligated rats restored GGT mRNA, protein, and activity in the initial segment to control levels. Furthermore, these investigators proposed that fibroblast growth factor may elicit its effects on Ggt_pr4 gene expression via activation of the ras–raf–mitogen-activated protein kinase pathway and downstream activation of the ETV4 transcription factor *(60–62)*. Most recently, studies by these investigators suggest that, not surprisingly, not all testis-regulated genes respond the same to changes in ETV4 transcriptional activity. The administration of an ETV5-dominant negative plasmid by in vivo electroporation to the rat initial segment resulted in the downregulation of Etv5, Etv4, and Etv1 mRNAs in the initial segment as well as putative target genes γ-glutamyl transpeptidase (Ggt_pr4), steroid 5 α reductase (Srd5a1), and glutathione peroxidase (Gpx5; ref. *30*). However, although the testis-regulated genes CRES*(cst8)* and MEP17*(len8)* contain ETS-binding sites within their promoters, they did not respond to the dominant negative, suggesting that there either may be several testis factors, each differentially regulating specific subsets of genes, or that one or a few testis factors may

mediate different downstream effects via the activation of multiple signaling pathways and subsequent effector molecules *(30)*.

4. NOVEL MECHANISMS FOR DELIVERY OF EPIDIDYMAL PROTEINS TO SPERMATOZOA

During epididymal transit, spermatozoa acquire new surface-associated proteins synthesized and secreted from the epididymal epithelium. Most of these proteins possess the typical signal sequences indicating trafficking through the Golgi and subsequent packaging and release from secretory granules (merocrine secretion). However, several studies have shown that epididymal sperm also acquire proteins that lack signal sequences, suggesting an unusual secretion pathway in the epithelium. Differential extraction of spermatozoa indicates these proteins act like integral membrane proteins and in fact, some of these proteins are thought to be anchored to the sperm plasma membrane by a glycosylphosphatidylinositol *(63,64)*. In the epididymal lumen, several of these proteins are associated with membranous vesicles known as epididymosomes. Although previously thought to be an artifact of fixation, these small, membrane-bound vesicles originate from the epididymal epithelial cells in a process known as apocrine secretion. This type of secretion involves the formation of apical blebs containing various-sized vesicles from the epithelial cells. Once the blebs have detached, they are thought to fragment and release the small vesicles *(65)*. Although similar types of vesicles have been known for some time to be secreted by the prostate (prostasomes) and are present in the semen, where they have proposed roles as protection for sperm against complement, enhancement of motility, and stabilization of the sperm membrane, Yanagimachi was the first to describe such vesicles in the epididymal lumen and show interaction of these vesicles with spermatozoa *(66)*. Analysis of proteins associated with the epididymosomes reveal protein profiles quite different from that of proteins in the lumen. Proteins associated with epididymosomes include P26h, believed to be involved in zona pellucida binding, HE5, macrophage migration inhibitory factor, ubiquitin, and glutathione peroxidase, all of which have been shown to be transferred to spermatozoa in the epididymis *(64,67–69)*. The studies examining the transfer of P26 from epididymosomes to spermatozoa demonstrated that transfer was pH-dependent and required high zinc concentrations (consistent with endogenous levels in the epididymal fluid). These investigators also observed that not all proteins present in the vesicles were transferred to spermatozoa, suggesting that only some proteins have the ability to be transferred or

that complete fusion and transfer of vesicles to spermatozoa does not occur *(70)*. Other studies examining epididymosomes in the cauda fluid from the ram epididymis identified different subsets of proteins present in these vesicles, including dipeptidyl peptidase V, neprilysin, mannosidase, and actin, but observed no interactions of such vesicles with spermatozoa, also suggesting that transfer of proteins may be by a subtle exchange rather than complete fusion *(71)*. It is also possible that epididymosomes are heterogeneous with different protein compositions depending on downstream functions. The functional significance of epididymosomes in sperm maturation remains to be elucidated. It is possible that epididymosomes are designed to ensure the safe delivery of some proteins to the sperm cell and perhaps to particular sperm domains without possible damage by luminal proteases. Alternatively, given the complexities of sperm maturation and the vast multitude of cellular and extracellular events the epididymis must carry out for maturation to occur, it is possible the epididymis has developed new strategies to deliver cellular proteins and their associated functions to the sperm surface rather than synthesize a secretory protein that carries out the same function as its cellular form. Along this line of thinking, studies by Sutovsky et al. *(68)* showed that ubiquitin is associated with epididymosomes and that during epididymal transit these vesicles delivered ubiquitin to damaged or defective spermatozoa, thus flagging them for removal. Thus, by the mechanism of apocrine secretion, a cellular process, ubiquitination, is now carried out extracellularly.

5. SELECTIVE REMOVAL OF PROTEINS FROM THE EPIDIDYMAL LUMEN

Although much focus has been spent on studying secretory proteins within the epididymal lumen and how they may affect maturation, how these secretory proteins are removed from the lumen is quite overlooked. Although some proteins secreted by the proximal epididymis remain in the luminal fluid to the cauda, many proteins are highly segment-specific in their localization, suggesting that their continued presence in downstream segments may be detrimental and that selective mechanisms must be in place for their removal. Furthermore, many proteins are secreted into the epididymal lumen by default, because they function within the secretory pathway rather than extracellularly. If these proteins are not removed, they might interfere with proteins that do have extracellular roles, thus affecting sperm maturation. A recent conceptual paper has proposed the existence of an extracellular quality control system that is analogous to the intracellular machinery responsible

for the removal of misfolded proteins from the endoplasmic reticulum and cytosol *(72)*. The extracellular quality control system has been proposed to consist of chaperones, proteolytic enzymes, and endocytosis mediators. Because of the active secretory functions of the epididymal epithelium and the critical nature of the maturation process occurring within the epididymal lumen, it is quite likely that such a mechanism is also active within the epididymal luminal compartment.

CRES is detected in the lumen of mouse segments 1 and 2 but abruptly disappears in segment 3 *(73)*. Protein analysis of epididymal fluid using Tricine gels to detect protein degradation products did not distinguish any smaller forms of CRES, rather only an abrupt disappearance of the majority of the protein from the lumen by suggesting proteolytic degradation within the lumen is likely not responsible for its turnover *(74)*. CRES may then be one of the proteins that could be potentially harmful if it is enriched beyond a critical threshold level within the extracellular compartment. Indeed, cystatins are amyloidogenic and, given the correct local environmental conditions, will oligomerize. In the worse situation, cystatin C will form large protein aggregates or amyloid fibers that are associated with the neurodegenerative disease amyloid angiopathy *(75)*. Based on its relationship with cystatins, we theorize that CRES may self-aggregate within the epididymal lumen, making it a target for removal by quality control machinery in the luminal fluid.

Biochemical analyses of caput luminal fluid demonstrated that CRES was part of high-molecular-weight soluble complexes in the lumen *(74)*. The formation of high-molecular-weight but soluble protein complexes in the lumen may be a means to control protein function before the removal of such complexes by endocytosis. Indeed, cystatin C dimers are not functional cysteine protease inhibitors *(76)*. Although the noncovalent aggregation of CRES seems to be a natural event, it bears the potential danger of amyloid fiber formation, particularly in light of the dramatic concentration of epididymal secretory proteins that occurs as a consequence of the active fluid reabsorption being carried out by the epididymal epithelium. Therefore, we hypothesized that the equilibrium of CRES aggregation and disaggregation needs to be tightly modulated by chaperones and other quality control proteins. Examination of fluid phase liquid chromatography fractionated caput luminal fluid by SDS-agarose gels, indentified CRES complexes that were resistant to denaturation by SDS *(74)*. Further studies revealed that CRES is a substrate for transglutaminase, a calcium-dependent enzyme that catalyzes isopeptide bond formation between glutamyl and lysinyl residues and thus can covalently crosslink proteins. The transglutaminases are known

modulators of protein aggregation and may well be part of extracellular quality control machinery that is involved in the removal of proteins from the epididymal luminal fluid *(72)*. Our studies suggest that both properties inherent in CRES protein as well as the presence of transglutaminase participate in the formation of stable CRES protein complexes that may mediate CRES function and ultimately may target it for nonspecific adsorptive endocytosis.

In addition to our studies with CRES, other investigators have noted a different type of high-molecular-weight protein complex in epididymal fluid. Studies by Ecryod et al. identified prion protein in large-molecular-weight soluble complexes in ram luminal fluid *(78)*. Prion protein, unlike CRES which is a secretory protein, is typically attached to the cell plasma membrane by a glycosylphosphatidylinositol anchor, thus its presence in "soluble" form in epididymal fluid is perplexing. Further examination of prion protein complexes by these investigators identified the presence of five other proteins that copurified with prion protein, including clusterin, a known chaperone, bacterial permeability-increasing protein (BPI), α mannosidase, cauxin, and β-galactosidase. These proteins appeared to associate with one another by hydrophobic interactions rather than through protein–protein interactions *(78)* and as such form a lipoprotein-like vesicle. It was proposed that such a mechanism may allow clearance of hydrophobic proteins without precipitation. Taken together, it appears that the epididymis has developed highly specialized mechanisms for the removal of luminal proteins, whether it is via protein crosslinking or formation of stable lipid-like structures.

6. CONCLUSION

In summary, the maturation of spermatozoa in the epididymis is an incredibly complex process requiring the exposure of spermatozoa to a series of individual microenvironments created by the epididymal epithelium. Understanding the multifaceted nature of each microenvironment is required to fully define epididymal function in sperm maturation and provide new therapies for infertility as well targets for contraception.

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IV Clinical Applications of the Study of the Genetics of Male Infertility

14 The Structure of the Y Chromosome and Its Role in Male Infertility

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Summary

Male infertility accounts for a significant proportion of reproductive failure. Although ejaculatory disorders and impotence can be successfully treated with medical therapy, disorders of genetic origin are more difficult to successfully overcome. Recent advances in our knowledge of Y chromosome structure and function have provided insight into many aspects of the genetic basis of spermatogenic failure and its possible inheritance when assisted reproductive technologies are used to produce offspring.

Key Words: Y chromosome; azoospermia; *AZF*; pseudo-autosomal region male infertility.

1. BACKGROUND

Infertility affects roughly 15% of reproductive aged couples. Although the background prevalence has not changed, changes in lifestyle and delayed childbearing have resulted in an increased number of couples seeking medical attention. Infertility can be categorized as 50% female in origin, 35% male, and 15% unexplained. Female factor infertility can be further separated into disorders of ovulation (20%), tubal disease (15%), cervical/uterine factors (5%), and endometriosis (10%; ref. *1*). Ovulation induction methods are used to treat cases of oligomenorrhea/amenorrhea. Combined ovulation induction with intrauterine insemination and/or in vitro fertilization (IVF) is used to treat other ovulatory problems and unexplained infertility. Tubal damage associated with infection and endometriosis requires surgical interventions in combination with IVF.

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Sperm disorders are the single most common cause of male factor infertility *(2)*. Ejaculatory disorders and impotence also result in infertility but are effectively treated with medical therapy. Sperm disorders may be separated into oligozoospermia and azoospermia; in cases of sperm dysfunction, the likelihood of natural conception is low.

Oligozoospermia (sperm density <20 million/mL) is sometimes caused by androgen deficiency and may associated with a decrease in motility, known as asthenozoospermia. Asthenozoospermia may be caused by sperm structural defects, prolonged periods of sexual abstinence, genital tact infection, antisperm antibodies, partial duct obstruction, and/or varicocele. Oligoasthenozoospermia is the most common identifiable anomaly found in the semen analysis *(3)*. The most frequent causes are cryptorchidism, endocrinopathy, drugs, excessive heat, toxins, infection, autoimmunity, trauma, and varicocele *(3)*. Drugs are ineffective for idiopathic oligozoospermia and the role of varicocele ligation is uncertain *(4)*.

Azoospermia, the lack of sperm in the ejaculate, can be divided into obstructive and nonobstructive categories. Obstructive azoospermia is characterized by normal sperm production and is often caused by congenital absence or anomalies of the vas deferens. Nonobstructive azoospermia is characterized by a varying degree of spermatogenic failure and is likely to be associated with an increased number of chromosomal abnormalities *(5,6)*.

With the development of intracytoplasmic sperm injection (ICSI), microsurgical epididymal sperm aspiration, and testicular sperm extraction more than a decade ago, men with sperm disorders are now able to father children. These techniques do not correct deficient spermatogenesis but provide a means of bypassing the problem. In standard IVF, human oocytes are harvested from hyperstimulated ovaries and incubated in a culture dish with sperm. Couples with severe male factor infertility reported poor results with standard IVF because approx 150,000 motile sperm per oocyte are required for proper fertilization. To overcome this requirement, micromanipulation techniques were developed. Zona drilling, partial zona dissection, and subzonal insertion of sperm, the microinjection of spermatozoa into the space between the zona pellucida and the plasma membrane, have been used to overcome severe male factor infertility. Typically only three to four sperm were inserted per oocyte; however, the high rate of polyspermy with partial zona dissection and subzonal insertion of sperm have proved lethal to the developing embryo. These problems are not encountered with ICSI, which requires the injection of only a single sperm per egg. ICSI is now the standard of care for treatment of severe male factor

infertility, because it produces higher clinical pregnancy rates and has broader applicability.

2. THE STRUCTURE OF THE Y CHROMOSOME

It is believed that more than 60% of men with idiopathic male infertility may have a genetic basis for their subfertility *(2)*. This naturally turns attention to the Y chromosome. The human Y chromosome is one of the smallest chromosomes in the genome. It contains more than 60 million nucleotides, with the least number of genes compared with any other chromosome, and acts as a genetic determinant of male characteristic features. Approximately 95% of the sequence is nonrecombining, and is present only in males. This nonrecombining region, also known as the male-specific region, MSY, represents a mosaic of heterochromatic sequences and three classes of euchromatic (X-transposed, X degenerate, and amplionic) sequences *(7)*. The heterochromatic region contains about 30 Mb of sequence and the euchromatic region contains about 24 Mb of sequence *(8)*. There are two pseudoautosomal regions, PABY1 and PABY2, on the short (Yp) and long (Yq) arms of the Y chromosome, respectively, with homologs found on the X chromosome *(7)*. This comprises 5% of the chromosome and is the only region that participates in meiotic recombination.

Because of its haploid status and absence of recombination, sequence variations in the Y chromosome are largely to the result of accumulation of *de novo* mutations *(9)*. Variations also arise from polymorphisms detected as single-nucleotide polymorphisms (SNPs) or micro- and mini-satellites *(10)*.

There are several genes on the Y chromosome associated with genetic anomalies and linked to infertility (Table 1; ref. *11*). One gene controlling spermatogenesis is referred to as azoospermia factor (*AZF*), located in the Yq11.23 region. It was originally established by Tiepolo and Zuffardi in 1976 that deletions of the long arm of the Y chromosome are associated with spermatogenic failure *(7)*. Vogt et al. observed that Y-chromosome microdeletions follow a certain deletion pattern, with three recurrently deleted, presumably nonoverlapping, subregions in the proximal, middle, and distal Yq11. These are designated *AZFa*, *AZFb*, and *AZFc*, respectively. It has since been shown that *AZFb* and *AZFc* regions are not independent, but show overlap and that the *AZFa* region, which spans 0.8-Mb of sequence is independent of the other two regions *(13)*. Approximately 20% of men suffering from infertility of nonobstructive (curable) oligo- or azoospermia with normal chromosomes have been found to show microdeletions of *AZF* sequences *(12)*.

Table 1
Y Chromosome-Linked Genes Associated With Genetic Disorders *(11)*

Gene abbreviation	*Gene*	*Locus*	*Disorder*
AZF1	Azoospermia factor 1	Yq11	Possibly? Sertoli cell only syndrome
DAZ	Deleted in azoospermia	Yq11	Possibly? Sertoli cell only syndrome
SRY	Sex-determining region Y	Yq11.3	Gonadal dysgenesis, XY type
USP9y	Ubiquitin-specific protease 9, Y chromosome	Yq11.2	Azoospermia

Deletion of the *AZFc* is the most common known cause of spermatogenic failure *(14)*. This region was completely sequenced *(15)* and found to contain massive palindromes spanning as much as 3-Mb sequences. The expression of the *AZF* gene is found to be testis-specific in human and several other mammals. The identification of the palindromic complexes P1 to P5 encompassing azoospermia factors may lead to a better understanding of the biological roles of various repeat elements *(13)*.

The deleted azoospermia (*DAZ*) gene family was identified in a study in which 12 infertile men were found to have overlapping deletions on the Y chromosome *(5)*. *DAZ* is reported to have at least four copies, is transcribed in the adult testis, and encodes an RNA-binding protein *(16)*.

The sex-determining region on chromosome Y (*SRY*), also known as testis-determining factor, is involved in male sex determination and is one of the most highly characterized of the Y-chromosome genes. The gene encodes a transcription factor of about 204 amino acids from a single open reading frame (ORF) of about 669 basepairs *(17)*. *SRY* is a member of the high-mobility group DNA-binding protein with a highly conserved HMG-box domain. *SRY* was mapped to the human Y chromosome by molecular analysis of sex-reversed patients. Analysis of four XX males with testes were found to have a minute portion of the Y translocated to the X chromosome. This fragment was found to be critical in defining the sex-determining region on the human Y chromosome *(18)*.

Mutations within the gene *USP9Y* has been reported in two individuals with spermatogenic failure leading to infertility *(11)*. Four base pair deletions result in premature truncation of the encoded protein. The *USP9Y* gene has a homolog, *USP9X*, on the X chromosome and both are ubiquitously expressed.

3. CONCLUSION

Because many cases of male infertility have been shown to be of genetic origin, the potential risk of transmitting infertility to future generations is of concern. There has been no evidence currently that the physical health of children is affected by use of infertility treatment techniques. There have been studies, however, showing that the deletions involved in spermatogenesis disorders are transmitted to male offspring via ICSI. The fertility status of these children is not known because these children have not yet entered puberty *(19)*.

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15 Y Chromosome Microdeletions and Haplotypes

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Summary

The human Y chromosome contains a number of genes and gene families that are necessary for spermatogenesis. Many of these genes are embedded in repetitive elements that are subject to deletion events. Deletions of azoospermia factor (AZF) regions *AZFa*, *AZFb*, and *AZFc* are found in approx 10–15% of men with either unexplained severe oligozoospermia or azoospermia. These deletions fall on different Y chromosome backgrounds and there is no evidence for a link between a Y-chromosome lineage and the presence or absence of an *AZF* deletion. Several partial *AZFc* deletions have been described. One of these, which removes around half of all the genes within the *AZFc* region, appears to be present as in inconsequential polymorphism in populations of northern Eurasia. A second deletion, termed gr/gr, results in the absence of several *AZFc* genes and has been suggested to be a genetic risk factor for spermatogenic failure. However, the link between the gr/gr deletion and infertility is more complex. First, the gr/gr deletion is actually not a single type of deletion but a combination of deletions that vary in size and complexity and result in the absence of different members of the deleted azoospermia (*DAZ*) gene family as well as other *AZFc* genes, such as *CDY1*. Second, there are regional or ethnic differences in the frequency of gr/gr deletions. In some Y-chromosome lineages, these deletion appear to be fixed and may have little influence on spermatogenesis. Third, these observations have influenced a number of association studies aimed to determine the relationship between the gr/gr deletion and male infertility. Consequently, some studies suggest that the gr/gr

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deletion confers a strong genetic susceptibility to reduced sperm counts, whereas others suggest that the genetic susceptibility may not exist or be limited to specific Y-chromosome haplotypes.

Clearly there is need for additional studies that combine an analysis of a series of markers in the *AZFc* region together with the haplotype of the Y chromosome in well-defined case and control populations. Many of the genes in the *AZFc* region present in multiple copies and gr/gr deletions can be associated with reciprocal duplication events. Therefore, there is a need to determine gene dosage if the relationship between gr/gr deletions and infertility is to be completely understood.

Key Words: Y chromosome; haplotype; haplogroup; male infertility; spermatogenesis; microdeletion; gr/gr; DAZ.

1. Y-CHROMOSOME POLYMORPHISMS DEFINE DISTINCT LINEAGES

The vast majority (57 of 60 Mb) of the Y chromosome does not recombine with the X chromosome and is transmitted as a single block from father to son with all functional variants and neutral polymorphisms being linked. A Y-chromosome lineage or haplogroup (Hg) is a monophyletic group of Y chromosomes defined by stable binary markers, such as single-nucleotide variants or insertion/deletions. More than 200 binary markers have been characterized on the human Y chromosome *(1)* and it is likely that many others will be available in the near future that can be used to further refine Y lineages. Y-chromosome microsatellites are variable in all populations and a particular combination of allelic states can be used to define a Y-chromosome haplotype within a defined Hg. The population distribution of Y chromosome variation indicates that most lineages are largely confined to particular human populations, which has important implications for association studies *(2)*. A number of association studies have been published that measure the frequencies of Y-chromosome haplotypes in case and control groups of men with different male-specific phenotypes, including infertility. The distinct population affinities displayed by many Y-chromosome haplotypes and their high degree of geographical specificity means that an association between the Y-chromosome background and a phenotype in one population or country may not be relevant for another. Conversely, the absence of an association in one population does not imply its absence in other populations. However, these studies are useful because they may identify classes or lineages of the Y chromosome that may be at increased risk of developing infertility. Furthermore, an increased awareness of the physical structure and detailed organization of Y chromosomes within a lineage is essential to aid in the interpretation of data

generated by association studies. This problem has been highlighted by attempts to correlate different types of microdeletions of the Y chromosome with reduced sperm counts and/or infertility. These studies will be the object of this chapter.

2. AZF DELETIONS ARE ASSOCIATED WITH MALE INFERTILITY

Three regions of the long arm of the Y chromosome, termed azoospermia factor (AZF)a, AZFb, and AZFc are associated with reduced or absent sperm counts *(3)*. Men presenting with AZF deletions appear to be otherwise healthy, although it should be stressed that there does not appear to be any longer-term studies to determine if these men are at risk from other health problems. All of the AZF microdeletions are the result of intrachromosomal exchange between regions containing highly repetitive sequences. Intrachromosomal exchange between repetitive elements derived from the HERV15 class of endogenous retroviruses cause deletions of the AZFa region *(4,5)*. Complete AZFb deletions are associated with recombination between palindromic sequences, are 6.23 Mb in length, and extend 1.5 Mb into the proximal portion of AZFc *(6)*. Likewise AZFb+AZFc deletions are also a consequence of recombination between palindromic sequences. These deletions are 7.66 Mb in size and do not include the distal portion of AZFc *(6)*. Deletions of the AZFc region are estimated to occur in 1 in 4000 males and are the most common class of deletion (~80% of the total) in men with a more severe phenotype (azoospermia or severe oligozoospermia [$<1 \times 10^6$/mL]) and are considered to be the consequence of homologous recombination between two direct repeats of 229 kb in length (termed b2/b4; ref. *7*) in a region of the Y chromosome that is made up almost entirely of long direct and inverted repeats termed amplicons. Within the AZFc region there are several candidate fertility genes, including three copies of basic protein on Y chromosome, 2 (*BPY2*), two copies of *CDY1a* and *CDY1b*; Chromodomain protein, Y chromosome 1 (*CDY1*), and four copies of the deleted in azoospermia (*DAZ*) gene *(3,7)*. It is still not clear if each of these factors contribute to infertility or if there is a key infertility gene. The different AZF deletions arise on various Y-chromosome lineages and there does not appear to be a Y Hg that either protects against deletion formation or is more sensitive to deletions *(8,9)*. However, it should be noted that studies to date have focused on AZFa or AZFb and AZFc deletions and it cannot be

ruled out that AZFa deletions, although rare, may fall on specific Y-chromosome backgrounds.

3. PARTIAL AZFc DELETIONS AND Y-CHROMOSOME VARIANTS

The relationship between partial AZFc deletions and reduced sperm counts (or fertility) is unclear. Some of these deletions appear to have little effect on fertility, whereas others appear to be associated with significant risk for developing spermatogenic failure. However, the relationship is complex and, as indicated previously, data that may be applicable to one population may not be applicable to another. To understand the depth of this problem we must first consider the intricate sequence organisation of the AZFc region.

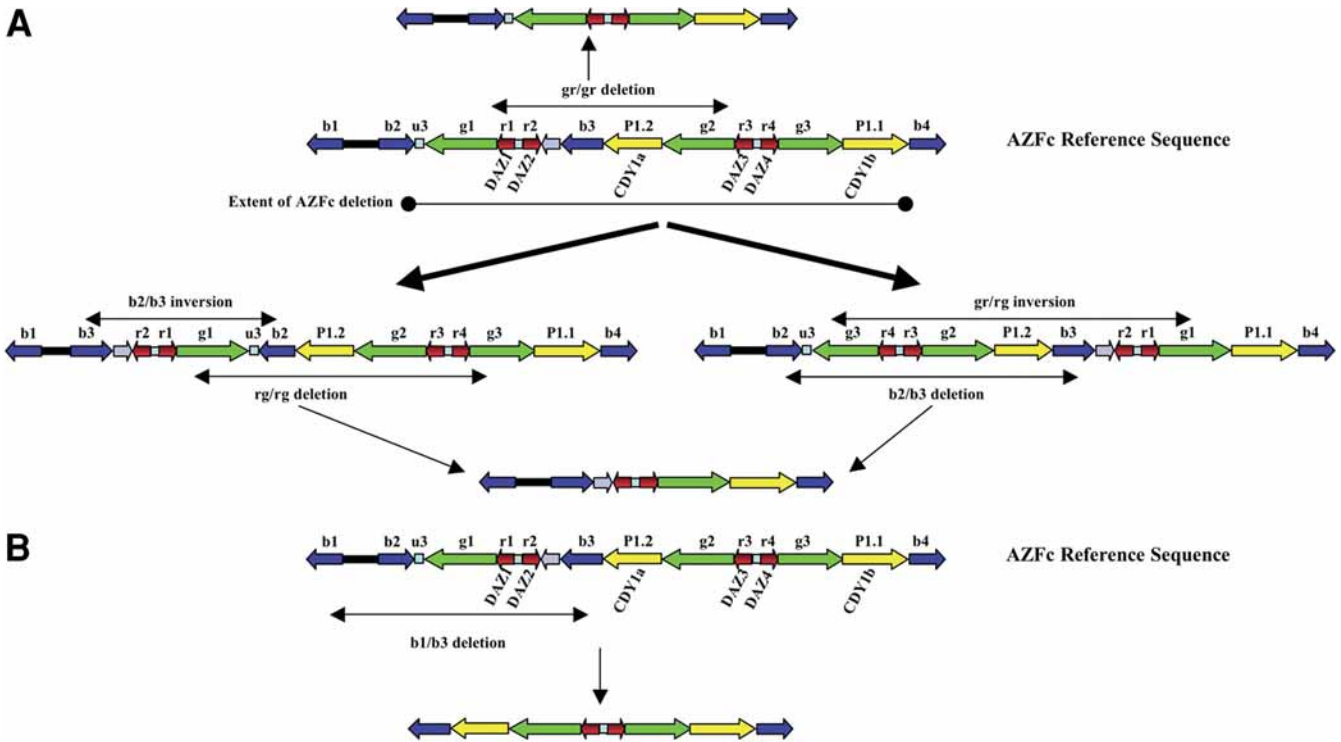
The entire AZFc region spans 4.5 Mb and consists of three palindromes with six distinct families of amplicons, which may have resulted from a complex series of tandem duplication and inversion events *(7)*. These amplicons were termed turquoise, gray, yellow, green (g), blue (b), and red (r). Other sequences (u1, u2, and u3) occur once each in the region but share a high degree of sequence identity to other Y chromosome loci. The four *DAZ* genes on the human Y chromosome exist in two clusters and each cluster consists of an inverted pair of *DAZ* genes (*DAZ1/DAZ2* and *DAZ3/DAZ4 [10]*). Changes in *DAZ* gene copy number have been demonstrated using the techniques of fluorescence *in situ* hybridization, quantitative polymerase chain reaction and the analysis of sequence-family variants (SFVs) that can distinguish between *DAZ* copies and between *DAZ* clusters. SFVs are defined as subtle differences between closely related but nonallelic sequences. In particular, the SFV sY587 (also known as DAZ-SNV V) can distinguish between *DAZ1/2* gene pair and *DAZ3/4* gene pair and has proven to be particularly informative.

Yen 2001 *(11)* predicted that recombination could occur between any pair of amplicons that are present in the same orientation (resulting in the deletion of the intervening sequences) and that such deletions may have an effect on fertility. Using Southern blot-based techniques, Yen and colleagues have determined that most men have four *DAZ* copies (similar to the original individual used to determine the structure of the *DAZ* genes *[12]*). In the same study, a small number of men (6%) were observed to carry six copies of the *DAZ* gene on different Y chromosome Hgs. The significance of this observation will be discussed later.

A large partial deletion of the AZFc region has been reported and it is suggested that this deletion does not to affect fertility. Vogt and colleagues

described a large deletion of the AZFc region that includes the sequences u3 and *DAZ3/4* (termed g1/g3; ref. *14*). This deletion occurs on haplogroup N3, a Y-chromosome lineage that is found at high frequencies in northern Europe (52% of Finns) and in other populations of northern Eurasia. Further work by Mitchell and colleagues indicated that this deletion also includes the *CDY1b* gene (they termed the deletion u3-gr/gr *[12]*). This deletion has been suggested to originate from an inversion of sequences mediated by the b2/b3 amplicons followed by recombination between the g1 and g3 sequences resulting in the loss of intervening sequences, including *DAZ3/4 (13)*. Repping et al. *(15)* suggested a second pathway by which this deletion could have arisen, namely a gr/rg inversion (g1, r1, r2 recombining with r3, r4, g3) followed by a b2/b3 deletion (Fig. 1). In reality, both mutational pathways are probably occurring and this can be tested in the general male population, because each pathway requires an intermediary step. In an analysis of interphase nuclei from men carrying Y chromosomes representing 44 different lineages, 4 of 44 individuals carried the predicted gr/rg inversion and 3 of 44 carried the b2/b3 inversion *(15)*. This deletion was also present in low frequencies in three other Y chromosome lineages (H*, O*, and O3*). Machev et al. *(14)* also found the deletion in the Y chromosome Hgs Y*(xD, E, J, K) and P. A large portion of AZFc, including 12 testis-specific genes or transcripts, is removed by this deletion, raising the important question of whether it contributes to infertility. However, presence of this deletion (referred to variously as u3-gr/gr, g1/g3, or b2/b3) at high frequencies in some populations suggests that it may be a polymorphism with limited or no effect on fertility.

A second partial AZFc deletion removes a 1.6-Mb proximal segment of AZFc including two copies of the *DAZ* gene cluster (*DAZ1/2*). Vogt and colleagues identified this deletion in 5 of 63 oligozoospermic samples and not in 107 fertile control samples and suggested that it may be responsible for reduced sperm numbers *(16)*. This deletion has subsequently been termed gr/gr (g1/g2, r1/r3, r2/r4) in reference to the resulting organization of the amplicons *(17)*. Gr/gr deletions were detected in 22 individuals following an initial screen of DNA samples from 689 men *(17)*. In a subsequent association study by the same group, gr/gr deletions were found in 9 of 237 (3.7%) men with spermatogenic failure of unknown origin. In 148 men with normal spermatogenesis, this deletion was not detected, suggesting that the gr/gr deletion correlates with decreased sperm production and that the gr/gr deletion is a significant risk factor for spermatogenic failure *(17)*. However, the association between gr/gr deletions and infertility is not so simple. In the same study *(17)*, the authors screened a human Y-chromosome biodiversity panel that


A
gr/gr deletion
b1 b2 u3 g1 r1 r2 b3 P1.2 g2 r3 r4 g3 P1.1 b4
DAZ1 DAZ2 CDY1a DAZ3 DAZ4 CDY1b
AZFc Reference Sequence
Extent of AZFc deletion
b2/b3 inversion
b1 b3 r2 r1 g1 u3 b2 P1.2 g2 r3 r4 g3 P1.1 b4
rg/rg deletion
gr/rg inversion
b1 b2 u3 g3 r4 r3 g2 P1.2 b3 r2 r1 g1 P1.1 b4
b2/b3 deletion
B
b1 b2 u3 g1 r1 r2 b3 P1.2 g2 r3 r4 g3 P1.1 b4
DAZ1 DAZ2 CDY1a DAZ3 DAZ4 CDY1b
AZFc Reference Sequence
b1/b3 deletion

represented 43 different Y-chromosome lineages. Gr/gr-deleted Y chromosomes were found in 14 independent Y-chromosome lineages. This indicates that the deletion has occurred multiple times during human evolution. In one Y-chromosome lineage (D2b), all samples screened (12/12) carried the gr/gr deletion, suggesting that it may be fixed in this Y chromosome Hg *(17)*. The fertility status of the individuals contributing to this panel is unknown but it suggests that in some Y-chromosome lineages the gr/gr deletion may have a limited effect on fertility. The gr/gr-deleted D2b Y-chromosome lineage is present in about 30–40% of Japanese men and has been reported to be associated with reduced sperm counts *(18)*. It is important to note that the association with this Hg was not observed in a follow-up study. Although there may be a weak association between the D2b haplogroup and reduced sperm counts, the majority of the Japanese population with this lineage have sperm counts that are more than 40×10^6 *(13,18)*. Although some more recent association studies have indicated a strong association between gr/gr deletions and reduced sperm counts, others have questioned the validity of this link. de Llanos et al. detected gr/gr deletions in 12 of 283 (4.2%) consecutive intracytoplasmic sperm injection candidates with either azoospermia or oligozoospermia and deletions were not found in a control panel of 232 men *(19)*. Ferlin et al. *(20)* reported an increased frequency of not only *DAZ1/DAZ2* (gr/gr) deletions, but also *DAZ3/DAZ4* deletions in men with reduced sperm counts compared with normospermic individuals.

Fig. 1. *(Opposite page)* Models of the origins of the most frequent partial AZFc deletions. **(A)** The reference AZFc sequence is indicated and the organization of amplicons belonging to various families are shown in colors (b1 to b4 [blue], r1 to r4 [red], g1 to g3 [green]). Two small palindromes P1.1 and P1.2 (yellow) lie within a larger P1 palindrome. Sequences with the same color code exhibit more than 99.9% sequence identity. The relative position of the unique sequence u3 is also indicated. The relative location of *CDY1* and *DAZ* gene copies is indicated underneath their respective amplicons. The deletion of the entire AZFc region occurs as consequence of recombination between amplicons b2 and b4 and is indicated. The schematic representation of the gr/gr-deleted chromosome is indicated above the reference sequence. Under the reference sequence, two pathways are indicated that could give rise to the b2/b3 deletion (also referred to as u3-gr/gr and g1/g3 *[13,14]*). Both pathways predict an inversion–deletion model. In one scenario (right), homologous recombination generates a gr/rg inversion (g1, r1, r2 recombining with r3, r4, g3), which is followed by a deletion between b2 and b3 amplicons. In a second scenario (left), there is an inversion mediated by b2 and b3 amplicons followed by an rg/rg deletion. All of these events are a consequence of homologous recombination. **(B)** Schematic representation of the generation of a b1/b3 deletion. This deletion was predicted by Yen *(11)* and removes a proximal portion of AZFc. It has been observed in a very small number of normospermic and infertile individuals (*see* refs. *15* and *21*) and consequently the relationship of the b1/b3 deletion to infertility is unknown.

However, several studies have not detected strong associations. Hucklenbroich et al. *(21)* observed that the incidence of gr/gr deletions in a population of ethnic Germans from Westphalia was not significantly different between a case population of 348 men (4% gr/gr-deleted) with nonobstructive oligospermia/azoospermia and a control population of 170 normospermic men (1.8% gr/gr-deleted). A lack of association between the gr/gr with reduced sperm counts was also observed by Mitchell and colleagues *(13)*. In our own studies, we have failed to find an association between the gr/gr deletion and reduced sperm counts in a study of patients attending a clinical center in Paris *(22)*.

In the light of this apparently conflicting data, how can we clarify the impact of partial AZFc deletions on human spermatogenesis? In some cases, partial deletions are associated with a secondary duplication. In their initial study, Repping et al. *(17)* screened 20 of the 22 men with gr/gr deletions using interphase fluorescence *in situ* hybridization and detected two men with a secondary duplication. Such a b2/b4 duplication, which has also been inferred from the data of Mitchell and colleagues *(13)*, could rescue the gr/gr-deleted phenotype. This idea is supported by the observations of Yen and colleagues that around 6% of men actually carry six copies of the *DAZ* gene *(12)*.

The key to solving these problems may lie in three important factors: the markers used in the screening process to identify partial AZFc deletions, the phenotype that is studied, and the Y-chromosome lineage on which the deletion has arisen.

It is important to emphasize that not all gr/gr deletions are the same. In the study by Machev et al. *(13)*, novel SFVs that differentiate between the two copies of the *CDY1* gene in AZFc were used as part of the screening panel. Although an association between partial AZFc deletions and reduced sperm counts was not observed when they screened samples using markers used by other groups, they did observe a weak association between the absence of markers defining *DAZ3/4* and *CDY1a* ($p = 0.042$) and infertility. A stronger association was observed between the loss of the *CDY1a* SFV alone and infertility ($p = 0.002$). It is important to emphasize that the loss of this SFV does not necessarily infer a deletion, because gene conversion events could also explain this observation. However, the data suggest that the use of additional informative markers within the AZFc region may unmask novel deletions that could be genuinely associated with reduced sperm counts. This has also been suggested by the study of Hucklenbroich et al. *(21)*, who found novel partial AZFc deletions in their infertile cohort but not in samples from normospermic men.

The second factor that needs to be explored is the phenotype that is under study. An interesting survey of gr/gr deletions in men seeking

fertility treatment in Australia failed to detect an association between gr/gr deletions and reduced sperm counts *(23)*. However, the authors did note an increased incidence of gr/gr deletions in men who were normospermic and yet infertile. Most of the studies published to date have sought to determine an association between the gr/gr deletion and men with reduced sperm counts and obviously this type of association would not be detected. It also raises the possibility that the gr/gr deletions could be associated with defaults in sperm mobility or morphology rather than actual numbers.

The third confounding effect could be the Y-chromosome lineage on which the deletion arises. As mentioned previously, some Y lineages have partial AZFc deletions that appear to have little or limited effects on fertility and this may be to the result of compensatory alterations elsewhere on the Y, or perhaps on the X chromosome or autosomes. This concept is reinforced by the observations of Nathanson et al. *(24)* in a study of the frequency of gr/gr deletions in men with testicular cancer compared with the incidence of these deletions in various control groups. In this study, the difference in the incidence of gr/gr deletion between the control groups was statistically significant.

The definition of the Y-chromosome haplotype in cases of partial AZFc deletions is likely to play an essential role in understanding the contribution of the deletion to reduced sperm counts. There is a pressing need for large-scale studies on well-characterized normospermic and oligospermic/azoospermic individuals of different ethnic origins with multiple informative AZFc markers if the correlation between these deletions and the phenotype is finally to be resolved.

4. CONCLUSION

Different types of deletions on the Y chromosome have been reported that cause infertility. These include the deletions AZFa, AZFb, and AZFc. Several partial deletions of AZFc have been described that, in some cases, may be trivial polymorphisms that are present on specific Y-chromosome lineages and in other cases may be associated with a risk of infertility. The latter include the gr/gr deletions that have been proposed to be associated with reduced sperm counts. However, the relationship between the gr/gr deletions and infertility is not clear because there are several types of gr/gr deletions that show marked region/ethnic differences in frequency and in some cases may be associated with duplication events. There is a need for larger studies on well-defined populations that include a description of the Y chromosome lineages to fully understand the relationship of these deletions with fertility.

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16 The Genetics of Male Infertility
From Bench to Clinic

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Summary

This chapter provides an overview of the causes of male infertility and identifies the developments that have occurred in the identification of genetic causes of spermatogenic disorders. The approaches to the identification of new genetic causes of infertility are discussed, and provide an indication of the mechanisms that can be disrupted by targeted inactivation of genes in mice. The emerging genetic targets provide new directions for the development of tests to detect mutations in these genes in men with infertility.

Key Words: Male infertility; genetics; spermatogenesis; mutagenesis.

1. INTRODUCTION

For the clinician, the inability to define the cause of a spermatogenic defect and develop an evidenced-based treatment regime is a source of great frustration. In this chapter, a brief review of the defined causes of male infertility is undertaken to illustrate the lack of information concerning the cause of spermatogenic defects that form the

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Table 1
Classification of Causes of Male Infertility

Hormonal:
- Hypothalamic lesions:
  - Kallmann's syndrome
  - Opiate induced
- Pituitary lesions:
  - Tumors
  - Hemochromatosis
  - Luteinizing hormone and follicle-stimulating hormone suppression from androgen abuse, opiates
- Testicular:
  - Androgen biosynthetic defects
  - Androgen receptor mutations

Testicular:
- Anorchia, torsion
- Maldescent of testis
- Orchitis: nonspecific, mumps
- Irradiation, chemotherapy
- Drugs
- Heat exposure
- Varicocele
- Immunological: sperm antibodies
- Genetic
  - Klinefelter's syndrome
  - Y-chromosome deletions
  - Primary ciliary dyskinesia
- Metabolic
  - Renal failure
  - Liver failure
- Testicular tumors
- Idiopathic spermatogenic defects

Post-Testicular
- Obstructive
  - Congenital absence of vas
  - After sexually transmitted disease
  - Post-inguinal surgery
  - Vasectomy
  - Intraprostatic
- Epididymal maturational defects
- Accessory gland infection
- Sperm antibodies
- Coital
  - Infrequent intercourse
  - Erectile dysfunction
  - Ejaculatory disturbances
- Sperm–oocyte interactions

Table 2
Frequency of Causes of Male Infertility in Patients in a Tertiary Clinical Setting in Melbourne in 1986 (n = 1041)

Primary testicular	
• Klinefelter's syndrome	1.9%
• Past cryptorchidism	6.4%
• Past mumps orchitis	1.6%
• Past irradiation and chemotherapy	0.5%
• Varicocele	40%
• Idiopathic spermatogenic disorders	44.4%
Secondary testicular failure	
• Hypothalamic/pituitary disorders	0.5%
Genital tract obstructions	
• Vasal agenesis	0.6%
• Epididymal obstructions	3.5%
Coital disorders	0.5%

basis of infertility. This data will set the context in which the search for genetic causes of male infertility is proceeding.

Male infertility can be the result of abnormalities in hormonal control, spermatogenic disorders, disruption of sperm transport and maturation, failure of sperm–oocyte interactions and coital disorders that limit the exposure of the oocyte to sperm. It is not possible to provide a comprehensive discussion of each of these areas in this chapter, and readers should consult the remaining chapters of this book and other recent publications for further details *(1,2)*. An overview is provided in Table 1. A summary of the causes of male infertility in patients presenting to our reproductive medicine clinic in 1986 provides an indication of the frequency of the differing causes of male infertility (Table 2). It is evident that the etiology of their spermatogenic disorder was unknown in about 40% of the men. Further, 40% of men had a varicocele identified, and given the current controversy of the relationship between varicoceles and infertility and spermatogenic disruption *(3,4)*, it could be argued that in about 80% of these men, the cause of their spermatogenic disturbance was unknown.

Twenty years later, the situation has not changed greatly. In many countries where vasectomy is popular, requests for reversal or the achievement of pregnancies by sperm retrieval from the epididymis or testis now represent a new cause of male infertility. The major advance in the area of genetics is the accepted view that in about 3–5% of men with sperm counts of less than 5 million/mL, a Y-chromosome deletion can be identified as the cause of infertility *(5–7)*. In addition, in a small number of men, mutations in other genes have been causally related to spermatogenic

Table 3
Genes in Which Mutations May Cause an Impairment in Hormonal Stimulation of Spermatogenesis

- *Gonadotrophin-releasing hormone (GnRH)*
  - Kalig 1 gene
  - Fibroblast growth factor receptor
  - GnRH receptor
- *Follicle-stimulating hormone (FSH)*
  - β-subunit
  - α-subunit common to luteinizing hormone (LH) and thyroid-stimulating hormone (TSH)
  - FSH receptor
- *LH*
  - β-subunit
  - α-subunit common to FSH and TSH
  - LH receptor
- *Testosterone*
  - Steroid biosynthetic enzymes, some common to adrenal
  - Steroid acute regulatory protein gene
  - Androgen receptor gene including CAG repeat length

defects or duct abnormalities. Some examples of these include androgen receptor defects *(8)*, mutations in the cystic fibrosis transmembrane regulator gene *(9)*, genes related to primary ciliary dyskinesia *(10,11)*, the *SYCP3* gene that encodes a component of the synaptonemal complex *(12)*, and genes related to disturbed hormonal control of spermatogenesis (Table 3; refs. *13–22*). Unfortunately, in these genetic defects the pathophysiology involved is still poorly understood, preventing therapeutic developments. Consequently, spermatogenic disorders classified as idiopathic still represent between 30 and 35% of the infertile men.

In view of the dearth of therapeutic options, the last 20 yr has seen the development of assisted reproductive techniques to assist the infertile male, and in many clinics the use of intracytoplasmic sperm injection (ICSI) represents between 40 and 50% of all treatment cycles. The success of ICSI has raised the inevitability that we are transmitting genetic defects that cause infertility to the next generation. Indeed, several papers have reported the transmission of Y-chromosomal deletions to children conceived by the use of ICSI *(23,24)*. The unanswered question is whether there are other genetic causes of infertility that when transmitted to the next generation may cause diseases other than infertility. Data from Lamb's work suggests that some infertile men may carry and transmit mutations that may increase the risk not only of infertility, but also neurodegenerative diseases such as the cerebellar ataxias *(25)*.

There is now an emerging and reasonably well developed body of evidence that genetic defects are causally related to a major portion of the spermatogenic defects that are currently classified as "idiopathic spermatogenic disorders" *(26,27)*. There are several reports and reviews that have established that targeted disruption of genes can result in spermatogenic damage and infertility as the sole phenotype or accompanied by other pathology in mice *(28)*. In this chapter, the known and postulated genetic mechanisms are assembled into a framework that can assist the clinician in the application of these developments to patient management and facilitate clinical research into genetic mechanisms of male infertility.

2. PHYSIOLOGICAL FRAMEWORK

There are multiple control points in the physiology of spermatogenesis that could be disrupted by mutations in specific genes. The resultant defects are divisible into two major groups: those that are reproductive tract-specific and result in infertility as the only phenotype and others that involve alterations in many organ systems. A clear example of the latter are the mutations in the cystic fibrosis transmembrane regulator, which cause cystic fibrosis and congenital absence of the vas deferens. In some instances, however, the same mutation can cause absence of the vas without cystic fibrosis *(9)*.

Key processes in male reproduction that can be disrupted may be classified as follows:

1. Hormonal mechanisms.
2. Mechanisms controlling spermatogenic output.
   a. Migration of germ cells into developing gonad.
   b. Spermatogonial proliferation and survival.
   c. Meiosis.
   d. Spermiogenesis.
   e. Multiple checkpoints.
3. Leydig cell defects.
4. Sperm-transporting system.
5. Sperm–oocyte interactions.

3. THE APPROACH TO THE IDENTIFICATION OF GENETIC DEFECTS

The approaches that have been undertaken to identify genetic defects causing infertility have varied. Frequently, these have arisen from the identification of a crucial control mechanism with a subsequent targeted disruption of a key gene or genes leading to the phenotype in

mice (*see* reviews in refs. *28–30*). This has been followed by a search for the equivalent human phenotype. For all of the key processes listed previously, it would be possible to list functional sets of genes that should be examined in men exhibiting the phenotype that might be expected if the control system was disrupted. To date, examples of the successful transition from mouse mutations to human mutations are rare but will hopefully increase rapidly in the future. One example relates to our understanding of the need for Sertoli cells to produce stem cell factor that acts, through its receptor c-kit on spermatogonia, to stimulate spermatogonial mitosis and survival. This knowledge has been gained by studying naturally occurring mutations in the c-kit and stem cell factor genes *(31,32)*. A mutation in the c-kit gene in mice results in the white-spotted mutant where failure of normal melanocyte migration (creating the white spots), anaemia, and infertility coexist, indicating crucial actions in several systems. A similar mutation in the *c-kit* gene in certain families results in the condition of human piebaldism, resulting from failure of normal melanocyte migration, but did not result in infertility or anaemia *(32)*. A further example is the mutation in the *SYCP3* gene where mutations in the mouse and human cause disruption of the meiosis *(12)*.

To date, most of the specific gene defects causing infertility have arisen from identification of a disorder with a clear familial transmission with a subsequent search for the specific gene defect. The elucidation of the genetic mechanism of androgen insensitivity syndrome arose from the identification of the hormonal mechanism of androgen insensitivity and the identification of the X-chromosomal location of the androgen receptor gene. Subsequent identification of mutations in this gene demonstrated the genotype–phenotype linkage. Although defective spermatogenesis was not the issue that gained clinical attention in the early reports of patients who were dominated by the male genotype with female external genitalia, there was no doubt that spermatogenesis was disrupted. More recent studies have identified specific mutations in the androgen receptor gene that cause defective spermatogenesis and infertility without altering the male external genitalia *(8,33)*.

Further, expanded CAG repeat length (polyglutamine tract) in exon 1 of the androgen receptor has also been associated with low sperm counts. Several studies have linked a high risk of azoospermia/severe oligospermia to expansions of the CAG repeat beyond 26 (mean for populations 20.7 [United States], 22.4 [Singapore], 21.8 [Australia], 21.8 [Denmark]). These and other observations suggest that the androgen receptor gene with an expanded CAG repeat has low intrinsic

androgen receptor activity *(18–22)*. Others have argued against this linkage *(34)*, but often these studies aggregated all infertile men in their populations, thereby potentially obscuring the specific mechanism. The majority of those studies that identified a linkage were done on infertile men in whom other known causes of infertility, including Y-chromosome deletions, were excluded. There is also the possibility that the size of the CAG repeat influences the response to spermatogenic suppression by androgen–gestation combinations *(22)*. Additionally, targeted disruption of the gene encoding the steroid acute regulatory protein established a phenotype in mice that affected all steroid-producing glands, such as the adrenal, testis, and ovary, producing a complex phenotype *(17)*. Mutations in the human gene have largely replicated the findings in mice.

A second approach arose from observations of chromosomal abnormalities in karyotypic studies. The association of small Y chromosomes observed in karyotypes of azoospermic men led to the identification of Y-chromosome deletions as a cause of severe spermatogenic disruption *(5)*. Subsequent studies have defined the nature and mechanisms of Y-chromosome deletions and these are considered in detail in other chapters in this book *(6,7)*. The frequency of Y deletions in reports has varied significantly, ranging from as high as 37% to as low as 2% *(35,36)*. This variation most likely arises from the selection of patients and may also arise from technical issues, such as the poor quality of DNA leading to failure of polymerase chain reactions. Improvements in our ability to localize defects has culminated in the identification of the nature of the deletions and their frequency, which is conservatively estimated to be 6–10% of men with idiopathic seminiferous tubule failure and about 2% of all men with sperm counts less than 10 million/mL.

In a further example of such an approach, Olesen et al. *(37)*, used digital differential display to identify testis-expressed transcripts, and compared their chromosomal mapping position with the breakpoints found in men with balanced reciprocal translocations found in 265 infertile men. They identified several "hot spots" at 1p31-33, 6p21, 6p22.1, Xq28, 7q 31, and 3p21.1-9. Some of these foci represent regions where known testis-expressed genes are located and others may represent the sites of novel genes with respect to testicular function.

A third area demonstrates the extraordinary power of modern genomics. It has been recognized for decades that the immotile cilia syndrome (now termed primary ciliary dyskinesia) was an inherited disorder. The phenotype of immotile cilia causing infertility and respiratory disorders such as bronchiectasis and dextrocardia (Kartagener's syndrome) is associated with abnormalities of the

axoneme, where either the dynein arms (a protein complex with adenosine triphosphatase activity), radial spoxes, or nexin linkages were usually absent on electron microscopy *(38)*. Recognizing that the structure of cilia was highly conserved, even to algal organisms such as chlamydomonas, recent studies have used mutant algal forms to identify some of the key genes. Subsequent examination of the human genome identified two genes, *DNAI1* and *DNAH5* with very high sequence homology (~90%) to the orthologs in chlamydomonas *(10,11)*, both of which show point mutations in patients with primary ciliary dyskinesia lacking outer dynein arms.

It is surprising that more examples of this approach leading to the elucidation of mechanisms are not available given the ever-increasing number of targets that arise from gene knockout experiments in mice. In part, this is no doubt a result of the relatively small number of academic institutions focusing on andrology that can collect detailed clinical information and DNA samples. It is also complicated by the fact that many men are now progressing to ICSI without a detailed clinical evaluation, underscoring the need for the training of more andrologists and the education of gynecologists in andrology. Third, family sizes have decreased in developed countries, limiting opportunities for the identification of family histories of infertility. Finally, there is a crucial need for adequate numbers of DNA samples from men who are fertile to ensure that an identified mutation is not found in the control populations. Further studies have identified the need for controls from the same population as those in whom the mutation had been identified especially with regard to Y haplotypes *(39–41)*.

Our own approach has been to develop a DNA collection with accurate clinical information and quality data, such as semen analysis, testicular histology, and electron microscopic imaging of sperm (from men with motility disorders). This has led to a collection of more than 2000 samples of DNA. Accurate histological reporting on testis biopsies when performed is crucial to defining the type of spermatogenic disruption. The latter requires fixation of biopsies in fluids such as Bouin's and not formalin, which causes cell shrinkage and the loss of chromatin patterns essential for identification of germ cell type. In addition, we have also collected DNA from the cord blood of infants born by ICSI and DNA from their fathers and latterly from their mothers, enabling us to trace the transmission of genetic defects by the use of assisted reproduction technology *(23,24,42)*.

The utility of our DNA repositories in helping define the clinical place of a potential genetic test is evident from our studies of the small Y-chromosome deletion termed gr/gr *(42)*. In a collection of 1387

infertile men, we showed that the prevalence of gr/gr deletions was 3.96% compared with 0.4% in 234 controls. This study also showed that the frequency of the gr/gr deletion was 3.6% in 504 men with sperm counts of less than 5 million/mL, 3.3% in 122 men with sperm counts of 5–20 million/mL, and 2.46% in 162 infertile men with sperm counts of more than 20 million/mL; all these frequencies were significantly greater than the control population of 0.4%. These frequencies are similar to the original study describing this deletion *(43)*.

The failure of a specific association of the gr/gr deletion with sperm density raises the possibility that the deletion causes sperm functional abnormalities that have not been recognized previously. The definition of the control groups has been critical in determining the significance of the gr/gr deletion. Some studies have failed to ensure that they have a control group in which all the parameters of the semen analysis are normal *(39)*. The availability of large numbers of men with normal semen parameters combined with demonstrated fertility is crucial for such studies and even more important in the search for rarer genetic defects that cause infertility, such as the immotile cilia syndrome with a frequency of 1 in 10,000 men.

4. MUTAGENESIS MODELS

As indicated in Table 3, it is possible to construct a logical screening program of genes for mutations causing infertility on the basis of known physiological control mechanisms. However, there are many other unknown mechanisms involved in the control of spermatogenesis. Identification of these pathways may well open up new avenues for therapeutic and diagnostic approaches. The use of random mutagenesis in inbred mouse strains using *N*-ethly-*N*-nitrosourea for example, when combined with phenotypic screening for infertility, can establish cohorts of infertile mice *(44,45)*. By the use of these breeding strategies and linkage analysis, the genetic mutation causing the infertility phenotype can be identified and, by inference key, point to key genes for the establishment of fertility *(45)*. (An example of lines arising from such a repository can be seen in refs. *44* and *45* and the Jackson Lab website [http://reprogenomics.jax.org].)

4.1. Can a Greater Understanding Be Gained From Gene Knockout Models?

In the current quest to publish in high-impact-factor journals with constraints on space, many papers concerning gene knockout phenotypes have limited data concerning specific details of testicular phenotypes.

This is especially true if there are abnormalities in multiple organ systems. Some excellent examples that illustrate a thorough characterization of a phenotype can be seen in the studies by Baarends et al. *(46–48)*, who have explored the function of the XY body in primary spermatocytes and its role in the silencing of unpaired DNA during male meiosis. They showed that the XY body forms a transcriptionally silenced chromatin domain and that targeted inactivation of genes encoding several DNA repair-related proteins that localize to the XY body interrupts meiosis *(46–48)*. Unfortunately, detailed cytological analysis is often not performed because of the lack of specific expertise, time taken, and number of animals needed, particularly if developmental studies are required.

For instance, the phenotype in the Bcl-w knockout mice would have been inadequately characterized if quantitative studies had not been performed demonstrating increased apoptosis before the profound "collapse" of spermatogenesis at 6 wk and thereafter *(49,50)*. Follow-up studies identified that Bcl-w and the other prosurvival molecules, Bcl_2 and Bcl-x_L , were present in spermatogonia and Sertoli cells but their expression declined in the adult testis and only Bcl-w persisted *(51)*. Thus, in the Bcl-w knockout mice, there are no prosurvival molecules left in the adult, hence the profound germ cell and Sertoli cell depletion.

It was noted that targeted disruption of the β-subunit of follicle-stimulating hormone (FSH) led to disruption of folliculogenesis but maintenance of some fertility in males, albeit with lower testicular volumes *(52)*. More detailed morphometric studies determined that the lower testis volume and decreased sperm output were in part to the result of a decrease in Sertoli cell number as a consequence of the absence of the proliferative action of FSH. However, these studies also showed that the number of germ cells that could be supported by an individual Sertoli cell decreased, indicating a metabolic requirement for FSH to maintain the "carrying capacity" of the Sertoli cells *(53)*.

5. CONCLUSION

A number of other chapters have explored some of the approaches used to identify genes causing infertility in men. A detailed consideration of them in this chapter is unwarranted within the space allocated. Rather, this chapter has assembled many of the genetic defects disrupting fertility into a format that allows a clinical approach to the identification of known genetic defects in patients and facilitates confirmation of genetically determined spermatogenic defects in mice relevant to man. Tables 4–6 use the physiological framework identified earlier to categorize the knowledge to date in some logical arrangement.

Table 4
Examples of Some Genes Involved in Mechanisms Controlling Spermatogenic Output

- *Migration of germ cells into developing gonad*
  - C-kit (stem cell factor receptor) mutations
  - Stem cell factor mutations
  - RNA-binding protein TIAR knock-out
- *Spermatogonial proliferation and survival*
  - Bax (proapoptotic)
  - Apoptosis protease-activating factor (Apat-1)
  - DFFRY
  - AZFa deletions
  - DNMT-3L: spermatogonial loss; Sertoli cell-only phenotype
- *Defects in meiosis*
  - Bcl 16 (antiapoptotic): ↑ apoptosis in M1 (meiosis 1)
  - Ataxia telangiectasia mutant (ATM): chromosome fragmentation
  - Cyclin A1: desynapsis abnormalities at M1
  - Deleted in azoospermia-like (Dazl): loss in M1
  - Dmc1-meiosis-specific RecA: zygotene arrest
  - HSP70.2: synaptonemal complex desynapsis failure
  - MLH1-DNA mismatch repair enzyme: meiosis arrest
  - RAD6b (hr6b)-ubiquitin conjugating enzyme: postmeiotic
  - Chromatin condensation
  - Synaptonemal complex protein 3 (SCP3): chromosome synapse failure
  - Translocated in liposarcoma (TLS): failure of synapsis
  - Microorchidia (morc): zygotene-leptotene arrest
  - Siah 1a: failure of M1 metaphase to anaphase transition
  - Mouse vasa homolog gene (Mvh): zygotene arrest
  - AZFb deletions: meiosis arrest
- *Specific defects in spermiogenesis*
  - Casein kinase II catalytic subunit (CK2): globozoospermia
  - Cyclic AMP-responsive element modulator (CREM): early spermatid arrest
  - Ca^{++}/calmodulin dependent protein kinase IV (Camk4): elongating spermatid defect
  - Transition nuclear protein1 (TP1): decreased sperm motility
  - Apolipoprotein B (apo B): decreased sperm motility and survival
  - DNAI1: loss of outer dynein arms in primary ciliary dyskinesia
  - DNAI2: candidate for primary ciliary dyskinesia
  - MDHC7 (mouse dynein heavy chain): KO → ciliary dyskinesia
  - DNAH5: absence of outer dynein arms, primary ciliary dyskinesia
  - Sperm calcium ion channel; involvement with hyperactivation of sperm

Table 5
Hypospermatogenesis

- *Generalized germ cell loss (oligospermia)*
  - Bclw (antiapoptotic): progressive germ cell loss
  - Aromatase (cyp19): progressive germ cell loss
  - Complementation group A: age-dependent decrease
  - AZFc deletions: severe oligospermia
  - Hormone sensitive lipase (HSL): oligospermia
  - Leydig insulin-like hormone (Ins 13): cryptorchidism
  - Occludin: progressive germ cell loss
  - Type 1 protein phosphatase Cγ2 (PP1 c γ2): spermatocyte and spermatid loss
- *Generalized germ cell loss (low normal/oligospermia)*
  - Decreased Sertoli cell numbers and "carrying capacity"
  - Follicle-stimulating hormone β-subunit knockout
  - Activin type IIA receptor knockout

Table 6
Other Defects Genetic Mechanisms With the Potential to Cause Infertility

- *Leydig cell agenesis, loss or dysfunction*
  - Desert hedgehog (Dhh): Leydig cell agenesis, peritubular cell defects
  - Macrophage colony-stimulating factor (M-CSF): knockout → absent testis macrophages and absent Leydig cells
  - Steroid acute regulatory protein mutations
- *Sperm transport defects*
  - Estrogen receptor α knockout: efferent duct back pressure
  - Cystic fibrosis transmembrane regulator: vas agenesis
  - PEA3 (ets-transcription factor): ejaculatory dysfunction

The large number of potential genetic targets and the costs associated with screening will cause the clinician problems in trying to identify the most appropriate tests that should be ordered in any clinical investigation. Until clear genotype–phenotype associations are defined, the clinician must still rely on careful analysis of the clinical features of the patient and information from routine tests such as semen analyses, FSH, luteinizing hormone, and testosterone and testicular histology to assist in the choice of the emerging genetic investigations. It is also clear that a karyotype should be undertaken in men with sperm counts of less than 10 million/mL. Further, given the frequency of Y deletions in men with sperm counts of less than

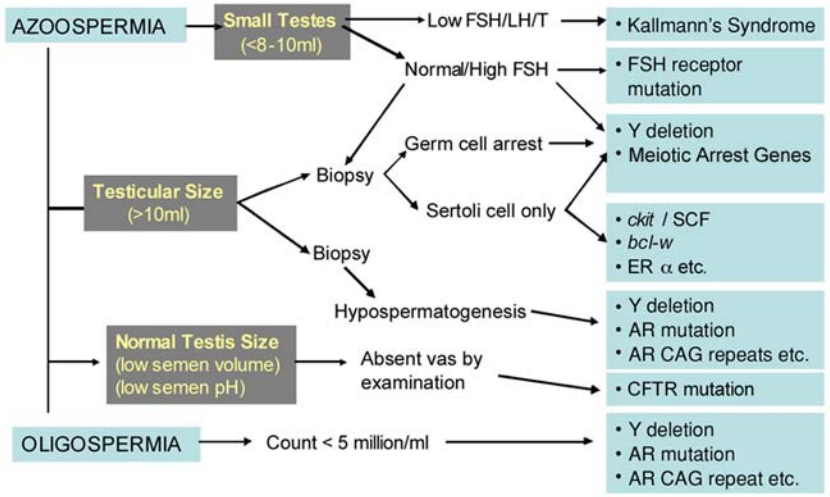

Fig. 1. Schematic approach to identifying groups of patients with a potential genetic basis of infertility.

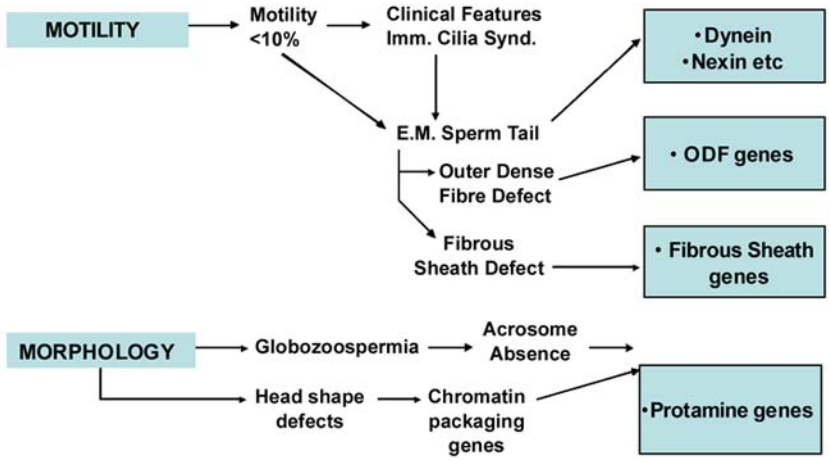

Fig. 2. Schematic approach to identifying groups of patients with a potential genetic basis of infertility for motility or morphology disturbances.

5 million/mL, karyotype should be a routine investigation. Given the data that gr/gr deletions are found in infertile men with a wide variety of spermatogenic abnormalities, the availability of a simple and cheap test could make such an investigation routine for most infertile men.

An outline of an approach to the choice of genetic evaluations for the common phenotypic presentations in infertile men is provided in

Figs. 1 and 2. However, the list of potential genetic targets cannot be listed exhaustively and these figures should be read with reference to Tables 3–6 and recent reviews *(28,30)*.

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17 The Future of the Diagnosis of Male (In)Fertility

Christopher De Jonge, PhD, HCLD

Summary

Traditionally, the diagnosis of male (in)fertility has relied on the results from clinical evaluation and semen analysis. Although these approaches have undeniable merit, often times causes for subfertility are more covert and are not readily identified by these traditional approaches. In these instances, the diagnosis is simply written as idiopathic or undiagnosed. The genomic and proteomic eras have promised great potential to remedy those cases defined as undiagnosed and further advance our understanding of the complex processes that combine and contribute to the so-called fertile and infertile male.

Key Words: Male infertility; semen analysis; diagnosis; genetics; clinical prognosis.

1. INTRODUCTION

In the absence of clinical pathology, accurate diagnosis of male infertility is further complicated if one must rely solely on results from semen analysis. Although there are strong correlates between abnormalities in one or more semen parameters and subfertility, there remains a substantial gray or indeterminate range for which diagnostic ability is at best equivocal.

The genomic and proteomic eras have promised great potential to offer remedy for those cases defined as undiagnosed and further advance our understanding of the complex processes that combine and contribute to the so-called fertile and infertile male (*see* accompanying chapters in this book).

This chapter briefly reviews some of the traditional approaches to male infertility diagnosis, what the new molecular technologies offer in the way of diagnostic promise and, finally, what the future clinical realities might be for diagnosing male (in)fertility.

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2. DIAGNOSIS AND MALE INFERTILITY

Before delving into the practical aspects of infertility diagnosis, perhaps it would be instructive to define what is meant by "diagnosis." Essentially three levels of complexity can be identified in defining the term diagnosis. The simplest definition is the *identification* of the nature or cause of some phenomenon. A more complex definition for diagnosis is the *process of identifying* a disease by its signs, symptoms, and results of various diagnostic procedures. Last, the most complex definition of diagnosis is the *comparison of the condition* of the patient to patterns from diseases sharing similarities, based on the examination of the specific clinical condition and on results from additional assays.

The clinician and clinical andrology laboratory collaborate in an attempt to offer the male patient answers as to why they are unable to conceive with their presumably fertile partner. The clinician relies on typical history and physical evaluations to determine whether follow-up investigation, such as sonography, radiography, endocrine, and chromosome testing, is warranted. Often, through one of these investigations, a specific diagnosis (cause) can be identified. For example, based on simple physical examination, bilateral cryptorchidism identifies the cause for the infertility (first definition presented earlier). However, although identification is made for infertility, it does not identify the cause for the undescended testes. That process of identification may result after implementing other diagnostic procedures.

The preceding analysis may appear to be arbitrary, yet it weighs significantly on exactly what is meant, perhaps semantically, by "diagnosis of male infertility." For instance, is the identification of one or more variances in semen parameters from established reference values diagnostic, or merely the manifestation of an etiology yet to be uncovered?

3. SEMEN ANALYSIS AND DIAGNOSIS

It can safely be asserted that the ability of semen parameters to distinguish between the subfertile and fertile male in the general population is suspect *(1–5)*. Indeed, investigators *(6)* performed a structured review of the literature focusing on articles published in English between 1983 and 2002 and using semen parameters (i.e., concentration, motility, and morphology) to establish thresholds that would distinguish between fertile and subfertile populations. For this study, the investigators were able to identify only 4 out of 265 articles that fit their structured review criteria. This small portion of directly comparable clinical research publications, in terms of design and methods, seems rather astounding when considered on balance with the heightened

awareness and practice of global standardization for semen analysis in the andrology laboratory (e.g., ref. *7*). The purpose of this example is to point out the overwhelming heterogeneity that exists in the clinical research database regarding semen parameter attributes that characterize the fertile and/or infertile male, thereby making it difficult to formulate diagnostic thresholds.

In the face of the aforementioned rather dire situation, there exists a plethora of publications in which semen analysis is demonstrated to have irrefutable merit. For example, there are those situations in which the number of countable or motile or normal spermatozoa is so small that a conclusion regarding fertility potential can be made with relative confidence (e.g., severe oligozoospermia, necrozoospermia, globozoospermia). Results from semen analysis such as these provide clear indication that therapeutic intervention is required for fertility, albeit no cause of the abnormality has been diagnosed. These results may also provide indication for additional diagnostic testing, such as Y-chromosome microdeletion analysis. Thus, perhaps the truer merit of semen analysis lies more squarely not so much with strict diagnostic ability but rather as a "signal" assay for stimulating further diagnostic investigation (e.g., refs. *8–10*).

Recent advances in the andrology research laboratory have shed light on the importance of sperm chromatin and DNA packaging as an additional attribute of fertile spermatozoa (*see* Chapter 20). Several different assays to evaluate DNA fragmentation have been developed, all of which have been purported to have clinical diagnostic merit *(11,12)*. Indeed, numerous reports, mostly from the in vitro fertilization laboratory, have detailed that if sperm DNA is fragmented and/or if protamine deficiency is evident, then the likelihood for term birth is decreased (e.g., ref. *13*). These DNA assays have not, however, made the transition into the clinical andrology laboratory for routine global application, primarily because of assay complexity and data diversity *(14)*.

It serves well to mention the World Health Organization (WHO) manual for semen analysis *(7)*. The WHO manual is the only text globally accepted to be the standard on which procedures and rationale for conducting examination of semen is performed. The manual has undergone several revisions since its first appearance in 1980. Perhaps the most conspicuous change that is evident today is the content dedicated to quality control. Sections pertaining to improvements in actual diagnostic ability have improved only marginally. Morphology assessment and its potential implications stand out as perhaps the only parameter that has gained in relative clinical significance. That said, morphology assessment (and all other semen parameters for that matter) is only as reliable as the technician, their training, and persistent proficiency

training *(15)*. Standardized training and methods for proficiency testing still remain somewhat enigmatic worldwide.

The aforementioned statements are in no means meant to slight the tremendous significance of the WHO manual, for without it we would be lost on the foggy mountaintop without a map and compass. I believe the content and relative stasis of the manual is reflective more of the disparate nature of the clinical research reports, as discussed earlier, rather than a failing by the editors who have written the manual over the years. The forthcoming version (5th edition in preparation) will prove to be the most transitional and reflective of the current global clinical pulse.

4. CLINICAL REALITIES

What then is the current global clinical pulse? Regrettably, the answer to this question involves far greater discourse than is reasonable for present purposes. Suffice to say that consumer demand, cost, health care systems, and insurance weigh considerably on how much effort will be placed on diagnosis vs that expended on treatment. Although it is inarguable that a patient expects a diagnosis for their malady, that desire is balanced keenly against their desire for a result, and for present context that means a healthy baby, and the sooner the better.

In the WHO manual for the standardized investigation, diagnosis, and management of the infertile male *(16)*, it is written that with the exception of azoospermia and congenital bilateral absence of the vas deferens there should be a second semen analysis to serve as comparison for the first. So, a diagnosis is made for congenital bilateral absence of the vas deferens, not from semen analysis *per se*, but what about for the azoospermic male? The manual recommends genetic testing for these and severe oligozoospermic males. That leaves a majority of men who might have one or more abnormal semen parameters and for whom the only diagnostic test recommended is a repeat semen analysis. But what is being diagnosed in a third or fourth repeat semen analysis—perhaps nothing! No infection, no varicocele…only the symptoms of low sperm count, motility, or morphology have been revealed. No clear reason (diagnosis) can be determined yet. This is what proteomics and or genomics might offer—the diagnosis.

However, the subfertile male that has a moderately diminished total sperm number, for example, 30×10^6, 40% of which are motile and whose strict morphology criteria score is 6%. What is his diagnosis? In fact, can one even be made? So, let's return to global pulse and finances.

The advent of intracytoplasmic sperm injection (ICSI) largely negated strong impetus to advance diagnostic ability in the clinical andrology

laboratory, with exception made to genetic testing. The perfect assisted reproductive technology universe now exists in the unity of one sperm and one egg. Who now cares whether there is 10 or 20 million sperm? The patients want a baby, so let us try a couple rounds of superovulation with intrauterine insemination and if that fails we will go straight to ICSI. The patient (couple) will likely get their baby, but is this the most cost-effective approach? The answer is not straightforward, nor is it likely to be globally unanimous. Cost-effectiveness is regionally dependent on whether the health care is government-sponsored, self-paid, state-mandated, or variations in between.

Cost also includes the emotional expense for the patients. It is well described that infertility is emotionally expensive. A more protracted diagnostics in lieu of expedited therapy might not be the option of choice for most patients. Applying the example of the oligozoospermic male with a presumed fertile female partner, would this infertile couple, by definition, be willing to go home and have timed intercourse for yet 1 yr more because the odds favor that they will conceive—at some point? No, at least probably not in the majority, these consumers will demand and likely their insurance or their own finances will urge a more rapid route straight to therapy rather than diagnosis. So, once again, superovulation with intrauterine insemination will likely be attempted and, if unsuccessful after several tries, then in vitro fertilization, with or without ICSI, will be the therapy of choice (therapy is a very loose term in this situation because no therapy is actually applied to remedy the oligozoospermia).

5. THE MOLECULAR GENETIC REVOLUTION

Without question, the unraveling and deciphering of the human genome has opened a treasure trove of potential diagnostic and therapeutic possibilities for both male and female factor infertility. The cause for a man's hypospermatogenesis may be determined using DNA microarray analysis, proteomics, and other technologies (e.g., refs. *17* and *18*). However, will a better ability to diagnosis male infertility lead to better therapeutic strategies? This is the million dollar question. Because, to reiterate, there is therapeutic ICSI and for male factor infertility this is the *panacea du jour*, and is not likely to be replaced any time soon.

6. CONCLUSION

It is important to heavily underscore that because of ICSI, and assisted reproductive technology in general, the importance of molecular genetic diagnostics in the field will likely blossom as greater understanding of these technologies in association with epigenetics is

gained *(19)*. Whether or not these technologies become routinely applied in the clinical andrology laboratory as diagnostic tools for unraveling the cause of a man's infertility remains to be seen.

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18 Polymorphisms and Male Infertility

Csilla Krausz, MD, PhD

Summary

The analysis of polymorphisms in genes involved in spermatogenesis represents one of the most exciting areas of research in the genetics of male infertility. These studies are not only important for identifying genetic risk factors for male infertility, but they may also represent an important starting point for searching for genes involved in spermatogenesis through linkage analysis. Despite many efforts, we often face frustrating situations in which initial promising data are not confirmed in later studies. Discrepancies between association studies are rather frequent and can be related to different factors, such as inadequate sample size, the pathogenetic heterogeneity of infertility, inappropriate control subjects, positive publication bias, and ethnic and geographic differences.

It is likely that some polymorphisms only lead to testicular dysfunction when associated with a specific genetic background or with environmental factors. The role of genetic background seems to be especially relevant for one of the most promising genetic risk factors, the gr-gr deletions of the Y chromosome. Certain gene variants may cause specific phenotypes and consequently only the analysis of a specific subgroup of patients is able to identify their clinical significance. To obtain reliable and clinically useful data, much more attention should be focused on the correct study design, which is still the major weakness of association studies.

Key Words: Polymorphism; spermatogenesis; gr-gr deletion; infertility genetics; Y chromosome.

1. INTRODUCTION

Despite our increasing knowledge of the physiology of male reproduction and the availability of new diagnostic tools, the pathogenesis of testicular failure remains undefined in about 50% of cases and is referred to as "idiopathic infertility" *(1)*. Idiopathic testicular failure is likely to be of genetic origin because the number of genes involved in human spermatogenesis is possibly thousands or more and only a small proportion of them has been identified and screened in infertile men.

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Table 1
Polymorphisms and Male Infertility

Polymorphisms	*More than one study*	*Single study*
in GENES involved in:		
Endocrine regulation of spermatogenesis	Androgen receptor[a,b] Follicle-stimulating hormone receptor Estrogen receptor α	Estrogen receptor β Combined single-nucleotide polymorphisms°
Specific spermatogenic functions	DAZL[a,b] PRM1[a]	USP26 GRTH CREM
Different cell functions (metabolism, cell cycle, mutation repair)	POLG[a,b] MTHFR[a]	GSTM1 PHGPx BRCA2
in DNA sequences:	Y-chromosome haplogroups[a] gr/gr deletions[a]	Mitochondial DNA haplogroups

[a]Data in the literature are contradictory.
[b]Polymorphisms that are not considered risk factors in the Caucasian populations.

Several reports have focused on the role of certain haplogroups, allele variants, and single-nucleotide polymorphisms (SNPs) in male infertility (Table 1). In many cases, only sporadic data are available, or alternatively, when more studies are published on the same polymorphism, the results are often contradictory. Inadequate sample size, pathogenetic heterogeneity of infertility, inappropriate control subjects, and ethnic and geographic differences (probably also related to environmental factors) may be responsible for discrepancies among case–control studies. On the other hand, these studies are not only important for identifying genetic risk factors, but they may also represent an important starting point in searching for genes involved in spermatogenesis through linkage analysis. In this regard, association studies dealing with human leukocyte antigen haplotypes are relevant for the identification of candidate major histocompatibility complex genes *(2,3)*, and mitochondrial DNA (mtDNA) haplogroups for genes involved in mitochondrial functions *(4,5)*. Similarly, Y-chromosome-related factors (other than azoospermia factor [AZF] deletions) can be determined indirectly by the definition of Y-chromosome haplogroups predisposing to male infertility *(6,7)*. In this chapter, recent findings concerning polymorphisms and male infertility are discussed.

2. POLYMORPHISMS IN GENES INVOLVED IN THE ENDOCRINE REGULATION OF SPREMATOGENESIS

The crucial role of androgens, gonadotropins, and estrogens in the endocrine regulation of spermatogenesis is well known, thus genes of their receptors represent a logical target for mutational analysis in the infertile male.

2.1. The Androgen Receptor

The androgen receptor (AR) is a ligand-activated transcription factor that is encoded by the *AR* gene located on the long arm of the X chromosome (Xq11-q12). The *AR* gene has been the object of a large quantity of studies, and both mutation screenings of the entire coding sequence and the promoter region have been reported (for review, *see* ref. *8*). The first exon of the *AR* codes for the transactivation-regulating domain and contains two polymorphic tracts: a CAG and a GGC repeat sequence. The polymorphic (CAG)n codes for a polyglutamine, whereas the (GGC)n repeat for a polyglycine stretch. It has been demonstrated in vitro that the length of the polyglutamine tract, while remaining within the polymorphic range, is inversely correlated with the transactivation activity of the receptor *(9)*. According to this observation, the first association studies dealing with CAG repeat length and male infertility have shown a significant association between relatively long CAG repeats and impaired sperm production. However, subsequent studies gave rather contradictory results, which in part can be the consequence of ethnic differences (the association seems to be more consistent in the Asiatic populations), although the heterogeneity of the control (unselected men or proven fertile men or normospermic men) and of the infertile (different inclusion criteria) groups (for review, *see* ref. *8* and references therein) may play also an important role. A repeat number more than 23 has been reported as significant risk factor only in 5 out of 11 studies, mainly involving Singaporean, Australian, North American, and Japanese subjects, whereas this association is not evident in the European studies *(10)*.

Two groups from Europe attempted to evaluate the joint effect of both exon 1 and polymorphic microsatellites on male infertility *(11,12)*. Although the two populations were both Caucasian, the "protective" and "at risk" CAG/GGC haplotypes were different. In the Swedish study, the <21CAG and GGN = 23, whereas in the Italian study the >23CAG and <16GGC combined haplotype confers a lower risk of infertility to the carriers. Because of these discordant association

data and the lack of in vitro expression studies on the effect of varying GGC length in combination with different CAG repeats, the clinical utility of the CAG/GGC haplotype definition remains unclear.

In summary, if only data based on large study populations are considered, the CAG repeat length polymorphism is an unlikely risk factor for male infertility. However, its role in modulating androgen action is evident in patients affected by Klinefelter syndrome *(13)*, in hypogonadal men undergoing T-replacement therapy *(14)*, and in hypoandrogenic males *(15)*. It is therefore possible that the mild functional effect of a long polyglutamine strech can be compensated by a relatively high serum testosterone level, ergo the polymorphism should not be evaluated in isolation but always in the context of environmental factors.

2.2. The Follicle-Stimulating Hormone Receptor

Besides testosterone, follicle-stimulating hormone (FSH) is another fundamental hormone for normal gametogenesis. FSH stimulates spermatogenesis through its specific receptor (FSHR) that is a member of the G protein-coupled receptor family. The receptor consists of 10 exons, located on chromosome 2 (2p21-p16). Mutation screening of the FSHR gene revealed various SNPs, among them the SNP in the core promoter at position -29 and two others situated in exon 10 (for review, *see* ref. *16*). Exon 10 codes for the C-terminal part of the extracellular, transmembrane, and intracellular domains. The two SNPs in exon 10 correspond to amino acid positions 307 and 680 of the mature protein. The two SNPs result in two major, almost equally common allelic variants in the Caucasian population: Thr^{307}-Asn^{680} and Ala^{307}-Ser^{680}. Studies comparing the distribution of the two SNPs in normal and infertile men did not show significant differences *(17)*, whereas the combination of the exon 10 SNPs with the -29 SNP evidenced specific allelic combinations, which can be considered as a new genetic factor for severe spermatogenic impairment *(18)*. Further confirmation of these promising data is awaited in other populations.

2.3. Genes Involved in the Estrogenic Pathway

Although the physiological role of estrogens in spermatogenesis is not clearly defined, human and animal models evidenced an association between estrogen insufficiency and abnormal spermatogenesis. Although recent studies suggest a role as a survival factor *(19)*, the excess of this hormone during the neonatal period or adulthood can impair sperm production in rats *(20)*. The physiological responses to estrogens are known to be mediated by at least two functional isoforms of estrogen receptors (ERs), namely ERα and ERβ, encoded by two

different genes in different chromosomes (6q25 and 14q23-24, respectively). Apart from estradiol, other compounds with estrogen-like activity (xenoestrogens) may bind to ERs and may account for the reported decline in sperm count as well as for the increased incidence of other components of the testicular dysgenesis syndrome (hypospadias, cryptorchidism, and testicular cancer) observed in the last 50 yr *(21)*.

Genetic screening of the ERα and ERβ genes has revealed the existence of several polymorphic sites in both genes and some of them have been the object of association studies dealing with male infertility. In the ERα gene, the most widely studied are the PvuII (T397C) and XbaI restriction fragment length polymorphisms (RFLPs) in intron I and the $(TA)_n$ variable number of tandem repeats within the promoter region. To date, four studies have been published in four different populations: Greek *(22)*, Japanese *(23)*, Spanish *(24)*, and Italian *(25)*. A significant association between male infertility and the XbaI RFLP *(22)* or the exon 4 codon 325C-G *(23)* polymorphisms was reported, however, the interpretation of these results is difficult because of the small sample size, especially in the Japanese study (only 31 patients). The Spanish *(24)* and our own study *(25)* on the Italian population aimed to define the role of TA repeats in the promoter region. Both investigations reached the conclusion that the distribution of TA genotype is not different between controls and patients; therefore, this polymorphism cannot be considered a risk factor for male infertility. However, further analysis in the Italian population showed a significant effect of this polymorphism on sperm output in both the control and the infertile groups. The number of TA repeats showed a significant inverse correlation with sperm count and the subdivision of the allelic combinations into two major genotypes (genotype A and B) revealed that men with higher TA repeat number on both alleles (genotype A) have significantly lower sperm production. Because previous studies on lumbar bone mineral density observed that allelic combinations with higher TA repeats are functionally more active *(26)*, our finding indicates that allelic combinations, which confer a stronger estrogen effect, may negatively influence human spermatogenesis. A plausible explanation would be that not only a deficit of estrogens, but also an exaggerated estrogen action related to this genetic variant (eventually combined with environmental factors), can be deleterious. Whether the observed negative effect reflects the expression of a disturbance in the early testis development or in the adult testis, and whether it is related to xenoestrogens, remains to be established. In the Spanish study, besides ERα, other estrogen-related genes have also been analyzed for polymorphic markers (ERβ, FSHR, CYP19A1, and NRIPI). The results support a

relevant role for the estrogenic pathway, especially for SNPs in the ERα gene, but also indicate that the combination of different allelic variants in the five genes may protect or predispose to male infertility. However, again because of the limited sample size, these data need to be confirmed in a larger study population.

Thus far, only one association study has been performed for ERβ gene SNPs (RsaI [G1082A] and AluI [G1730A]) and male infertility. The frequency of the heterozygous RsaI AG-genotype was three times higher in infertile men than in controls, indicating that this polymorphism may have modulating effects on spermatogenesis *(27)*.

In summary, preliminary data suggest that ERα and ERβ polymorphisms may influence male fertility and spermatogenic efficiency. It will be of interest to verify the effect of the aforementioned ER polymorphisms on spermatogenic potential in a selected group of subjects with different levels of exposure to xenoestrogens.

3. TWO EXAMPLES OF POLYMORPHISMS IN CANDIDATE AUTOSOMAL SPERMATOGENESIS GENES: PROTAMINE 1, PROTAMINE 2, AND DAZL

A number of spermatogenesis, autosomal, candidate genes have been identified and represent the most obvious targets for mutation analysis. Among them the Protamine (PRM) 1 and PRM2 and deleted azoospermia-like (DAZL) genes were the object of several studies, finally leading to the conclusion that PRM1 polymorphism is relevant in a specific subset of patients, whereas the DAZL gene polymorphism is relevant only in a specific ethnic group.

After the completion of meiosis, in the late phases of spermatogenesis, the haploid genome is compacted within the sperm head by two DNA-binding proteins, PRM1 and PRM2. This remarkable repackaging event is related to the requirement for a unique chromatin architecture that would enable a specific transcription schedule after fertilization. Premature translation of PRM1 messenger RNA causes precocious nuclear condensation and arrests spermatid differentiation in mice *(28)* and the disruption of either the *Prm1* or *Prm2* gene in mice leads to haploinsufficieny, abnormal cromatin compaction, sperm DNA damage, and male infertility.

Although reduction of PRM2 has been reported in infertile men, mutations in the PRM2 gene have not been reported in association with reduced PRM2 content *(29)*. Nishimune et al. *(29)* identified a number of SNPs in both the PRM1 and PRM2 genes in a large number of infertile men presenting mainly with azoospermia and proven fertile controls. No

association between any of the SNPs and infertility was observed. However, one mutation in the PRM2 gene induced a nonsense codon C248T and was present in heterozygosity in one azoospermic patient. Unfortunately, the testis histology of the patient is unknown and this makes difficult to interpret the consequences of this mutation. In a recent study, a highly selected group of infertile male patients were screened for PRM1 gene mutations *(30)*. A novel SNP, G197T, in a highly conserved region of the gene was identified in 3 out of 30 patients. Based on the absence of this SNP in more than 700 individuals, it appears to be a promising new genetic risk factor for a specific subgroup of infertile patients with normal sperm count associated with abnormal sperm DNA fragmentation and/or teratozoospermia.

The *DAZL* gene is an autosomal homolog of the Y-chromosomal *DAZ* gene cluster and is mapping to chromosome 3p24 *(31)*. *DAZ*, *DAZL*, and *BOULE* are members of the same family and encode RNA-binding proteins with important role in spermatogenesis *(32)*. No clinically relevant mutations for the *BOULE* *(33,34)* and the *DAZL* genes have been reported so far, except one SNP in the *DAZL* gene sequence in exon 3 (T54A), which was reported as a susceptibility factor to oligospermia/azoospermia in the Chinese population *(35)*. This SNP is situated within the highly conserved RNA-recognition motif domain of the DAZL protein and it may lead to functional consequences such as reduced RNA binding. Despite this promising finding, subsequent studies in Caucasian populations *(36–38)* and in the Japanese population *(39)* failed to detect the T54A mutation in more than 900 men tested, strongly contrasting with the relatively high frequency of this mutation (7.4%) in the Chinese patients. This remarkable difference represents an example of how ethnic background is also important for polymorphisms involved in spermatogenesis.

4. THE POLG GENE POLYMORPHISM

Normal function of sperm mitochondria is a prerequisite for normal spermatogenesis and motility. The mtDNA polymerase γ (POLG) is the sole polymerase for mtDNA and an impaired activity of this protein leads to mitochondrial dysfunction through accumulation of mtDNA mutations. The gene maps to 15q24-15q26 and its first exon contains a polyglutamine tract encoded by a motif $(CAG)_{10}$ CAACAGCAG *(40)*. The length of the CAG repeat is polymorphic with a major allele at 10 repeats.

Rovio and colleagues *(41)* proposed an association between the absence of the common 10 CAG allele and male infertility in a relatively small group of infertile (n = 99) and fertile (n = 98) men. This

finding has been extensively debated by subsequent larger studies. One study on the Danish population observed a significantly higher frequency of homozygous not10 CAG repeat allele in a subgroup of men affected by unexplained infertility (i.e., normal sperm count, motility, and morphology; ref. *42*). However, this conclusion was based on an interpretation bias (i.e., the seven unexplained infertile men with the homozygous not10/not10 CAG genotype were not normospermic, with the exception of one subject). The recalculated real frequency, 1 of 42 (2.38%) instead of 7 of 49 (14.3%), shows no significant difference in respect to the control fertile group (0.8%). In the same year, based on our own study on the Italian population (n = 385), we concluded that there is no relationship between the polymorphic CAG repeat in the POLG gene and idiopathic male infertility *(43)*. The same conclusion was achieved later in another large study on the French population *(44)*.

It is therefore clear that the POLG CAG polymorphism has no clinical significance, neither for idiopathic, nor for unexplained male infertility. Considering the importance of mitochondria for sperm motility, the question of whether pure asthenozoospermia (with the sole symptom of reduced sperm motility) can be the consequence of the not10/not10 CAG genotype, remains to be addressed. However, before performing such a study, it would be important to clarify if the length of the CAG tract has any functional effect on the polymerase activity (for review, *see* ref. *45*).

5. Y-CHROMOSOME POLYMORPHISMS: THE GR/GR DELETIONS

Apart from the classical AZF deletions, a new type of Yq deletion has recently attracted the attention of geneticists and andrologists. A partial deletion in the AZFc region, termed gr/gr has been described specifically in infertile men with varying degrees of spermatogenic failure *(46)*. This deletion removes half the AZFc gene content, including two copies of the major AZFc candidate gene called DAZ *(47)*. In the last 2 yr, an intensive search for gr/gr deletions in infertile and control normospermic men has started to define their frequency and clinical significance *(48–53)*. From the first studies, it was clear that in contrast to the classical AZF deletions, gr/gr deletions can be found also in normospermic men *(49,51,52)*, although at a significantly lower frequency. Therefore, rather than a specific cause, this genetic anomaly represents a risk factor for spermatogenic failure. Among a number of plausible explanations for the heterogeneous phenotype, we hypothesized the presence of polymorphisms or mutations in the autosomal homolog of

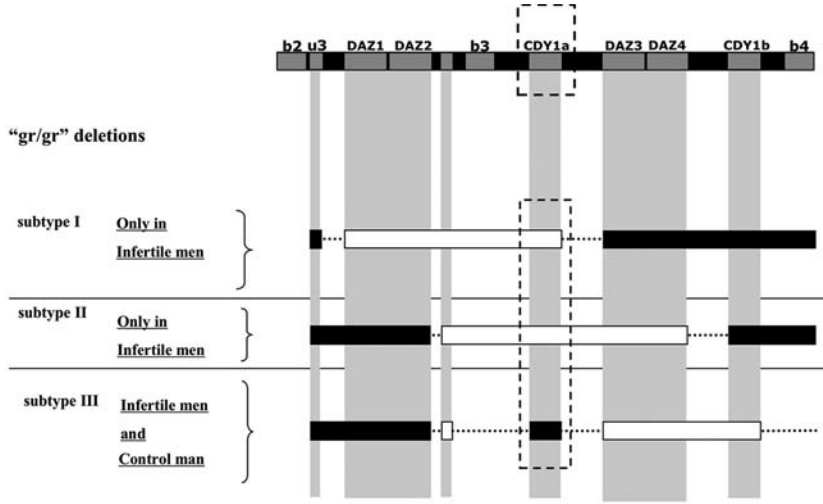

Fig. 1. Following molecular characterization of the gr/gr deletions (gene copy dosage and type of DAZ and CDY1 gene deletion), three subtypes of gr/gr deletions were identified in a large group of infertile and control men of Italian origin *(52)*. CDY1b copy seems to be specifically deleted in infertile men, suggesting that different deletion patterns may have different phenotypic effects.

the DAZ gene, *DAZL (36)*. However, similar to the AZFc-deleted patients, we found no new mutations in the entire coding region of the DAZL gene except the polymorphic Thr12-Ala change (T12A), which, because of its relatively high frequency in the normospermic group, does not seem to have any modulating effect *(36,52)*. The currently used method for the detection of gr/gr deletions is based on STS plus/minus type of analysis, which alone does not provide information about the type of missing gene copies. This analysis may also detect false deletions as a result of rearrangements of the STS-containing sequence, and is also unable to rule out a duplication of the nondeleted part of the AZFc region. The majority of gr/gr studies lack a detailed molecular analysis (i.e., the reduced gene dosage is not confirmed and the type of deleted gene copies is also unknown; refs. *49–51* and *53*). Mitchell et al. *(48)* developed a method able to detect the type of the missing CDY and DAZ copies, whereas Vogt et al. *(54)* developed an RFLP-based DAZ copy analysis. Using the first method, our preliminary data indicate that gr/gr deletions associated with the loss of CDY1a copy are found only in infertile men and not in normospermic controls (Fig. 1; ref. *52*). However, the number of controls is low and further combined studies are needed.

Aside from the gr/gr deletions, the AZFc region predisposes to a number of other possible partial deletions *(55,56)*. The Y-chromosome background seems to play an important role in the pathogenic consequence of these deletions. For example, another deletion named b2/b3 *(57)* or u3-gr/gr *(48)* or g1/g3 *(58)*, which removes a similar quantity of AZFc genes compared with the gr/gr deletion, seems to have no effect on fertility status in association with a certain Y-chromosome background commonly present in Northern Eurasian populations (Y haplogroup N; refs. *57* and *58*). A similar conclusion can also be drawn for the gr/gr deletion found in association with Hgr D2b, which is present in 20% of Japanese subjects *(7)*. Consequently, a combined molecular characterization (haplogroup, gene dosage, and gene copy type definition) of the gr/gr deleted patients and controls will probably allow the distinction between pathogenic and neutral deletions. In the meantime, the screening for gr/gr deletions can be advised for patients undergoing assisted reproductive techniques, because this test is able to provide the identification of a transmissible genetic risk factor (odds ratio = 10.2, confidence interval 1.28–80.3) for reduced sperm count *(52)*.

6. VARIOUS OTHER POLYMORPHISMS

A number of other association studies dealing with different polymorphisms (SNPs or microsatellites) in autosomal and X-linked genes have been published in the recent years. Among them are genes that encode for proteins involved in protection against oxidative stress, such as glutathione S-transferase M1 (GST M1) and Phospholipid hydroperoxide glutathione peroxidase (PHGPx; refs. *59* and *60*). Polymorhisms in these genes are not associated with idiopathic infertility, however, they may have a role in specific conditions associated with oxidative stress (e.g., varicoceles; ref. *60*). Similarly, another gene polymorphism C677T in the methylenetetrahydrofolate reductase gene seems to have clinical relevance only in specific environmental conditions characterized by low dietary intake of folates, which is more common in Indian, African, and Southeast Asian populations *(61–64)*.

New variants in a number of genes with proven or potential role in human spermatogenesis were recently identified and are of interest for future case–control association studies. The common variant N372 in *BRCA2* gene *(65)*, polymorphisms in the *USP26 (66)*, and the gonadotropin-regulated testicular helicase (*GRTH*; ref. *67*) genes have been recently proposed as potential risk factors for severe spermatogenic failure. Among the most relevant spermiogenesis candidate genes, the cyclic adenosine monophosphate-responsive element modulator *(CREM)* gene has been the object of mutation screening in a

specific group of men with round spermatid arrest *(68,69)*. In this pilot study, a number of genetic changes have been identified and it seems that certain patterns of homozygous and heterozygous alterations could exert pathological effects *(69)*.

7. CONCLUSIONS

The analysis of polymorphisms in genes involved in spermatogenesis represents one of the most exciting areas of research in the study of the genetics of male infertility. However, we are often facing frustrating situations in which initial promising data are not confirmed in later studies. Discrepancies between association studies are rather frequent and can be related to different factors. Low sample size represents one of the most frequent causes for lack of replication and there are many evidences in the literature showing the critical value of the sample size. According to a meta-analysis by Joannidis et al., a minimum of 150 subjects (controls and cases) should be required for association studies *(70)*. This problem is especially important when subjects are further divided into subgroups according to different allelic combinations.

Apart from the sample size bias, genuine ethnic and geographic differences can also contribute to the lack of confirmation of results in different populations. The recently described DAZL gene polymorphism represents a remarkable example of ethnic differences *(36)*. Finally, control group bias is also a common weakness of many association studies. It is important to distinguish between normal spermatogenesis and fertility because a control group selected on the basis of fertility status may contain up to 10% of men with severe spermatogenic failure *(71)*. If the expected effect of a polymorphism is spermatogenic failure, the correct control group should be normospermic men, whereas if the polymorphism is predicted to influence the sperm fertilization capacity, the most appropriate controls should be proven fertile men.

Polymorphisms should be considered as risk factors rather than direct etiological causes for spermatogenic disturbances or male infertility. Although data are controversial for the majority of polymorphisms, some of them, such as POLG, AR, and the DAZL gene, clearly cannot be considered as genetic risk factors for male infertility.

It is likely that some polymorphisms lead to testicular dysfunction only in association with a specific genetic background or with specific environmental factors. The role of genetic background seems to be especially relevant for one of the most promising genetic risk factors, the gr-gr deletions. Environmental factors may be relevant for ER polymorphisms, MTHR, and genes involved in oxidative stress. Certain gene variants may cause specific phenotypes (e.g., PRM1 and CREM)

and consequently only the analysis of a specific subgroup of patients is able to identify their clinical significance. To obtain reliable and clinically useful data, much more attention should be focused on the correct study design, which is still the major weakness of association studies.

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19 The Genetics of Abnormal Protamine Expression

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Summary

During spermiogenesis, the sperm chromatin undergoes dramatic remodeling. The testis-specific protamine proteins facilitate these nuclear changes by replacing the somatic cell histones, a process that produces highly condensed, transcriptionally silent chromatin. In humans, there are two forms of sperm protamine: protamine 1 (P1) and protamine 2 (P2), which occur in a strictly regulated 1:1 ratio. Sperm protamine-deficiency and P1:P2 ratio deregulation have been implicated in male infertility. The details of the underlying genetic basis of abnormal protamine expression are just emerging. This chapter summarizes our current knowledge of the sperm protamines, their relationship with male infertility, and what is currently understood regarding the genetic basis of abnormal protamine expression.

Key Words: Chromatin; genetics; expression; protamine; sperm.

1. INTRODUCTION TO THE SPERM PROTAMINE PROTEINS

During spermiogenesis, the haploid sperm chromatin undergoes a dramatic remodeling *(1)*. Two classes of sperm-specific nuclear proteins, the transition proteins and protamines, are responsible for packaging the sperm chromatin into a highly compact transcriptionally silent form *(2)*.

The protamine proteins replace the somatic cell histones in a two-stage process (Fig. 1) *(3)*. The first step occurs in haploid round spermatids and involves replacement of the histones with the transition proteins (TP1 and TP2). Subsequently, in elongating spermatids, the protamine proteins (P1 and P2) replace TP1 and TP2. The resulting chromatin is highly condensed and transcriptionally silent.

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A
TP1
P1
DAPI
TP1 P1 DAPI
B
TP1
P1
DAPI
TP1 P1 DAPI
C
TP1
P1
DAPI
TP1 P1 DAPI

The protamine proteins are ubiquitous in the sperm of all mammals, a testament to their importance during sperm development *(4)*. P1 is present in all mammalian species, whereas P2 has been detected in spermatozoa of mouse hamster, vole, rat, stallion, and man *(4)*. Although the P2 protein is not present in some other mammals, such as the bull and boar, the gene encoding P2 is present and transcribed in these species.

From an evolutionary standpoint, mammals have likely inherited the *P1 gene* from a common ancestor because it is present in all species studied to date *(5)*. There are two possible explanations for the origin of the *P2 gene*. The most plausible is that P2 derived from P1 through a gene duplication event *(6)*. Alternatively, P1 and P2 may have been inherited from a single common ancestor and successive species have subsequently lost the ability to express P2 *(6)*.

The human *P1* and *P2 genes* encode a 50-amino acid protein and a final processed protein of 57 amino acids, respectively *(7,8)*. Overall, there is roughly 50% identity between human P1/P2. In various mammalian species, the P2 content may vary from 0 to 80% *(4)*. However, within a given genus the ratio of P1 to P2 (P1:P2) is strictly regulated and in humans, is approx 1:1 *(4)* (Fig. 2).

From a structural standpoint, the protamines are relatively small proteins but are highly basic in character because of a high level of arginine residues present in their amino acid sequences (~50% of the total amino acid compliment; refs. *7*,*8*). This aspect of the protamine structure promotes their association with the negatively charged DNA backbone. In addition, P1 and P2 contain copious amounts of cysteine residues (~10%), which foster a highly stable chromatin structure resulting from inter- and intramolecular interactions via disulfide bond formation *(7,8)*.

During the elongating stage of spermatogenesis, P1 is synthesized as a mature protein product, whereas P2 is synthesized as a precursor protein of 103 amino acids and undergoes proteolytic cleavage of its amino-terminus to produce a mature P2 protein *(3)*. Like many other proteins, phosphorylation of the protamines is required for their proper DNA incorporation and subsequent processing. P1 is rapidly phosphorylated

Fig. 1. *(Opposite page)* Immunofluorescence micrographs showing protamine 1 (P1) and transition protein 1 (TP1) expression in round and elongating spermatids. **(A)** Early round spermatid displaying TP1 nuclear localization without detectable P1 immunofluorescence. **(B)** Early elongating spermatid showing TP1 nuclear localization in concert with P1 cytoplasmic production. **(C)** Later elongating spermatid showing P1 nuclear localization with minimal residual TP1. Bar = 10 μm for A, B; 25 μm for C.

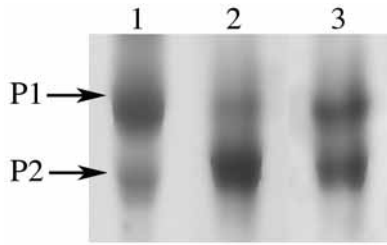

Fig. 2. Protamine detection via gel electrophoresis. Protamine 1 (P1) and P2 bands are located on the top and bottom rows of the gel, respectively. The banding patterns show examples of patients with elevated (lane 1), reduced (lane 2), and normal (lane 3) P1:P2 stoichiometry.

after translation by the serine/arginine protein-specific kinase (SRPK) 1 *(9)*. An intermediate form of P2, one derived by proteolysis of the precursor P2, is also rapidly phosphorylated by another protein, the Ca^{2+}/calmodulin-dependent protein kinase (Camk) 4 *(10)*.

The protamine genes, which contain only a single intron, are highly conserved in the sperm of all mammalian species *(4)*. The haploid genome encodes a single copy of the human P1 and P2 genes mapped to chromosome 16p13.3. In addition, TP2 is mapped to the same locus on chromosome 16p13.3. This *P1–P2–TP2* locus spans a 28.5-kb region and is organized in a linear array, a structural feature affording concurrent expression of the P1, P2, and TP2 genes *(11)*. This multigenic locus, therefore, represents a single coordinately expressed chromatin domain.

2. THE RELATIONSHIP BETWEEN ABNORMAL PROTAMINE EXPRESSION AND MALE INFERTILITY

Numerous reports have emerged in the last decade establishing a relationship between abnormal protamine expression and male infertility. These studies indicate aberrations in the P1:P2 ratio are related to impaired sperm quality and male infertility *(12–19)*. The majority of studies highlight populations of infertile men with abnormally elevated P1:P2 ratios *(12–19)*. Two of these reports document small populations of infertile males with undetectable levels of P2 *(16,17,19)*. Taken together, these data have led to the assumption that abnormal expression of P2 accounts for aberrant P1:P2 ratios in infertile men.

A population of infertile males was also identified with abnormally low P1:P2 ratios *(12,20,21)*. The identification of infertile men with abnormally low P1:P2 ratios raised the possibility that P1 is also abnormally

expressed in patients with deregulated protamine stoichiometry. Indeed, protein quantification data strongly suggests P1 underexpression underlies the majority of cases involving abnormally reduced P1:P2 ratios *(12,20)*. Conversely, P2 underexpression appears responsible for the majority of abnormally elevated P1:P2 ratios *(12,20)*. Taken together, these data suggest deregulated expression of both P1 and P2 underlie aberrant sperm P1:P2 ratios. However, it appears that P2 underexpression is more common than P1 underexpression, evidenced by the increased incidence of patients with elevated P1:P2 ratios vs those with reduced P1:P2 ratios.

Sperm functional ability and semen quality parameters, including sperm count, motility, and head morphology, are significantly diminished in patients with abnormal P1:P2 ratios vs patients with normal P1:P2 ratios *(12,13,19,22)*. The ability of these sperm to successfully penetrate oocytes appears to be compromised, evidenced by significantly impaired oocyte penetration abilities *(12,19)*. Furthermore, patients with abnormal protamine expression also display reduced sperm DNA integrity and increased chromatin fragmentation *(21)*. Sperm functional ability, semen quality parameters, and sperm DNA integrity are markedly reduced in patients with abnormally reduced P1:P2 ratios, even vs patients with abnormally elevated P1:P2 ratios *(12,20,21)*. These data suggest P1 deficiency may be particularly detrimental to spermatogenesis in humans.

Animal knockout studies further highlight the importance of the protamines during mouse spermatogenesis and embryogenesis *(23,24)*. Consistent with the human data, mouse P1 and P2 haploinsufficiency results in severely abnormal spermatogenesis and increased DNA damage and sperm cell apoptosis *(23,24)*. Additionally, this induced protamine deficiency directly impairs in vitro embryonic development in mice *(23)*.

It is somewhat surprising that sperm concentration is significantly lower in patients with aberrant P1:P2 ratios, because late spermiogenesis events are not closely linked to events regulating sperm concentration. One possible hypothesis may be that patients lacking P1 or P2 reflect severely abnormal spermatogenesis. Thus, protamine deregulation may occur because of generalized spermatogenetic problems and/or an early aberration in the spermatogenic pathway that results in downstream deregulated protamine expression. Alternatively, the link between semen quality and protamine levels may also be the result of a generalized defect during late spermiogenesis, the point at which stored protamine transcripts undergo translation and posttranslational modifications *(25)*. Coincidentally, this is the stage at which numerous sperm function attributes are acquired, some of which are defective in protamine-deficient sperm.

Another alternative explanation for the link between protamines and semen quality is that protamine transcription and/or translation may act as a "checkpoint" during spermatogenesis, with spermatogenesis directly tied to the relative quantities of P1 and P2. The role of protamines as a checkpoint may seem unlikely, but two facts may indicate that it is not an unreasonable hypothesis. First, protamines are ubiquitous in mammals and critical for normal fertilization ability *(4)*. Second, the mouse knockout studies have shown that protamine haploinsufficiency leads to complete male infertility, including a diminished sperm count *(23,24)*. Although it is possible that the mouse model is not reflective of human spermatogenesis, that study, along with the human data, indicates a strong relationship between protamines and spermatogenesis.

3. THE GENETIC BASIS OF ABNORMAL PROTAMINE EXPRESSION

The etiology of human sperm protamine deficiency has remained elusive. Protamine expression deregulation may occur at multiple points along the expression pathway, including mutations in the protamine genes, aberrant transcription regulation, unfaithful translation repression or activation, and incomplete posttranslational protein processing. A number of studies have emerged in the last decade that have sought to elucidate the genetic basis of abnormal protamine expression.

Genetic sequencing approaches targeting the testis-specific nuclear proteins P1, P2, TP1, and TP2 suggest it is unlikely that aberrations in the coding and intronic regions underlie the majority of cases involving abnormal protamine expression *(16,26–33)*. Initial genetic screens of the protamine genes failed to reveal any pathogenic mutations in the small populations of infertile men *(28,32)*. More recent studies have led to the identification of a number of polymorphisms in the protamine and transition protein genes *(27,29–31,33)*. However, the majority of these studies have failed to correlate direct measures of protamine content with the incidence of these various polymorphisms. In general, there appears to be a very low incidence of these protamine gene polymorphisms. Thus, it does not appear that protamine gene mutations and polymorphisms underlie the majority of abnormal protamine expression cases.

This conclusion appears to be confirmed by a study from our laboratory comparing the identity and frequency of nuclear protein gene polymorphisms in protamine-deficient populations, severely infertile populations, and fertile controls *(42)*. A total of 15 single-nucleotide polymorphisms (SNPs) were identified in the P1, P2, TP1, and TP2

genes. A number of these SNPs resulted in amino acid changes, but occurred in similar frequencies within protamine-deficient patients, severely infertile men, and the fertile control population. Although unlikely, it may be possible that three of the intronic P2 variants may influence aberrant P2 expression, because their presence was limited to only the protamine-deficient population. However, the rare occurrence of these intronic SNPs suggests they do not contribute to the majority of cases involving P2 deficiency.

One of the most exciting discoveries was the elucidation of a potential mechanism of protamine expression deregulation via protamine transcript quantification *(20)*. Semiquantitative real-time RT-PCR revealed abnormally high levels of sperm P1 messenger RNA (mRNA) retention in cases of P1 protein underexpression *(20)*. This abnormal accumulation of P1 transcripts strongly suggests that protamine mRNA, although produced normally, fails to undergo translation. Therefore, defects in translation regulation may underlie the abnormally reduced P1:P2 ratios associated with P1 underexpression.

During spermiogenesis, protamine transcription and translation are temporally uncoupled *(25,34,35)*. Translational regulation is one of the more important aspects of protamine biology and accounts for this delay in protamine protein production *(12)*. If protamine transcription and translation are allowed to occur concurrently, the chromatin undergoes precocious compaction and sperm development is arrested *(36)*.

Under normal circumstances, protamine translation regulation begins immediately with RNA processing via intron splicing and mRNA polyadenylation *(25)*. Polyadenylation serves a dual function to both protect the mRNA from degradation and provide a binding site for translation repressor proteins. As the poly-A mRNA enters the cytoplasm, it is translationally repressed via storage in messenger ribonucleoprotein particles and binding by specific translation repressor proteins, which target the 5′-untanslated region, 3′-untanslated region, and poly-A sequences *(25)*.

Translation repression is removed a few days later, during the elongating spermatid stage, by covalent modification of the messenger ribonucleoprotein particles, release of translatable mRNA, and removal or migration of the translation repressor proteins, leaving the poly-A tail susceptible to degradation. Subsequently, the protamine proteins are translated, phosphorylated, and incorporated into the chromatin. The increased P1 mRNA retention observed in patients underexpressing P1 protein may arise as a result of defects in any of these translation repression/activation steps or abnormalities in the primary mRNA regulatory sequences.

In the case of P2 underexpression, the mRNA data suggests a multifaceted deregulation pattern *(20)*. Although the majority of cases involving P2 deficiency are associated with decreased mRNA levels, nearly 25% of the P2-deficient patients appear to have an abnormal retention of P2 transcripts, similar to observed scenario for P1-deficient patients *(20)*. This dichotomy in P2 mRNA levels within P2-deficient patients suggests various mechanisms potentially underlie deregulated P2 expression.

Abnormal posttranslational P2 processing may play an important role in P2 underexpression. After translation, P2 is rapidly phosphorylated by Camk4 *(10)*. This phosphorylation step is an absolute requirement for proper binding of P2 to the DNA *(10)*. After the full-length phosphorylated form of P2 is bound to the DNA, it undergoes proteolytic cleavage to produce a mature shortened P2 protein *(37)*. Thus, P2-deficient patients with abnormally elevated P1:P2 ratios may possess defects in the Camk4 protein itself or defective signaling pathways that activate the kinase. Indeed, support for this hypothesis is provided by a study demonstrating an abnormal accumulation of P2 precursors in patients with reduced P2 levels *(17)*.

The decreased P2 levels associated with low P2 mRNA levels may also derive from diminished levels of P2 transcription. Intuitively this scenario does not appear likely, given P1 and P2 are transcribed from a single coordinately expressed gene cluster with similar upstream regulatory elements *(3,5,38,39)*. However, a variable length GA-repeat specific to the P2 promoter has now been identified that may serve to modulate transcription efficiency *(30,38)*.

Finally, there may also be a relationship between disruptions of the hypothalamic–pituitary–gonadal axis and abnormal protamine expression. Two studies in particular highlight a specific relationship between normal endocrine signaling pathways and the normal expression of the sperm nuclear proteins *(40,41)*. The first study evaluated the effects of mouse follicle-stimulating hormone (FSH) receptor disruption on sperm nuclear protein expression patterns *(40)*. A dramatic reduction of TP1, TP2, and P2 expression was observed in 21-d-old mice. Furthermore, in sexually mature mice there was a 50% reduction in total protamine (P1 + P2) levels with an abnormal retention of mono-ubiquitinated histone 2A.

The second study demonstrated cyproterone acetate mediated disruption of testosterone binding to the androgen receptors resulted in a downstream reduction of protamine expression *(41)*. Taken together, these studies indicate endocrine-signaling pathways play an important role in protamine expression. Although the mechanisms are not well understood, FSH is thought to regulate protamine expression through its activation of the cycle adenosine monophosphate response element modulator, which

in turn, upregulates protamine transcription. Nevertheless, a generalized malfunction of the hypothalamic–pituitary–gonadal axis, defects in the FSH and androgen receptors, or disruption of the proper signal cascades may also be at the root of abnormal protamine expression.

4. CONCLUSION

The protamine proteins play a critical role during spermatogenesis in the proper remodeling and packaging of the sperm chromatin. Populations of infertile men have now been identified that abnormally express the protamine proteins. In particular, these patients display abnormally protamine stoichiometry, either in the form of reduced or elevated P1:P2 ratios. These abnormal P1:P2 ratios have been shown to arise as a result of deregulated expression of the P1 and P2 proteins, respectively. Aberrations in protamine expression are associated with diminished semen quality, compromised sperm functional ability, and reduced sperm DNA integrity. Deregulated protamine expression does not appear to be a principle result of nuclear protein gene mutations, but instead may arise because of mechanisms of aberrant translation regulation, posttranslational processing defects, or improper endocrine signaling.

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20 Chromatin Damage and Male Infertility

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Summary

There is accumulating evidence linking sperm chromatin damage to poor reproductive outcome. The chromatin damage is associated to sperm anomalies that manifest themselves as breaks in the sperm nuclear DNA, aberrant ratios of protamine and histones in the chromatin, and the presence of apoptotic marker proteins in the ejaculated spermatozoa. This chapter examines the mechanisms involved in generating chromatin damage during spermatogenesis in the human, the techniques used to test sperm chromatin and how they may affect reproductive outcome, and how to reduce the risk of using spermatozoa with chromatin damage.

Key Words: Sperm chromatin; apoptosis; intracytoplasmic sperm injection; DNA damage; spermatogenesis.

1. INTRODUCTION

The classic semen parameters (sperm concentration, motility, and morphology) have long been seen as a true indication of the fertility potential of a man. Although these will always provide a frontline indication of fertility potential, the advent of new assisted reproduction technologies (ARTs) has caused a rethinking of how spermatozoa is assessed. With the use of intracytoplasmic sperm injection (ICSI), in vitro fertilization (IVF), and to a lesser extent intrauterine insemination (IUI), many of the deficiencies revealed in a classic semen analysis (e.g., a low sperm count) can be overcome. Subsequently, the classic semen analysis is now proving less conclusive in some cases and has led us to examine the ejaculated spermatozoa for more subtle characteristics that may affect reproductive outcome.

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One key characteristic has been a more thorough examination of the sperm nucleus. This has led to an abundance of articles being published indicating that chromatin damage in the form of DNA strand breaks and aberrant protamine:histone ratios are clearly detectable in ejaculated spermatozoa and their presence is heightened in the ejaculates of men with poor semen parameters *(1)*.

Damaged chromatin in sperm heightens the risk that the paternal genome is compromised. The impact an abnormal paternal genome may have on reproductive outcome *(2)* is unquestionably less when compared with its female counterpart's role. Egg quality is clearly the major driving force in respect to the chances of a couple achieving a pregnancy. In contrast, the influence of the human sperm on reproductive outcome has been less well characterized, however we recently showed that in 10–15% of IVF cycles a paternal factor may exist *(3)*. The factors present in the paternal genome that impact on poor reproductive outcome are still hypothetical. However, in the last decade the quality of the sperm nuclear chromatin has been more rigorously examined.

2. MECHANISMS INVOLVED IN THE ORIGIN OF CHROMATIN DAMAGE

Spermatogenesis is a complex process of proliferation and differentiation transforming spermatogonia into mature spermatozoa. This unique process involves a series of mitoses and a meiotic division followed by marked changes in cell structure, in addition to proliferation and differentiation. It is clear that spermatozoa can arise in the ejaculate possessing various nuclear anomalies related to chromatin damage. These arise because of the failure of a number of processes that can either cause and/or fail to detect or eliminate the abnormal spermatozoa. Two processes that may fail in their task during spermatogenesis, leading to abnormal sperm in the ejaculate, are apoptosis and chromatin remodeling.

3. APOPTOTIC MARKERS PRESENT ON EJACULATED SPERMATOZA

Apoptosis may play two putative roles during normal spermatogenesis: limitation of the germ cell population to numbers that can be supported by the Sertoli cells and selective depletion of abnormal spermatozoa.

In mouse models, numerous pro- and antiapoptotic proteins have been found to play key roles in spermatogenesis. The Bcl-2 family includes both prosurvival and proapoptotic members, and provides a signaling pathway that appears imperative in maintaining male germ cell homeostasis *(4)*. Bcl-2 and Bcl-x_L are pro-survival members of the

Bcl-2 family. Transgenic overexpression of Bcl-2 and Bcl-x_L results in blockage of cell death at a critical stage, and results in disruption of normal spermatogenesis and infertility in male mice *(5,6)*. Concurrently, knockout models of the pro-apoptotic factor Bax results in germ cell death and testicular atrophy *(7)*. These studies show that an increase in proapoptotic or antiapoptotic proteins can result in disruption of normal spermatogenesis and suggest that apoptosis plays an important role in male gametogenesis by regulating the size of the spermatogenic cell population. Meanwhile, although it seems plausible, the role of apoptosis in selective depletion of abnormal spermatozoa is yet to be proven.

In the human, we originally found that men with abnormal sperm parameters display higher levels of the apoptotic protein Fas on their ejaculated spermatozoa *(8)*. The presence of Fas on ejaculated spermatozoa correlated strongly with a decreased sperm concentration and sperm with abnormal morphology. More recently, we and others also found that other apoptotic markers such as Bcl-x, p53, caspase, and annexin V are also present on ejaculated human spermatozoa and show distinct relationships with abnormal semen parameters *(9–12)*. Double-labeling experiments have shown that ejaculated human sperm expressing apoptotic marker proteins can also display chromatin damage and show signs of immaturity, such as cytoplasmic retention *(10,13)*.

4. CHROMATIN REMODELING DURING SPERMIOGENESIS

A key change in chromatin structure occurs during spemiogenesis when histones are replaced by protamines to confer the compacted chromatin/DNA packaging seen in mature spermatozoa *(14)*. McPherson and Longo demonstrated the presence of endogenous DNA strand breaks in elongating rat spermatids, when chromatin structure and nucleoproteins are modified *(15)*. They proposed that the presence of endogenous nicks in ejaculated spermatozoa might be indicative of incomplete maturation during spermiogenesis. They also postulated that chromatin packaging might involve endogenous nuclease activity to create and ligate nicks during the replacement of histones by protamines, and that an endogenous nuclease, topoisomerase II, may play a role *(16,17)*. Topoisomerase II functions by transiently introducing DNA double-strand breaks, allowing the passage of one double helix through another, and resealing the double-strand break *(18)*. Although the role of topoisomerase II in spermatogenesis is yet to be clarified, it is expressed in the human testis *(19)*. The transient presence of DNA breaks has been reported in both mouse and human *(20,21)*. An abundance of articles have been published indicating

that DNA strand breaks are clearly detectable in ejaculated spermatozoa and their presence is heightened in the ejaculates of men with poor semen parameters *(2,22–25)*.

5. GENERATING SPERMATOZOA WITH DAMAGED CHROMATIN

The presence of spermatozoa with damaged chromatin in the ejaculate is likely influenced by a failure of one or both of the apoptosis and chromatin remodeling mechanisms to function effectively. We have previously hypothesized that the presence of spermatozoa with apoptotic marker proteins in the ejaculate is the result of a process we have termed *abortive apoptosis (8,26–28)*, which is when germ cells are earmarked for apoptosis during spermatogenesis but fail to be cleared and remain in the spermatogenic pool. In addition, failure of the chromatin remodeling process to proceed correctly may allow the production of spermatozoa with DNA breaks. Indeed our own studies have shown that DNA strand breaks are not always present in the same spermatozoa that show apoptotic markers *(10)*. Further failure in other systems to detect abnormal sperm during spermatogenesis, such as DNA repair pathways, will also contribute to this pool of abnormal spermatozoa in the ejaculate. Additionally, we believe that sperm arising from these inadequacies during spermatogenesis will be more susceptible to reactive oxygen species (ROS).

5.1. Reactive Oxygen Species

Further to the events affecting sperm DNA in the testes are posttesticular DNA damage and the possible implication of ROS. Greco et al. recently reported that DNA fragmentation in ejaculated spermatozoa detected by the terminal deoxynucleotidyl transferase [TdT]-mediated dUTP nick end-labeling (TUNEL) assay is significantly higher compared with that in testicular spermatozoa (23 vs 4.5%; ref. *29*). They also found higher pregnancy rates using testicular sperm compared with ejaculated spermatozoa *(29)*. Steele et al. *(30)* found similar results when they compared epididymal sperm with testicular sperm using the Comet assay. One of the theories attempting to explain the differences in DNA damage between testicular and epididymal spermatozoa implicates ROS. Ollero et al. *(31)* suggested that high levels of ROS production and DNA damage observed in immature spermatozoa may be indicative of derangements in the regulation of spermiogenesis and that DNA damage in mature spermatozoa may be the result of oxidative damage harming immature spermatozoa during sperm migration from the seminiferous tubules to the epididymis. Although there

are indications that post-testicular events affect sperm DNA integrity, it is still unclear whether it is a testicular failure to generate normal spermatozoa that makes them more susceptible to the effects of ROS.

6. TECHNIQUES USED TO TEST SPERM CHROMATIN AND HOW THEY MAY AFFECT REPRODUCTIVE OUTCOME

6.1. Testing Sperm

A number of tests have been reported in recent years as indicators of sperm nuclear chromatin integrity; however, their effectiveness is increasingly being questioned *(32)*. One of the tests most commonly used to detect DNA strand breaks is TUNEL. The TUNEL technique labels single- or double-stranded DNA breaks, but does not quantify DNA strand breaks in a given cell. Other tests of sperm nuclear DNA integrity include *in situ* nick translation *(33)* and the Comet assay *(34,35)*.

The most widely used test relating to pregnancy data in the human is the sperm chromatin structure assay (SCSA™). This test has its origins in the large animal field where it was clearly shown to have an impact on providing evidence of fertility potential and was proposed as early as 1980 as a test of sperm integrity in the human *(36)*. The SCSA is a flow-cytometric test that measures the susceptibility of sperm nuclear DNA to acid-induced DNA denaturation *in situ*, followed by staining with acridine orange *(37–39)*. Acridine orange is a metachromatic dye that fluoresces red when associated with denatured (fragmented) DNA and green when bound to double-stranded (normal) DNA. Therefore, an increase in the percentage of cells with a high ratio of red to green fluorescence indicates an overall increase in DNA fragmentation in the spermatozoa from that ejaculate. Because the SCSA is a quantitative (on a continuous scale), as opposed to a qualitative measurement, it has the potential to better define thresholds associated with reproductive outcome *(38)*.

6.2. Sperm Chromatin Assessment and Predicting Pregnancy

The SCSA has now been proposed clinically as a service. It provides two main measures: the DNA fragmentation index (DFI; i.e., the sperm fraction with detectable denaturable single-stranded DNA mainly resulting from DNA breaks) and the highly DNA-stainable (HDS) cells (i.e., the sperm fraction showing increased double-stranded DNA accessibility to acridine orange mainly because defects in the histone-to-protamine transition process) *(38,39)*. Because these parameters are not correlated to each other, they represent independent

aberrations of the human mature male gamete in the ejaculate. DFI has been shown to influence normally initiated pregnancy *(40,41)*: increasing levels of DFI (>30%), independently from World Health Organization standard semen parameters, were associated with a decreased probability to father a child. Studies have been set up also to challenge the SCSA prediction power in the context of ART. In the first one, a small (24 men) pilot study showed that when DFI was more than 27%, no pregnancies could be obtained after IVF/ICSI *(42)*. Last year, two other studies reinforced this finding. Larson-Cook et al. *(38)* examined 89 couples undergoing IVF/ICSI. The end point was clinical pregnancy 14 d after embryo transfer as assessed by positive serum human chorionic gonadotrophin (hCG) and ultrasound detection for a fetal sac. They showed that all patients who achieved a pregnancy had a DFI less than 27% (on the other hand, HDS was not correlated to pregnancy). Saleh et al. *(43)* considered 19 couples undergoing IUI, 10 couples undergoing IVF, and 4 couples undergoing ICSI. In this study, levels of DFI (but not of HDS) were negatively correlated with biochemical pregnancy. The highest DFI value in biological fathers was 28%. Although quite consistent in their finding, some discrepancies emerged from these two studies: sperm concentration, % motility, and % morphology were significantly lower in patients who failed to initiate a clinical pregnancy in the Saleh et al. *(43)* study but not in the Larson-Cook et al. *(38)* study; the fertilization rate was related to DFI in the Saleh et al. *(43)* study but not in the Larson-Cook et al. *(38)* study. It is worthwhile to note that Benchaib et al. *(44)*, who assessed the clinical pregnancy rate in a cohort of 50 IVF patients and 54 ICSI patients by positive plasma hCG and ultrasound detection of a fetal heartbeat, also showed that higher (>10%) sperm DNA fragmentation levels (this time evaluated by TUNEL assay on the discontinuous gradient centrifugation selected sperm) was a negative factor to obtain pregnancies via ICSI (but not via IVF) and no pregnancies were started if DNA fragmentation was more than 20%.

The enthusiasm originated by these original studies, on the existence of an upper DNA fragmentation threshold above which no pregnancy can be obtained after ART, has been cooled down as more investigations have been published. First, Gandini et al. *(45)*, in a study involving 34 couples (12 IVF and 22 ICSI) did not note any difference between patients initiating pregnancies or not and, above all, they reported healthy full-term pregnancies even with high levels of DFI (up to 66.3%). Pregnancy rates were 25% for IVF and 40.9% for ICSI. HDS was not correlated either with pregnancy or delivery. No association between the SCSA parameters and the fertility rate

was found. Second, Bungum et al. *(46)* investigated 306 consecutive couples undergoing ART (131 IUI, 109 IVF, and 66 ICSI), taking into account biochemical pregnancy (positive plasma hCG), clinical pregnancy (intrauterine gestational sac with a heartbeat 3 wk after a positive hCG test), and delivery. Delivery rate was 15.3% after IUI, 28.4% after IVF, and 37.9% after ICSI. They reported that, for IUI, the chance of pregnancy/delivery was significantly higher in the group with DFI less than 27% (and HDS <10%): only one delivery was obtained in the 23 males having a DFI more than 27%. The combination of DFI and HDS gave a higher predictive value regarding the outcome of IUI. On the other hand, no statistically significant difference in the outcome after IVF/ICSI was noted by dividing patients according to the DFI level of 27%. However, the results of ICSI were significantly better than those of IVF: for example, in the group with DFI more than 27%, the authors reported higher clinical pregnancy (52.9 vs 22.2%), implantation (37.5 vs 19.4%), and delivery (47.1 vs 22.2%) rates when comparing ICSI with IVF performances. In addition, by restricting the analysis to IVF patients only, the group with less than 27% DFI level consistently showed better clinical pregnancy (36.6 vs 22.2%), implantation (33.3 vs 19.4%), and delivery (29.7 vs 22.2%) rates as compared with the group of men with DFI more than 27%. Finally, Virro et al. *(47)* studied 249 couples undergoing IVF/ICSI and noted that men with DFI less than 33% had a significantly greater chance of initiating a clinical pregnancy (positive hCG), lower rate of spontaneous abortions, and an increase of ongoing pregnancies at 12 wk (47 vs 28%). HDS and standard World Health Organization parameters were not related to pregnancy outcomes. Further, Gardner et al. did not find a difference in the implantation and pregnancy rates between two groups that had 16 vs 40% fragmentation rates detected by SCSA *(48)*. All these studies demonstrate that high levels of DNA damage were compatible with pregnancy and delivery after IVF/ICSI.

The increasing number of publications in this field indicates that the relevance of sperm nuclear DNA is not completely black and white. A number of conclusions from the ever-increasing wealth of data collected about the various sperm nuclear DNA integrity tests and their predictive power in ART *(49)* can be made. Briefly, the conclusions can be summarized by the following points:

1. An increased fraction of sperm showing DNA damage is a negative trait that reduces the chances to father a child.
2. An absolute number or percentage of DNA strand breaks not compatible with pregnancy is far from being established.

3. The predictive power of the current sperm DNA integrity tests seem to lose their strength from natural conception to ICSI, passing through IUI and IVF.

7. REDUCING THE RISK OF USING SPERMATOZOA WITH CHROMATIN DAMAGE

Human semen is heterogeneous in quality, not only between males but also within a single ejaculate. Differences in quality are evident, both when examining the classical parameters of sperm number, motility, and morphology and in the integrity of the sperm nucleus. Potentially the greatest risk of using spermatozoa with damaged chromatin is present when using ICSI.

The ability to improve the efficiency of preparation techniques to eliminate spermatozoa with nuclear anomalies, selection of the best spermatozoa prior to ICSI, and/or selection of embryos that may have an abnormal paternal complement may all assist in eliminating the risk of using spermatozoa possessing damaged chromatin and making ICSI a safer technique. A number of studies have shown that spermatozoa prepared using a density gradient centrifugation technique significantly improves the quality of spermatozoa in the preparation *(33,50)*. In our own study, we showed that there was a significant ($p < 0.001$) decrease in both chromomycin A3 positivity and DNA strand breakage in sperm samples from different men after preparation by density gradient centrifugation. Chromomycin A3 indirectly demonstrates a decreased presence of protamines. These results indicated that density gradient centrifugation can enrich the sperm population by separating out those with nicked DNA and with poorly condensed chromatin. Another technique we proposed in 1999 was culturing ICSI embryos post-embryonic genome activation to the blastocyst stage *(51)*. This was based on the idea that the extent of nuclear DNA damage in spermatozoa is related to embryo development to the blastocyst stage, a time when the embryonic genome is activated, transcriptional activity has begun, and the paternal genome plays a significant contributory role in embryo function. Subsequently, we have shown that a significant negative correlation exists between the extent of DNA strand breaks in sperm samples and blastocyst development after IVF or ICSI *(52)*.

A final methodology is to improve the selection of spermatozoa before ICSI. One technique that has been reported is the selection of spermatozoa under high magnification. Bartoov et al. *(53)* reported that they were able to achieve a pregnancy rate of 58% in patients who had previously failed at least five consecutive routine cycles of IVF and

ICSI. They have also reported a follow-up study showing improved pregnancy rates with ICSI and morphologically selected sperm compared with conventional ICSI *(54)*. Aitken's group *(55)* also reported a novel electrophoretic sperm isolation technique for the isolation of functional human spermatozoa free from significant DNA damage. Briefly, the separation system consists of a cassette comprising two chambers. Semen is introduced into one chamber and a current applied that leads to a purified suspension of spermatozoa collecting on one side of the chamber within seconds. Suspensions generated by the electrophoretic separation technique contain motile, viable, morphologically normal spermatozoa that exhibit lower levels of DNA damage. A technique using magnetic cell sorting using annexin V-conjugated microbeads that eliminates apoptotic spermatozoa based on the externalization of phosphatidylserine residues has also been reported as a means of ameliorating sperm samples *(56)*.

Another promising sperm selection technique has recently been reported by Huszar and collaborators. They had previously reported that sperm that are able to bind to hyaluronic acid (HA) are mature and have completed the spermiogenetic process of sperm plasma membrane remodeling, cytoplasmic extrusion, and nuclear histone–protamine replacement *(57)*. Testing of a newly invented ICSI sperm selection method based on the binding ability of spermatozoa to HA has shown that the HA-bound sperm is less likely to show chromatin damage, apoptotic marker proteins, cytoplasmic retention, and aneuploidy compared with unselected sperm *(58)*. A limitation of this technique is that it may not be optimal for use in patients with very low sperm counts and motility.

8. CONCLUSIONS

There is now an abundance of studies showing a range of nuclear anomalies in ejaculated spermatozoa. It is also apparent that they are more likely to be present in men with poor semen parameters. The concern as to the level of this relationship is definitely greater in relation to ICSI, in which there is a higher chance that such a sperm will be chosen to fertilize an egg. The tests currently available only provide an inkling of the impact of sperm chromatin damage on reproductive outcome success. However, novel methods are being developed to limit the chance of using chromatin-damaged sperm in ARTs. More research is needed to improve our current knowledge in relation to how and why the chromatin damage arises in spermatozoa, how to detect and remove them more accurately, and how they may relate to failed reproductive outcomes.

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21 Clinical Evaluation of the Genetics of Male Infertility

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Summary

Clinical evaluation for genetic abnormalities is at the crossroads of investigational and practical patient management. Whereas scientific investigation begs for further evaluation of the likely genetic anomalies that underlie many cases of male infertility, clinical testing and consultation must, wherever possible, be practical and directed toward optimizing patient outcome. Men with congenital obstructive azoospermia as well as patients with severely defective spermatogenesis will commonly have identifiable genetic abnormalities. Men with bilateral congenital absence of the vas deferens are commonly cystic fibrosis (CF) carriers, and their female partner should be tested for CF gene mutations before conception. Severely defective spermatogenesis may reflect a karyotypic abnormality or partial deletion of the Y chromosome. Detection of Y microdeletions or specific chromosome anomalies may affect the chance of sperm retrieval in azoospermic men, whereas it appears that the use of sperm from men with genetic abnormalities does not affect intracytoplasmic sperm injection outcomes. Specific genetic testing is warranted for certain subsets of infertile men. Other genetic tests (sperm DNA integrity testing, sperm aneuploidy analysis) are being evaluated and may prove to be clinically useful in the future.

Key Words: Azoospermia; Y-chromosome microdeletion; diagnosis; treatment; kayotype; chromatin.

1. INTRODUCTION

For all men with infertility, a complete history and physical examination is recommended to identify potentially correctable causes of male factor infertility. The American Urological Association and American Society for Reproductive Medicine Practice Committees recommend that initial evaluation of the man and woman in an infertile couple proceed in parallel and be initiated at the same time *(1)*. The initial evaluation of the man involves semen analysis and reproductive

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history. If abnormalities are found on initial evaluation, then complete evaluation with additional history, complete physical examination, hormone testing, and genetic analysis may be indicated.

An infertile man is evaluated when the results will influence the treatment for a couple. In most cases, evaluation is aimed toward identification of conditions that may be treated and change the chance of achieving a pregnancy. Preferably, evidence that treatment is effective will have been demonstrated in randomized controlled clinical trials of that treatment. Unfortunately, relatively few treatments for male infertility have such a level of evidence to support treatment. Therefore, evaluation is directed toward identifying conditions that may affect male infertility (such as varicoceles, obstruction of the reproductive tract, and so on) despite the lack of strong evidence to support treatment. Medically important conditions associated with infertility (testis tumors, pituitary lesions) are also sought during this evaluation.

A second indication for evaluation exists where there is evidence that a genetic condition may be present in the infertile man that would affect the prognosis for treatment or a condition present in the infertile man could affect the health of offspring. Genetic testing is recommended because certain genetic conditions are found in subgroups of infertile men. The conditions where genetic testing is clearly indicated are discussed in this chapter. Other genetic testing that is considered investigational but not yet recommended for routine use is also discussed.

2. OBSTRUCTIVE AZOOSPERMIA

Men with obstructive azoospermia (OA) may be candidates for microsurgical reconstruction or sperm retrieval with assisted reproduction. Regardless of the approach for fertility treatment, the potential genetic etiology of congenital OA must be evaluated.

Mesonephric (Wolffian) duct anomalies are commonly associated with mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. CF is the most common fatal autosomal-recessive disorder in the white population, with an incidence of approx 1 in 2500 live births and a carrier frequency of 1 in 25 persons of Northern European descent. The CFTR gene was cloned in 1989, with more than 550 mutations described, 51 of which are commonly tested in a CF genetic screening panel. In most ethnic groups, these 51 mutations will define more than 70–90% of patients with CF. In approx 95% of men with CF, the Wolffian derivatives of the vas deferens and most of the epididymis do not develop, resulting in the condition referred to as

congenital bilateral absence of the vas deferens (CBAVD). Conversely, 50–80% men with CBAVD (but no digestive or pulmonary symptoms suggestive of CF) will have CFTR mutations definable. This relationship has been summarized nicely by Oates and Amos *(2)*. Jarvi et al. have also reported that up to 47% of men with idiopathic epididymal obstruction will also have CFTR mutations present *(3)*. Given the high prevalence of the CFTR mutation carrier state in women in North America, it is prudent to consider evaluation of the female partner for CFTR mutations when a man presents with Wolffian duct abnormalities including congenital unilateral absence of the vas deferens or idiopathic epididymal obstruction.

Of interest, men with CBAVD who also have renal anomalies (unilateral agenesis or anomalies of ascent) do not appear to be at any higher risk of carrying CFTR anomalies than men in the general population. For developmental or other genetic reasons, an early defect in mesonephric development may affect both the ureteral bud as well as Wolffian duct development. Therefore, not all men with CBAVD will have CFTR mutations. However, all men with CBAVD should be evaluated for renal anomalies, and the female partners of men with CBAVD should be evaluated for CFTR status, because many CFTR anomalies may not be detected with a standard panel of 51 mutations analyzed. Men with CBAVD and two kidneys should be assumed to be carriers of CFTR mutations and their female partners should be tested for CF gene mutations before treatment with assisted reproduction *(1)*.

3. SPERMATOGENIC FAILURE

Men with spermatogenic failure have decreased numbers and/or quality of spermatozoa in the ejaculate. In its most severe form, these patients will have no sperm in the ejaculate (nonobstructive azoospermia [NOA]). External heat or toxic effects or significant medical pathology may be the underlying cause of spermatogenic failure. In both cases, knowledge and removal/management of these conditions is critical before proceeding with assisted reproduction. In addition, genetic evaluation may provide significant prognostic value that can affect decision making for patients who are to be treated with assisted reproduction. Genetic testing (Y-chromosome microdeletion analysis and karyotype) are recommended for men with oligozoospermia (<5–10 million sperm/cc). CFTR gene mutations have also been associated with NOA or idiopathic infertility in anecdotal reports. Larger studies have not confirmed these assertions. Therefore, CF mutation screening of men with impaired spermatogenesis is not currently recommended.

4. GENETIC ABNORMALITIES AND TESTING

The frequency and clinical significance of certain genetic disorders associated with spermatogenic failure, including NOA are adequate to support screening for specific genetic abnormalities. These abnormalities include both chromosomal abnormalities, detectable with routine karyotype testing, and Y-chromosome microdeletions, so-called azoospermia factor (AZF) defects. Other rare genetic causes of male infertility have been reviewed previously *(4)*. For men with severe male factor infertility, including men with sperm concentrations less than 10×10^6/cc and NOA, karyotype evaluation and Y-chromosome microdeletion analysis is recommended before treatment with assisted reproduction.

4.1. Karyotype Evaluation

The most common karyotypic abnormality in men with severe male factor infertility is Klinefelter syndrome, affecting up to 7–13% of azoospermic men. Almost all men with the pure, classic form (47,XXY) of Klinefelter will be azoospermic, whereas limited sperm production is commonly found in men with a mosaic pattern of Klinefelter syndrome. It was previously felt that only spermatogonia with a 46,XY complement could produce spermatozoa; however, observations indicate that a significant proportion of 24,XY spermatozoa are present in the testes of men with Klinefelter *(5)*. General teaching has suggested that men with Klinefelter syndrome can be readily identified by their typical physical appearance of tall stature, gynecomastia, and small, firm testes. We now routinely detect men with nonmosaic Klinefelter syndrome who are normally masculinized, and between 5′6″ and 5′10″ in height. Observations reported by Oates et al. also confirm that some men with chromosomal abnormalities will have an otherwise normal phenotypic appearance, except for their infertility *(2)*.

The presence of Klinefelter syndrome provides a favorable prognostic feature for sperm retrieval using microdissection testicular sperm extraction (TESE) in azoospermic men. For these patients, the chance of sperm retrieval is 72%, and once sperm is retrieved, the chance of clinical pregnancy is more than 50% at our institution *(6)*. The rate of development of 47,XXY, or 47,XXX embryos after sperm retrieval for men with Klinefelter syndrome appears to be very low. None of the children born at our institution, and only one fetus reported in published literature, had Klinefelter syndrome, despite the slight increased risk of aneuploidy in sperm obtained from such men.

Other karyotypic abnormalities identified include Robertsonian translocations, chromosomal inversions, and non-Klinefelter sex

chromosome abnormalities. Detection of these structural chromosomal anomalies results in an increased risk of aneuploidy or unbalanced chromosomal complements in embryos. Couples with structural abnormalities should have genetic counseling, and the use of genetic preimplantation diagnosis (PGD) is warranted for these couples.

4.2. Y Chromosome (AZF) Microdeletions

Several genes have been identified in the distal portion of the long arm of Y (Yq) that are frequently deleted in men with NOA. The best described gene has multiple copies, and is referred to as deleted azoospermia (DAZ). Deletions involving DAZ were identified in 13% (12/89) of azoospermic men screened by Reijo et al. in 1995 *(7)*. In addition, Reijo et al. observed that men with severe oligozoospermia had a 6% (2/35) chance of DAZ deletions *(7)*. Other investigators have found longer deletions of the Y chromosome associated with male infertility. Vogt et al. have suggested that three relatively discrete regions of Yq, AZFa, AZFb, and AZFc, are deleted in severely infertile men *(8)*. We have further characterized the clinical significance of AZF deletions and found that the deleted region determines the chance of sperm retrieval *(9)*. The AZF regions have also been mechanistically described based on the pallindromic repeat regions that recombined to allow the deletions along the Y chromosome. In addition, short regions of deletion within AZFc that are commonly deleted in normospermic and azoospermic men have been described (gr/gr). Because there is inadequate evidence that gr/gr deletions occur at a higher rate in men with low sperm production or infertility than for men with normal sperm production and fertility, it is likely that such deletions reflect polymorphisms.

Several investigators have found that 3–18% of men with severe sperm production abnormalities, including azoospermia, have Y-chromosome deletions. However, the literature is difficult to evaluate because the data was generated in multiple laboratories, each looking at different patient populations and examining different regions and sites on Yq. For example, some investigators considered a microdeletion present when only a single sequence-tagged site was absent, whereas others considered a microdeletion present only if sequential sites on the Y chromosome failed to amplify with polymerase chain reaction-based analysis. Nevertheless, all investigators consistently found Y microdeletions of the AZFa, AZFb, or AZFc regions in a measurable proportion of severely subfertile men, with no detectable deletions in normal fertile men, or in the fathers or brothers of men with Y microdeletions.

Y-chromosome deletions affecting fertility usually involve deletion of one or more entire AZFa, AZFb, or AZFc regions. An additional region of the Y chromosome referred to as AZFd has been described, however, AZFd is within AZFc, has no prognostic significance, and is not associated with impaired sperm production. Therefore, such deletions appear not to have any clinical relevance. Approximately two-thirds of men with deletions involving only AZFc have sperm in the ejaculate (severe oligozoospermia). In azoospermic men with AZFc deletions, sperm production is commonly present within the testicle, and TESE is at least as successful as for other men with NOA, who have an overall sperm retrieval rate of 58%. At Cornell, sperm was found by TESE in 75% of men with AZFc deletions and azoospermia *(9)*. Once sperm are found from men with AZFc deletions, either in the ejaculate or by TESE, fertilization and pregnancy results following intracytoplasmic sperm injection (ICSI) are comparable to those obtained for other couples (matched for sperm production level and contemporaneously) treated at our center *(10)*.

For men with deletions involving the AZFb region, the chance of having sperm in the ejaculate or finding sperm with TESE is severely decreased *(11)*. Sperm were found in none of 23 men we evaluated with deletions involving AZFb who had a biopsy or sperm retrieval attempted with TESE *(9)*. Therefore, we do not recommend that men with deletions involving the entire AZFb region undergo TESE.

Overall, approx 9% of men with NOA and Sertoli cell-only pattern on diagnostic testis biopsy will have AZFa deletions. Deletions involving the entire AZFa region are also commonly associated with a Sertoli cell-only pattern on diagnostic biopsy *(12)*. It is important to discriminate between partial and complete deletions of an AZF region, given that at least one patient with a deletion of part of the AZFa region had germ cells on testis biopsy. However, to date, no sperm has been retrieved from men with complete deletions of AZFa or AZFb. Because the documented number of cases in the literature is limited, absolute predictive statements are not possible to make at this time. However, the prognosis for sperm retrieval is clearly different and dramatically worse for men with complete AZFa and AZFb deletions than for other patients with NOA *(9)*.

Because Y chromosome abnormalities, including deletions, will be passed on to any male child who is produced after assisted reproduction, these men must have genetic counseling before treatment. In an important preliminary study of fathers and ICSI-derived sons, Kent-First et al. found that 10% (3/32) of unselected ICSI-derived boys had detectable Y-chromosome microdeletions, however, only

one of the three boys had a father with Y-chromosome microdeletions detected on testing of his peripheral blood *(12)*. These results suggest that mosaicism with germline deletions on the short segments of the Y chromosome frequently develop in spermatozoa of men with severe male factor infertility. Because men with these genetic defects have rarely or never fathered children naturally, it is uncertain whether any medical conditions will be present in the offspring with Y-chromosome microdeletions, except for infertility. This knowledge makes genetic counseling difficult. On the other hand, common sense suggests that because the fathers are otherwise healthy and normal, the presence of a Y-chromosome microdeletion does not pose a high risk for major congenital defects in potential offspring. A definitive answer will not be available for many years, when the children born from this process are adults.

5. EFFECT OF GENETIC ANOMALIES ON TREATMENT CHOICE

Evaluation of a sequential series of 170 men with NOA who were candidates for TESE at our institution revealed that 17% of these men had definable genetic defects, be it Y-chromosome microdeletions or karyotype abnormalities *(6)*. We have found that the knowledge of having a genetic defect leads many men to pursue options other than TESE-ICSI and that, regardless of treatment choice, the majority of men find it reassuring to know the cause of their infertility.

6. OTHER GENETIC TESTS NOT CURRENTLY RECOMMENDED FOR CLINICAL APPLICATION

6.1. Androgen Receptor Defects

Defects of the androgen receptor (AR) result in phenotypic females (testicular feminization), whereas very mild mutations have been associated in a limited number of cases with a male phenotype and infertility. A variable-length region of exon 1 of the AR has been identified and characterized that contains multiple glutamine (CAG) sequences. This region, referred to as a polyglutamine or trinucleotide-repeat segment, has been associated with several disease states. Long extensions of the polyglutamine region (≥40 CAG repeats), is associated with Kennedy disease, a severe, degenerative, neuromuscular condition. Shorter numbers of CAG repeats are associated with an increased risk of prostate cancer. Men of African-American descent have shorter CAG sequences than Caucasian men, whereas Asian men have the longest CAG repeats.

Interestingly, black men are at the highest risk of developing prostate cancer, with Asian men having a limited risk.

Different studies of CAG repeats in infertile men have been reported. In an article looking at all types of AR mutations in infertile men, in addition to CAG-repeat length polymorphisms, no mutations were found in 35 patients or 32 controls *(13)*. Azoospermic men had significantly longer CAG repeats than controls (23.2 vs 20.5). The odds of having a CAG repeat length of 20 were sixfold higher for men with a defect in spermatogenesis. It was postulated that assisted reproductive techniques may lead to a trend toward higher CAG-repeat lengths and higher risk of Kennedy disease. Other studies have supported a difference in CAG-repeat length in severe male factor infertility *(14,15)*, whereas others have failed to confirm the association *(16)*. Taken together, CAG-repeat length in the AR may be a risk factor for some men with infertility. Several anecdotal reports have suggested that isolated men with longer CAG-repeat sequences associated with infertility have had increased sperm concentration and pregnancies after treatment with clomiphene citrate or exogenous testosterone. The increased serum testosterone provided by these treatments was postulated to overcome the AR defect. Nevertheless, these are uncontrolled case reports. Indeed, most men treated over prolonged periods with exogenous testosterone will have spermatogenesis suppressed because of decreased endogenous pituitary gonadotropin production and subsequent lowering of intratesticular testosterone levels. Therefore, AR receptor analysis is not currently recommended as a genetic screening test because it does not predict infertility, is not associated with an obvious increased risk of disease in offspring, and treatment has not been demonstrated to be of benefit for "affected" patients.

6.2. Sperm DNA Integrity Testing

Several tests allow assessment of DNA integrity within spermatozoa. The deoxynucleotidyl transferase [TdT]-mediated dUTP nick end-labeling (TUNEL) assay detects the number of DNA breaks within the head of spermatozoa. This test has been used to detect sperm that are undergoing apoptosis. The Comet assay similarly detects DNA fragmentation using an *in situ* detection technique. The sperm chromatin structure assay (SCSA™) is now commonly applied for analysis of DNA integrity in men with infertility. This test evaluates spermatozoa DNA as being single-stranded (abnormal) or double-stranded (normal). Unfortunately, its clinical role has not been clearly defined. Initial studies suggested that abnormal SCSA levels (>27% DNA fragmentation index) were associated with no

chance of fertility naturally or with assisted reproduction. Many further studies have been done to evaluate the effect of abnormal DNA integrity on pregnancy rates. Most studies have not controlled for female age or other factors that are known to affect the results of IVF/ICSI. However, most studies have suggested that abnormal DNA integrity has a significant negative effect on the chance of pregnancy after assisted reproduction *(17)*. Although it would be of concern to use spermatozoa with grossly abnormal DNA for IVF or ICSI, based on preliminary data, it appears that abnormalities of sperm DNA integrity rarely exist as an isolated finding. In most cases, sperm from semen samples with poor DNA integrity often have abnormal motility, low concentration, and abnormal morphology. However, 8% of infertile men with normal semen parameters will have abnormal sperm DNA integrity *(18)*. Therefore, the role of these tests of DNA integrity as a clinical tool for male factor infertility is yet to be fully defined *(19)*. It is possible that some couples who have poor embryo development or recurrent pregnancy loss may have abnormal DNA integrity as a cause *(20)*.

A more sophisticated test for DNA adducts allows detection of 8-hydroxydeoxyguanosine (8-OhdG), a measure of oxidative damage to spermatozoa *(20)*. Increased DNA damage is seen in spermatozoa from men with oxidative effects, including men who are smokers, and treatment of these men with antioxidants (e.g., vitamin C 250 mg/d) has been shown to decrease 8-hydroxydeoxyguanosine formation (consistent with decreased oxidation *(21,22)*.

6.3. Sperm Aneuploidy Analysis

Defective sperm production is associated with an increased risk of sperm aneuploidy. Whereas normospermic men have approx 1% sperm aneuploidy, oligozoospermic men have 8% aneuploidy and men with NOA have 15% aneuploidy *(23)*. Morphologically abnormal sperm are also more likely to have sperm aneuploidy, although the frequency of sperm aneuploidy is small even in men with 0% normal forms. Aneuploid sperm may fertilize oocytes, especially during assisted reproduction. Sperm aneuploidy may also play a significant role in recurrent pregnancy loss *(24)*. Sperm aneuploidy occurs at a much lower frequency than oocyte aneuploidy, our observation that limits the clinical value of routine sperm aneuploidy testing of any subgroup of infertile men. The lack of increased birth defect rates after assisted reproduction treatment of men with severe infertility supports the limited value of sperm aneuploidy testing. However, the prognostic role of sperm aneuploidy analysis in treatment of infertile men has

yet to be demonstrated, and couples that are appropriate candidates for sperm aneuploidy analysis are still being refined. Therefore, sperm aneuploidy analysis is not yet recognized as a useful clinical tool for management of infertile couples.

7. ROLE OF GENETIC ANALYSIS DURING AND AFTER ASSISTED REPRODCTION

Genetic analysis before ICSI can provide several distinct advantages. First, it may allow PGD of genetic defects in embryos. Second, it may provide important prognostic information before treatment. Third, it reassures men that they were born with their infertile condition and did not cause it through sexual or environmental exposures. PGD can detect chromosomal or specific genetic abnormalities in embryos before intrauterine transfer, but knowledge of the specific genetic abnormality is necessary to direct PGD analysis. Finally, it is evident that approx 20% of men with genetic anomalies detected and confirmed will choose to pursue other options rather than sperm retrieval to become fathers *(6)*.

8. CONCLUSION

Men with NOA or severe oligozoospermia (<5–10 $\times$ 10^6 sperm/cc) should be tested with karyotype analysis and evaluated for Y-chromosome microdeletion. This recommendation reflects the American Society for Reproductive Medicine Practice Committee and American Urological Association Male Infertility Best Practice Policy Guidelines published in 2001. Men with less than 10×10^6 sperm/cc should also be counseled regarding Y-chromosome microdeletion analysis.

Men with OA owing to congenital absence of the vas deferens or idiopathic epididymal obstruction are at high risk to be carriers of CF gene mutations. Therefore, their female partners should be tested before treatment with microsurgical reconstruction of the male or assisted reproduction with retrieved spermatozoa. The men are often commonly evaluated for CF gene mutations, because many couples want to know what caused their fertility problem.

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Index

The Genetics of Male Infertility

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Infertility is a common and severe health problem affecting roughly 7% of the male population. It is likely that the majority of those patients have genetic abnormalities that are the cause of their infertility. In *The Genetics of Male Infertility,* twenty-one researchers and clinicians review the study of the genetics of male infertility, the tools available in the laboratory and clinic, the current state of knowledge, and the future of research and translation into clinical diagnostics and treatments.

This volume gives special attention to the significant technological advances in the treatment of male infertility, including intracytoplasmic injection (ICSI) in conjunction with in vitro fertilization (IVF). In addition, new tools discussed include in vitro methodologies, in silico technologies, and new model organisms.

Providing a review of our current understanding of genetic causes of male infertility, *The Genetics of Male Infertility* serves as a guide to evidence-based clinical applications, and a preview of future possibilities.

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- **Comprehensive review of our current understanding of genetic causes of male infertility**
- **Written by world-renown researchers and clinicians in the field**
- **Technological advances in the treatment of male infertility**
- **Discussion of new tools available in the laboratory and clinic**

CONTENTS

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