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Male-biased genes of *Drosophila melanogaster* that are conserved in mammalian testis

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1. ABSTRACT

Male-biased genes have drawn considerable attention due to their relatively rapid rates of interspecies diversification. These genes are often involved in processes related to reproductive behavior, sexual competition, and gametogenesis. Despite this trend toward rapid evolutionary change, many core molecular pathways underlying spermatogenesis are conserved between *Drosophila melanogaster* and several mammalian species. Using BLAST search in the database, 22 testis-specific mammalian orthologues of 174 known *D. melanogaster* male-biased proteins were identified. They are related to a variety of molecular processes. Several also showed association with human male-factor infertility. These genes/proteins may find applications in the diagnosis and treatment of male infertility and the development of novel contraceptives.

2. INTRODUCTION

Males and females of several species show remarkable sexual dimorphism at the phenotypic level. Although members of the opposite sex may differ by a single sex-specific chromosome, the majority of their genes are shared. Therefore, the sexual dimorphism observed in these species must be attributed to differences in gene expression (1). Such sex-biased expression is common for genes involved in reproductive behavior, sexual competition, and gametogenesis. This places unique selective constraints on genes unique or highly biased to one sex. In particular, male-biased genes display relatively significant interspecies diversity. This diversity is reflected by numerous changes in gene sequence and level of expression among closely related species (1,2).

Over evolutionary time, male-biased genes are prone to rapid alterations in expression and sequence. Thus, conservation of such characteristics between distantly related species may elucidate the core genes required for proper sexual function in males. Testis-specific genes, in particular, provide a subset of genes that are likely to play essential roles in spermatogenesis, and may find applications in the diagnosis and treatment of male infertility and the development of novel contraceptives (3,4).

D. melanogaster has served as a model organism in genetics research for almost a century, and has provided a wealth of data on gene expression. Although phylogenetically distant from mammals, approximately 61% of the human proteome has identifiable homologues in *D. melanogaster* (5). There is evidence that the genetic regulation of spermatogenesis and the resulting sex characteristics are well conserved (6). For example, in *D. melanogaster, boule* mutation produces sterility, which can be restored by human *BOULE* ortholog (7). This suggests that several fundamental molecular mechanisms of spermatogenesis in mammals, including humans, can be inferred through studies of Drosophila male-biased genes.

The availability of sequence and expression data has facilitated identification of mammalian orthologues of *Drosophila* male-biased genes that have a role in spermatogenesis. In the present article, we searched for *Mus musculus* and *Homo sapiens* testis-specific orthologues within a set of *D. melanogaster* male-biased genes using the bioinformatics approach. In order to examine functional conservation, Gene Ontology (GO) annotations were obtained. The major objective of this study was to identify novel, testis-specific mammalian genes in the database that are evolutionarily conserved and have functional/sequential homologies with *D. melanogaster* male-biased genes. The long term goal is to investigate their utility in the specific diagnosis and treatment of male infertility and contraceptive vaccine development.

3. DISCUSSION

Male-biased genes from *Drosophila melanogaster* were obtained from the Sex Bias Database (SEBIDA) (8). The data set used contained only genes confirmed as male-biased by three separate microarray studies (9-11). All genes had a P-value of ≤ 0.030, and a false discovery rate of 10%.

Each gene from the SEBIDA data set (n=174) was used in a BLAST search to obtain homologous proteins in *M. musculus* and *H. sapiens* (9). BLAST results were parsed through custom Python scripts utilizing modules from the Biopython library (13). Those alignments with E-values < 0.0005 were cross referenced to NCBI's UniGene database to assess tissue expression patterns based on EST representation (14). Those showing EST expression exclusively in the testis were saved, and GO terms for each were obtained using a custom Python parser. Finally, duplicate entries within any single species were removed to obtain a list of unique, testis-specific genes.

The initial search identified 91 proteins within the *M. musculus* and *H. sapiens* combined set. After removing duplicate entries, the data set was reduced to 22 entries (Table 1), 7 corresponding to *H. sapiens* and 15 to *M. musculus*. All showed EST expression exclusively in the testis. However, the degree of expression and developmental stage of spermatogenesis during which they were expressed differed for each protein. The molecular functions of these proteins vary widely, though many appear to be involved in processes related to development and activity of spermatozoon microtubules and flagella. Several of the proteins completely lacked associated GO terms and/or any corresponding published information in the PubMed database. However, all of these did contain at least one annotated conserved domain that could be used to infer function.

The identified proteins can be classified into the following categories:

3.1. Flagellar Proteins

Testis-specific protein NYD-TSPG, also known as tubulin tyrosine ligase-like 2 (TTLL2), is a member of the tubulin tyrosine ligase (TTL) family. This gene family is implicated in apoptotic processes, but some members may also be necessary for proper flagellar and ciliar functions (15). Members of this family mediate addition/removal of tyrosine moiety on α-tubulin and may cause irreversible damage to microtubules through incorporation of the alternative substrate nitrotyrosine (16,17). Additionally, another member of the TTL family, TTL.6, was found to be preferentially expressed in the testis and to have undergone a period of rapid evolution due to positive selection in the human lineage (18). The specific roles of TTLL2 and TTL.6 within the testis is still unclear, though previous research has implied that they may have important roles in urogenital development, spermatogenesis, and male fertility, all of which depend on apoptosis (18). The UniGene entry for TTLL2 only indicated expression within the fetal testis, possibly indicating its role is limited to either sexual organ differentiation or development of spermatogonia. A second tubulin tyrosine ligase-like protein, TTLL8, was also identified as a homologue of Drosophila gene, *ACXC*. Like TTLL2, this tubulin tyrosine ligase has not received much attention for its role, although abundantly expressed in the testis (15). It is required for glycylation of tubulin by TTLL10, which is vital for activity of flagella and cilia (15). This may implicate TTLL8 in mechanisms leading to reduced sperm motility in asthenozoospermic men. The identification of two TTLL proteins as homologues of Drosophila proteins suggests that this family warrants further investigation for its role in spermatogenesis.

Outer dense fiber of sperm tails 3 (ODF3) was found as a 254 aa homologue in both mice and humans. It is present in the middle and principal pieces of sperm tail. It possibly has a role in preservation of elasticity and protection from shearing effects (19). Additionally, defects

Table 1. Testis-specific genes of *Drosophila melanogaster* and their *H. sapiens* and *M. musculus* homologues

***Drosophila melanogaster* Protein**			**Mammalian homologue**				
Name	**Size**	**Function**	**Species**	**Name**	**Size**	**Function**	**Locus**
1. Flagellar Proteins							
CG3964, isoform B	989 aa	Tubulin tyrosine ligase activity	*H. sapiens*	Testis-specific protein NYD-TSPG/Tubulin tyrosine ligase-like family member 2 (TTLL2)	592 aa	Tubulin tyrosine ligase activity	6q27
ACXC, isoform B	436 aa	Adenylate cyclase activity	*H. sapiens*	Tubulin tyrosine ligase-like family member 8 (TTLL8)	814 aa	Tubulin tyrosine ligase activity	22q13.33
CG10252	229 aa	Protein binding	*H. sapiens*	Outer dense fiber of sperm tails 3 (ODF3)	254 aa	Processes related to cell differentiation, multicellular organismal development, and spermatogenesis	11p15.5
CG10252	229 aa	Protein binding	*M. musculus*	Outer dense fiber of sperm tails 3 (ODF3)	254 aa	Processes related to cell differentiation, multicellular organismal development, and spermatogenesis	7 F5
Tektin A	585 aa	Protein binding in microtubule cytoskeleton organization	*M. musculus*	Tektin 3	490 aa	Protein binding in microtubule cytoskeleton organization	11 B2
2. Peptidases							
CG5282	451 aa	Dipeptidase activity, dipeptidyl-peptidase, metalloexopeptidase activity	*M. musculus*	Dipeptidase 3 (MBD-3)	493 aa	Dipeptidase activity, dipeptidyl-peptidase activity, metalloexopeptidase activity, zinc ion binding	8 D3
CG8564	504 aa	Metallocarboxypeptidase activity, zinc ion binding	*M. musculus*	Carboxypeptidase A5 (CPA5)	436 aa	Metallocarboxypeptidase activity, zinc ion binding	6 A3.3
3. Microtubule Associated Proteins							
Cytoplasmic dynein light chain 2, isoform B	89 aa	Microtubule motor activity, protein binding	*M. musculus*	Dynein light chain type 1 (DLC1)	89 aa	Microtubule motor activity	13 B3
CG10126, isoform B	227 aa	Calcium ion binding	*M. musculus*	Centrin 1 (Cetn1)	172 aa	Calcium ion binding, G-protein beta/gamma-subunit binding	18 A2
Metabolic Proteins							
CG14740	478 aa	Acyltransferase activity, lyase activity, transferase activity	*M. musculus*	Citrate synthase-like protein (CSLP)	466 aa	Citrate (Si)-synthase activity	10 D1
5. Heat Shock Proteins							
CG11035	231 aa	Heat shock protein binding, unfolded protein binding	*M. musculus*	Hypothetical protein LOC75015	234 aa	Heat shock protein binding	3 H2
6. Cation Transport							
ACXC, isoform B	436 aa	Adenylate cyclase activity	*M. musculus*	Solute carrier family 22 member 16 (Slc22a16)	649 aa	Cell differentiation, ion transport, multicellular organismal development, spermatogenesis	10 B1
7. Chromatoid Body Associated Proteins							
CG7082, isoform D	576 aa	RNA binding	*M. musculus*	Tudor domain-containing protein 6 (Tdrd6)	1941 aa	Nucleic acid binding, Protein binding	17 B3
8. Miscellaneous Uncharacterized Proteins							
CG14183	872 aa	Protein binding	*H. sapiens*	IQ motif containing with AAA domain 1 (IQCA1)	363 aa	ATP binding	2q37.2-q37.3
CG5315, isoform B	362 aa	Hormone binding, protein binding, receptor activity	*H. sapiens*	Progestin and adipoQ receptor family member 9 (PAQR9)	377 aa	Receptor Activity	3q23
CG8001	748 aa	No GO term	*H. sapiens*	WD repeat domain 42B (WD42B)	600 aa	No GO term	Xp21.3
CG8001	748 aa	No GO term	*M. musculus*	Plasmacytoma expressed transcript 2	747 aa	No GO term	X C1
CG6332	353 aa	No GO term	*M. musculus*	Testiscular haploid expressed gene (Theg)	375 aa	Protein binding, cell differentiation, multicellular organismal development, spermatogenesis	10 B5-C1
CG5565	240 aa	Phosphoglycolate phosphatase activity	*M. musculus*	Haloacid dehalogenase-like hydrolase domain containing 1A (HDHD1A)	234 aa	Catalytic activity	18 C
CG17838, isoform H	534 aa	Nucleic acid binding, nucleotide binding	*H. sapiens*	RNA binding motif protein 46 (RBM46)	485 aa	Nucleic acid binding, nucleotide binding	4q32.1
CG3483	391 aa	Isocitrate dehydrogenase (NAD+) activity	*M. musculus*	Hypothetical protein LOC243996	396 aa	Isocitrate dehydrogenase (NAD+) activity	7 B5
CG10999, isoform C	187 aa	No GO term	*M. musculus*	UPF0418 protein FAM164B	172 aa	No GO term	10 A2

within the ODF region constitute a major determinant of aberrant tails in the sperm of asthenoteratozoospermic men (19). This protein was originally characterized in rat sperm and was found to be expressed in the epididymis, brain, and testis. This indicates a generalized role in organization of

the cytoskeleton (20). Likewise, the Drosophila homologue, CG10252, has high expression in the testis.

Tektin 3 is a highly conserved member of the Tektin family, which consists of coiled-coil filamentous proteins that form part of the axoneme (21, 22). An early characterization of the protein postulated its use in male contraceptive development. However, a later study demonstrated that Tektin 3 null mice are fertile, though they produced sperm having defects in progressive motility, aberrant bending of flagella, and diminished midpiece width (21,22).

3.2. Peptidases

Membrane bound dipeptidase 3 (MBD-3) is only one of three membrane-bound dipeptidases that is exclusively expressed in the mouse testis (23). Like the other dipeptidases, MBD-3 is a membrane protein anchored via glycosylphosphatidylinositol (GPI). It has metalloprotease activity and can hydrolyze cystinyl-bis-glycine to cysteine. Its function in the testis is unknown and warrants further study. Interestingly, the knockout of another membrane-bound dipeptidase, testicular ACE, has been shown to reduce male fertility and ZP-binding, suggesting that MBD-3 might have a similar role (24).

Carboxypeptidase A5 (CPA5) is the only member of the metallocarboxypeptidase gene family which is exclusively expressed in testis in the mouse, but it also has limited expression in the pituitary in humans (25). It hydrolyzes aliphatic and aromatic amino acids. Its precise function remains unknown but it is speculated that it has a role in cleavage of β-endorphin and other prooppiomelanocortin-derived (POMC) proteins in the brain (25, 26). β-endorphins and other POMC products are known to have function in the testis (25-27).

3.3. Microtubule associated proteins

Dynein light chain type 1 (DLC1) is a component of the multisubunit dynein protein which forms complexes with microtubules and has motor activity (28). DLC1 also interacts with a transcription factor (nuclear respiratory factor 1) which serves as an initiator of cytochrome c expression and other nuclear-encoded proteins involved in respiration (28). Additionally, DLC1 directly inhibits neuronal nitric oxide synthase and binds to several other proteins, including apoptotic factor Bim (29). Although the exact role of DLC1 in spermatogenesis/spermiogenesis is not known, it is speculated that it has important roles in chromatin condensation, streamlining of round spermatid during spermiogenesis, and release of mature sperm into lumen of seminiferous tubule (29). Thus, aberrations in DCL1 function may also affect sperm morphology, motility, and chromosomal integrity. The precise role of these molecular processes during normal and abnormal spermatogenesis/spermiogenesis needs further investigation.

Centrin 1 (cetn1) is the testis-specific murine paralog to the ubiquitously expressed centrin 2 and centrin 3. Centrins are functionally associated with the centrosome, affecting its duplication and eventual segregation. However, recent findings suggest that centrins may also function in conjunction with the nuclear pore (30, 31). The precise function of testis-specific centrin remains unclear. It may have a unique germline purpose, or it may serve to compensate for depletion of the X-linked centrin 2 during spermatogenesis (31).

3.4. Metabolic proteins

Citrate synthase-like protein (CSLP) is similar to its paralogue, citrate synthase, which is important in the tricarboxylic acid (TCA) cycle, which occurs ubiquitously in every cell of the body. However, UniGene reports that CSLP is exclusively expressed in the testis. Although mature sperm primarily use glucose as an energy source, spermatocytes and spermatids require glucose, lactate, and pyruvate. Additionally, TCA cycle enzymes have lower activity in spermatocytes than in spermatids (32). The exact role of CSLP in spermatogenesis/spermiogenesis is unclear.

3.5. Heat Shock proteins

Hypothetical protein LOC75015 is linked to two UniGene profiles, one of which has high testis-specific expression and another that also has low expression in the brain. The protein contains a DnaJ domain, which characterizes it as a member of the HSP40/DnaJ family. Members of this family function ubiquitously as mediators of the HSP70/DnaK chaperone and are also upregulated within the mammalian testis. This suggests that they have a role apart from traditional responses to heat during spermatogenesis (33). Supporting this, it has been shown that Tsarg1, a DnaJ protein, which is preferentially expressed in the rat testis, may act with HSP70 to prevent apoptosis of spermatogenic cells (34). A second study found a novel DnaJ protein, rDJL, to be a participant in movement of vesicles during acrosome formation (35). Several studies have found other testis-specific DnaJ proteins in rodents and primates with possible essential roles in spermatogenesis (36-38). This protein and other members of the DnaJ/HSP40 family are interesting and need further investigation.

3.6. Cation transport

The murine solute carrier Slc22a16 is homologous to Drosophila ACXC, one of the first male germline specific adenylyl cyclases (39). Early characterization of the genomic region surround *ACXC* provided evidence for rapid evolution due to positive selection at this locus. According to UniGene, this protein has high, adult restricted, testis-specific expression. Its role in spermatogenesis has not been investigated. However, the human Slc22a16 orthologue, often called carnitine transporter 2 (CT2) has a role in carnitine transport during sperm maturation and is expressed in several cancer cell lines (40, 41). In humans, CT2 transports L-carnitine from the epididymal epithelium into the lumen during sperm maturation. It is also found in Sertoli cells (41). L-carnitine has been implicated in sperm motility, and its administration to asthenospermic men resulted in improved sperm motility. Inhibition of Slc22a16 mediated L-carnitine transport in the epididymis may provide an interesting target for the development of non-hormonal male contraceptives (41, 42).

3.7. Chromatoid body associated proteins

Tudor domain-containing protein 6 (Tdrd6) is highly expressed in the testis and seems to play essential roles in spermiogenesis and male fertility. It localizes to the chromatoid body (CB), which is thought to have a similar role in the somatic P-body as related to RNAi machinery (43, 44). Tdrd6 -/- mice were viable and appeared to have normal phenotypic development, including normal testis until puberty. When spermatogenesis began, testis had decreased concentration of elongated spermatids and no sperm were present in the epididymis (44). In the absence of Tdrd6, the CBs were not correctly formed, and several miRNAs were significantly up/down-regulated. This suggests a key role of Tdrd6 in maintenance of small regulatory RNAs (44). Although this protein also interacts with piRNA-associated proteins MIWI and MILI, its absence does not significantly alter expression of piRNA-regulated L1 or IAP retrotransposons (44). This may indicate: either (1) Tdrd6 does not play a major role in piRNA machinery or (2) piRNA-mediated retrotransposon silencing primarily occurs prior to Tdrd6 expression during the mid-pachytene stage, as suggested earlier (45). Tdrd6 may provide an exciting target for male contraception.

3.8. Miscellaneous Uncharacterized Proteins

IQ motif containing AAA domain 1 (IQCA1) has an IQ motif in addition to Walker A and Walker B motifs which characterize the AAA+ superfamily of ATPases. The IQ motif is involved in several diverse functions including Ca^{2+} independent calmodulin (CaM) binding (46,47). The IQ motif was originally characterized in myosins but later was also identified in outer sperm proteins, neuronal growth factors, phosphatases, proteins involved in Ras exchange, and proteins coupled with mitotic/meiotic spindle (47). The AAA+ ATPases can act as chaperones, proteases, and helicases (48). IQCA1 is localized in the cytoplasm and nucleus of the cell.

The progestin and adipoQ receptor (PAQR) 9 is part of the PAQR family, a group of proteins characterized by seven transmembrane passes. These proteins are distinct from previously described G-protein coupled receptors (49). They mediate progestin-induced signaling via nuclear-receptor independent pathways by coupling with inhibitory G-proteins (49). The UniGene entry indicates that PAQR9 is only expressed in the human fetus.

WD repeat domain 42B (WD42B) and its murine homologue, plasmacytoma expressed transcript 2 are testis-specific homologues of Drosophila protein CG8001. The murine homologue is expressed in plasmacytoma cell lines and testis (50). The human protein is named for its WD40 repeat domain, which plays a role in a variety of protein-protein interactions (51). Another testis-specific WD40 protein, WDC146, is preferentially expressed in the testis during the pachytene stage and is localized in the nucleus (52). Similar to WDC146, WD42B is expressed in many cancer cell lines and is localized in the nucleus (52). However, the molecular functions of Drosophila, murine, and human homologues remains unknown.

Testicular haploid expressed gene (*Theg*) has high spermatid-specific expression (53, 54). The THEG protein has also been identified in humans and has a similar expression pattern (54). While insertional mutation in the locus containing *Theg* gene results in spermatids lacking flagella or having severe flagellar malformations (55), *Theg* knockout mice showed no significant defect in spermatogenesis and produced fertile males (56). The discrepancy between these two studies may be due to the fact that the insertional mutation of the locus might have disrupted a nearby gene or interfered with a *cis*-acting element within the ~20kb region that was deleted. Drosophila homologue, CG6332, also has testis-specific expression (Unigene), although its function has not been investigated. The evolutionary conservation of this gene and its expression pattern suggest an important function worthy of selective pressure, perhaps processes beyond gamete production, such as sperm competition.

Additional homologues of *D. melanogaster* that were found included: haloacid dehalogenase-like hydrolase domain containing 1A (HDHD1A), hypothetical protein LOC243996, UPF04108 protein FAM164B, and RNA binding motif protein 46 (RBM46). The first three homologues were found in *M. musculus*, and the fourth one was found in *H. sapiens* (Table 1).

4. CONCLUSIONS

The conservation of sequence and expression patterns among distantly related species implies that the proteins identified in this study likely perform functions vital to reproductive success. This is particularly true for male-biased genes used in this study, as they rapidly acquire interspecies mutations and expressional differences. BLAST similarity searches of 174 male-biased *D. melanogaster* genes revealed 22 testis-specific mammalian orthologues. Although many remain largely un-or under-characterized, several have been found to be linked to male-factor infertility. The genes/proteins identified in this study may provide novel targets for non-hormonal contraceptive development and exciting molecular insight into etiology and treatment of male-factor infertility.

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