Published in final edited form as:

Genesis. 2006 November ; 44(11): 515–522. doi:10.1002/dvg.20241.

A Gene Trap Knockout of the Abundant Sperm Tail Protein, Outer Dense Fiber 2, Results in Preimplantation Lethality

Nicholas A. Salmon¹, Renee A. Reijo Pera¹, and Eugene Yujun Xu^{2,*}

¹ Center for Reproductive Sciences, Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California-San Francisco, San Francisco, California

² Division of Reproductive Biology Research, Department of Obstetrics and Gynecology, Center for Genetic Medicine, Feinberg School of Medicine, Northwestern University, Chicago, Illinois

Summary

Outer dense fiber 2 (*Odf2*) is highly expressed in the testis where it encodes a major component of the outer dense fibers of the sperm flagellum. Furthermore, ODF2 protein has recently been identified as a wide-spread centrosomal protein. While the expression of *Odf2* highlighted a potential role for this gene in male germ cell development and centrosome function, the in vivo function of *Odf2* was not known. We have generated *Odf2* knockout mice using an *Odf2* gene trapped embryonic stem cell (ESC) line. Insertion of a gene trap vector into exon 9 resulted in a gene that encodes a severely truncated protein lacking a large portion of its predicted coil forming domains as well as both leucine zipper motifs that are required for protein–protein interactions with ODF1, another major component of the outer dense fibers. Although wild-type and heterozygous mice were recovered, no mice homozygous for the *Odf2* gene trap insertion were recovered in an extended breeding program. Furthermore, no homozygous embryos were found at the blastocyst stage of embryonic development, implying a critical pre-implantation role for *Odf2*. We show that *Odf2* is expressed widely in adults and is also expressed in the blastocyst stage of preimplantation development. These findings are in contrast with early studies reporting *Odf2* expression as testis specific and suggest that embryonic *Odf2* expression plays a critical role during preimplantation development in mice.

Keywords

Odf2; sperm; gene trap; outer dense fibers

INTRODUCTION

Sperm development requires the production and assembly of numerous gene products. Outer dense fiber 2 (ODF2) protein was identified as a major component of the outer dense fibers of the sperm flagellum (Brohmann *et al.*, 1997; Schalles *et al.*, 1998; Shao *et al.*, 1997; Turner *et al.*, 1997). Nine outer dense fibers surround the microtubules of the axoneme and although they may not contribute directly to motility, they are believed to maintain the elastic properties of the sperm tail and protect it from shear stress during epididymal transport and ejaculation (Baltz *et al.*, 1990). Due to the absence of a genetic model that could be used to dissect the function of the outer dense fibers, direct evidence for their

*Correspondence to: Eugene Yujun Xu, Division of Reproductive Biology Research, Department of Obstetrics and Gynecology, Center for Genetic Medicine, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611. e-xu@northwestern.edu.

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requirement in male germ cell development and fertility is lacking. Such an association is, however, strongly implied by the observation that tail abnormalities in the sperm of asthenoteratozoospermic men are associated with abnormal development of the outer dense fibers (Haidl *et al.*, 1991).

Several studies have examined the structure and expression of ODF2 protein. ODF2 is a putative coiled coil protein containing two leucine zippers that mediate interaction with ODF1, another major component of the outer dense fibers (Donkor *et al.*, 2004; Shao *et al.*, 1997). A critical function of *Odf2* is implied by strong sequence conservation (Petersen *et al.*, 1999): for example, the amino acid sequences of human and mouse ODF2 share greater than 97% homology. Consistent with a major role in formation of the sperm tail, *Odf2* mRNA in the murine testis is first seen at low levels in pachytene spermatocytes (Horowitz *et al.*, 2005; Turner *et al.*, 1997) and shows highest expression in postmeiotic spermatids (Hoyer-Fender *et al.*, 1998). ODF2 protein is first detectable in postmeiotic spermatids (Schalles *et al.*, 1998). The protein is initially stored in granulated bodies before being sorted to the elongating sperm tail where it is incorporated into the outer dense fibers along with ODF1 and at least 12 other proteins (Oko, 1988; Schalles *et al.*, 1998).

Early reports consistently reported murine *Odf2* expression as testis specific, as determined by Northern blotting (Brohmann *et al.*, 1997; Hoyer-Fender *et al.*, 1998; Schalles *et al.*, 1998; Shao *et al.*, 1997; Turner *et al.*, 1997). However, in a recent publication, monoclonal antibodies directed against purified chicken centrosomes have identified ODF2 protein as a widespread centrosomal component (Nakagawa *et al.*, 2001). Further studies have also suggested that ODF2 protein at the centrosome is able to associate both with itself and with microtubules (Donkor *et al.*, 2004). Furthermore, an *Odf2* gene targeted knockout mouse F9 cell line has recently been described, in which a critical role for ODF2 at the centrosome for the generation of primary cilia was revealed (Ishikawa *et al.*, 2005). However, these cells did not appear to be defective in other aspects of centrosome function, such as centriolar duplication and the organization of the microtubule networks. In addition, cell cycle progression and cell proliferation appeared indistinguishable from controls.

Prior to this study there have been no reports of *Odf2* knockout mice. As a result, details of the in vivo role of ODF2 in formation of the outer dense fibers and in its recently identified role at the centrosome have been lacking. Such a model would be invaluable in determining whether *Odf2* is required specifically during spermatogenesis, as was originally suggested (Brohmann *et al.*, 1997; Hoyer-Fender *et al.*, 1998; Schalles *et al.*, 1998; Shao *et al.*, 1997; Turner *et al.*, 1997), or whether *Odf2* also plays key roles at the centrosome in vivo.

The publicly available gene trap resources, coordinated by the International Gene Trap Consortium (IGTC), generate gene trapped embryonic stem cell (ESC) lines that can be used by researchers to determine the functions of genes of interest (Nord *et al.*, 2006). We obtained an *Odf2* gene trap ESC line from BayGenomics (a member of the IGTC) (Stryke *et al.*, 2003) and have generated *Odf2* knockout mice.

METHODS

Generation of *Odf2* Knockout Mice

BayGenomics mouse (strain 129/a) embryonic stem cell (ESC) line RO072, carries an *Odf2* allele disrupted by the insertion of a gene trap vector (pGT2Lxf). pGT2Lxf carries a splice-acceptor sequence upstream of the reporter gene, β -*geo* (a fusion gene of β -galactosidase and neomycin phosphotransferase II). Injection of these cells into C57Bl/6 blastocysts at the University of California-San Francisco Transgenic Core Facility resulted in chimeric mice that were bred with further C57Bl/6 mice to obtain germline transmission of the *Odf2*

mutant allele. *Odf2* mutant mice were backcrossed three generations to C57Bl/6 mice. All mice were bred and maintained in the animal housing facility at the UCSF and were subjected to a 12-h day/night cycle. Progeny were weaned at day 21.

Characterization of Insertion Site and Genotyping Assays

The genomic insertion site of pGT2Lxf into the *Odf2* gene was determined using a long range PCR approach. Forward primers were spaced ~800 bp apart between exons 8 and 9 of the *Odf2* gene. The insertion site was successfully amplified from tail DNA as part of a 1.6 kb fragment by PCR using a forward primer from the 3' end of the intron and a reverse primer from the vector. Primer sequences were: forward, 5'-GGGCTTTTGGGTTTAGTTCC-3' and reverse, 5'-CGACGTTGTAAAACGACGGGATC-3'. The PCR product was run on a 1% agarose gel and extracted using a gel extraction kit (QIAquick, Qiagen, Germantown, MD). PCR product was then sequenced at the UCSF Genomics Core Facility. A PCR genotyping strategy differentiates between the mutant and wild-type alleles from DNA extracted from tail tips. A common forward primer was used alongside reverse primers specific to each allele. Primer sequences were: forward, 5'-CCGAGAGACTAATGGAGCAAC-3'; mutant reverse, 5'-CCACAACGGGTTCTTCTGTT-3'; and wild type reverse, 5'-CTGGTCCACTTCGCTCTCTC-3'. These primers amplified bands of 147 bp and 676 bp for the mutant and wild-type allele, respectively. Reactions were performed in 20 µl volumes containing 100 µM dNTP, 1 µM each primer, 2 µl 10× buffer, and 0.2 µl Taq DNA polymerase (Promega, Madison, WI), 11.8 µl dH₂O and 1 µl template DNA. PCR reactions began with a denaturing step at 95°C for 3 min followed by 35 cycles of 95°C for 20 s, 57°C for 20 s, and 72°C for 30 s. The validity of the PCR genotyping in distinguishing germ line transmission was confirmed by Southern blotting. Genomic DNA was digested with BamHI and hybridized to a probe specifically recognizing the *β-geo* gene.

Embryo Genotyping

DNA was isolated from blastocysts and post-implantation embryos using the PicoPure DNA extraction kit (Arcturus Bioscience, Mountain View, CA) according to the manufacturer's instructions, with the exception that embryos were lysed in 5–10 µl of proteinase K DNA extraction buffer and 2 µl were used for PCR. PCR reactions were as described above.

RT-PCR and Quantitative RT-PCR

Total RNA was extracted from adult mouse tissues using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA from blastocysts was isolated using the PicoPure RNA Isolation Kit (Arcturus Bioscience). This RNA was used as a template for cDNA synthesis using superscript II (Invitrogen, Carlsbad, CA) and was primed with 5 mM oligo dT. cDNA was purified using a DNA Clean and Concentrator–5 kit (ZYMO Research, CA). All RT-PCR reaction conditions were performed as described above. For experiments in which *Odf2* expression across a range of wild-type tissues was tested, *Gapdh* was also amplified as a control. Primer sequences were: *Odf2* forward, 5'-CAGAAAAGCTGGTCTCGGTG-3'; *Odf2* reverse, 5'-CCATCCATTTTCAGCCTCCAC-3'; *Gapdh* forward, 5'-GTGTTCTTACCCCCAATGTG-3'; and *Gapdh* reverse, 5'-TGTGAGGGAGATGCTCAGTG-3'. These primers amplified bands of 365 bp and 397 bp for *Odf2* and *Gapdh*, respectively. For experiments in which qualitative expression of the wild-type and mutant allele in the testis was assayed (Fig. 2a), two sets of primers were used: (1) Upstream ('Up') primers recognized both the wild-type and gene trap allele, forward, 5'-AGAAAAGCTGGTCTCGGTG-3' and reverse, 5'-CATCCATTTTCAGCCTCCAC-3', (2) 'Vector' primers spanned the insertion site from *Odf2* sequence to vector sequence, forward, 5'-TGAGAACACGGTGCTCAGAC-3' and reverse, 5'-GTTTTTCCAGTCACGACGTT-3'.

QPCR reactions were performed in a MyIQ thermal cycler using the iQ SYBR Green Supermix (BIO RAD, CA). Reactions were performed in 50 µl volumes, each containing 25 µl of supermix, 1.25 µl (500 nM) of each primer, 20.5 µl of water, and 2 µl of template. PCR reactions began with a denaturing step at 95°C for 3 min followed by 45 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 45 sec with fluorescence measured at the end of each cycle. Finally, a melting curve was generated by raising the temperature from 50 to 95°C at 0.2°C intervals. PCR efficiency was measured for every primer pair in every run and relative quantification was performed as described previously (Salmon *et al.*, 2004), with the exception that *Gapdh* was used as the reference gene.

Northern Blotting

Total RNA was prepared using TRIzol reagent (Invitrogen). This RNA was then used for purification of mRNA using the Oligotex mRNA Kit (Qiagen). Five hundred nanograms of mRNA for each sample was mixed with 2x gel loading buffer (Ambion, Austin, TX), heated to 65°C for 5 min and chilled on ice for 2 min. mRNA was run on a 1% agarose-formaldehyde gel at 60 V for 3 h in 1x MOPS. Gels were washed in distilled water to remove formaldehyde and transferred to Hybond-N+ nylon membranes (Amersham Biosciences, Piscataway, NJ) overnight in 20x SSC buffer. Membranes were UV-cross linked. Membranes were prehybridized for 2 h at 42°C in 10 ml of prehybridization buffer containing 5x SSC, 50% formamide, 5x Denhardt's solution, 1% SDS, and 100 µg/ml salmon sperm DNA. DNA probes specifically recognizing either *Odf2*, *LacZ*, or *Gapdh* were radioactively labeled with p32 using High Prime (Roche Applied Science, Indianapolis, IN). Prehybridization buffer was removed and replaced with hybridization buffer containing 5% Dextranulphate in place of salmon sperm DNA. Hundred nanograms of labeled probe was added and the membranes were incubated overnight at 42°C. Membranes were washed with 2x SSC and 0.1% SDS at 65°C until background was low and exposed at -80°C.

LacZ Staining of Frozen Sections

Expression of the *Odf2-LacZ* fusion protein was visualized using X-gal staining of frozen testis sections. Testes were fixed overnight in neutral buffered formalin at 4°C and frozen sections were prepared by the UCSF Research Morphology Core Facility. Sections were rinsed in PBS and incubated overnight at 37°C in solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, and 1 mg/ml X-gal. Slides were rinsed in PBS and immersed in ethanol (100%) for 3 h. Slides were stained with Eosin for 2 min, dehydrated in an ethanol series from 75 to 100% and cleared in xylene for 2 min before addition of cover slips.

Sperm Analysis

Sperm counts were performed on mature mice. Each epididymide was dissected out and placed in 1 ml of PBS for 1 h at 37°C. Sperm were then counted using a hemacytometer. Sperm counts were performed in parallel on a wild-type and heterozygous mouse. In total, eight pairs of mice were counted.

Sperm motility assays were performed on mature mice. Each caudal epididymide was dissected out and placed in 1 ml of MEM with Earles, supplemented with nonessential amino acids, 0.23 mM pyruvic acid, 75 mg/ml Penicillin G, 50 mg/ml Streptomycin sulphate, 0.01 mM tetra-sodium EDTA, 3 mg/ml BSA (Sigma-Aldrich, St. Louis, MO), and 5% FBS. The caudal epididymide was cut three times and incubated at 37°C for 1 h to allow the sperm to capacitate. Sperm were diluted 1:40, transferred to a hemacytometer and counted in 5 large squares. Sperm were classified as progressing if they were making clear progress across the square. Total motile sperm include both progressing sperm and sperm

that maintained a fixed position but were visibly twitching. Progressing and motile sperm were recorded as a percentage of total sperm.

RESULTS

Generation of *Odf2* Knockout Mice Using Baygenomics ES Cell Line RRO072

The BayGenomics gene trap database was screened for ESC lines containing insertions within the *Odf2* gene. ESC line RRO072 contained an appropriate insertion on the basis of 5' RACE data that identified proximity to exon 8 of the 17-exon *Odf2* gene. Chimeric mice were generated by injection of RRO072 ES cells into C57Bl/6 blastocysts. Chimeric mice were then mated with C57Bl/6 females and germline transmission was confirmed by PCR amplification of the β -*geo* gene in tail DNA from progeny.

The precise location of the RRO072 genomic insertion was mapped via a long range PCR approach. A common reverse primer on the vector was used in a series of PCR reactions with one of 10 forward primers, spanning the full 8.5 kb of intron between exon 8 and 9 (Fig. 1a). Although the highly repetitive nature of intronic sequences led to some false positives as identified by sequencing (Fig. 1a, lanes 5 and 6), one primer consistently amplified a band (Fig. 1a, lane 10) that DNA sequencing confirmed as an integration site by the gene trap vector within exon 9 of the *Odf2* gene (Fig. 1b,c). Based on this sequence information, a PCR genotyping strategy was developed that clearly differentiated between wild-type and mutant alleles (Fig. 1d). The validity of this genotyping approach was confirmed by Southern blotting for the *LacZ* gene (Fig. 1e).

Characterization of *Odf2* Mutant mRNA Transcript

RT-PCR was used to confirm the presence of a fusion mRNA transcript in heterozygotes. A forward primer on the *Odf2* mRNA, upstream of the insertion site, and a reverse primer on the β -*geo* gene amplified the fusion transcript in heterozygotes only (Fig. 2a). DNA sequencing of this product showed that the first 30 bp of exon 9 are included in the fusion transcript (Fig. 2b). A cryptic splice site within exon 9 permits fusion with the splice acceptor on the gene trap vector 26 bp upstream of the insertion site.

Nucleic acid probes were designed that recognized *Odf2*, β -*geo* and *Gapdh* transcripts and were used to probe a northern blot containing testis mRNA from wild-type and heterozygous mice (Fig. 2c). As well as a 2.5 kb transcript representing wild-type *Odf2* mRNA, a second *Odf2* transcript was detected, 4.9 kb in size, that was the predicted size of a fusion transcript, containing exons 1 to 9 of *Odf2* and the β -*geo* gene. This 4.9 kb transcript was specifically recognized by a β -*geo* probe, confirming its identity as a fusion transcript. Interestingly, the fusion transcript appeared to be expressed at lower levels than the wild-type transcript in heterozygous mice. Furthermore, the wild-type transcript in heterozygous mice appeared to be expressed at higher levels than the wild-type transcript in wild-type mice. This observation was confirmed with quantitative RT-PCR, using primers that specifically distinguished these three transcripts (Fig. 2d). This confirmed that the fused *Odf2*: β -*geo* transcript is expressed at very low levels and that the wild-type allele is over-expressed in heterozygotes, implying a compensation mechanism.

Odf2 mRNA Expression and ODF2: LACZ Expression in the Testis

RT-PCR analysis of a range of wild-type adult mouse tissues demonstrated high *Odf2* mRNA expression in the testis (Fig. 3a). Weak *Odf2* expression was detectable in all other tissues examined; however, *Odf2* expression in these remaining tissues was highest in the epididymis. RT-PCR analysis of blastocyst RNA showed that *Odf2* is also expressed in the preimplantation embryo (Fig. 3b).

Heterozygous mice express a fusion protein containing the translated regions upstream of the insertion site fused to LacZ expressed from the β -geo gene on the gene trap vector. X-gal staining was used to assay for expression of the fusion protein in frozen testis sections from heterozygous mice. LacZ expression was first seen in round spermatids in the seminiferous tubule (Fig. 3c). At low magnification, LacZ staining was also apparent in the region of the sperm tails within the lumen. At high magnification, LacZ expression within the elongating spermatid was restricted to a single spot within the cytoplasm and was not seen in the developing sperm tail (Fig. 3d). In individual spermatozoa within the seminiferous tubule, LacZ expression was seen not in the tail portion itself, but within the cytoplasmic droplet attached to it (Fig. 3e). Therefore, it would appear that the ODF2:LacZ fusion protein is not incorporated into the outer dense fibers of the sperm tail. To confirm this, LacZ activity was assayed within the caput epididymis. Although endogenous LacZ activity was found within portions of the epithelium of the epididymis (data not shown), no LacZ staining was seen within the epididymal sperm tails (Fig. 3f), suggesting that the truncated ODF2 protein lost its ability to be assembled into the sperm tail.

Mice Homozygous for the RRO072 Insertion Are Not Viable

Heterozygous matings were established with both the F₁ and the F₃ generations, following backcrossing (Table 1a,b). No homozygous progeny were recovered from a total of 286 pups. However, backcrossing experiments established that both males and females transmit the *Odf2* knockout allele, suggesting that the absence of homozygotes is due to embryonic lethality. Interestingly, there was a strong transmission ratio distortion (TRD) in favor of the mutant allele when transmitted by F₁ mice (Table 1a). This TRD was particularly significant when the mutant allele was transmitted from F₁ males. However, TRD was not apparent in backcrosses with F₃ mice, implying that this phenomenon was due to genetic background effects rather than as a direct result of knocking out *Odf2* (Table 1b).

In order to further investigate the embryonic lethality, we set up timed heterozygous matings and collected embryos at specific stages of development (Table 1c). An F9 cell line homozygous for a targeted insertion within the *Odf2* gene has previously been shown to lack primary cilia (Ishikawa *et al.*, 2005). We genotyped embryos between e6.5 and e8.5 as primary cilia are first seen in the ventral node around this time. However, no homozygotes were recovered among these embryos. We next isolated and genotyped blastocysts at e3.5 but again recovered no homozygotes. Therefore, it is likely that a homozygous insertion in the *Odf2* gene results in lethality during pre-implantation stages of development, prior to the formation of the blastocyst.

Sperm Counts and Motility Are Unaffected in RRO072 Heterozygotes

ODF2 is a major component of the outer dense fibers of the sperm tail. Heterozygous mice carrying a gene trap insertion in exon 9 of *Odf2* had normal sperm counts when compared with wild-type mice of the same genetic background (Fig. 4a). Furthermore, sperm motility was assayed, with sperm classified as either progressing—making forward progress across the field of view—or motile—including sperm that were progressing as well as sperm that were twitching regularly. No significant difference in motility was seen between sperm from wild-type and heterozygous mice.

DISCUSSION

We have generated *Odf2* knockout mice using an *Odf2* gene trapped ESC line. Mice carrying a homozygous insertion in exon 9 of the *Odf2* gene are inviable as determined by the absence of homozygous embryos by the blastocyst stage of development. This suggests

that *Odf2* expression plays a crucial role during early preimplantation development of the mouse embryo.

The *Odf2* knockout mutation we have generated is likely to be a strong, if not null, mutation. There are two predicted isoforms of the *Odf2* gene: these have 17 and 23 exons, respectively. The RRO072 insertion disrupts both. Unlike most gene trap lines, which have insertion sites in intronic regions, the RRO072 insertion lies directly within exon 9. Interestingly, a cryptic splice site within exon 9 was used to generate a chimeric transcript from the partial *Odf2* gene and β -*geo* on the vector. The truncated transcript lacks sequence encoding most of the highly conserved coil-coil forming domains as well as both leucine zippers required for binding to ODF1, another component of the outer dense fibers of the sperm tail. This would be predicted to severely disrupt ODF2 function as supported by our finding that the truncated protein failed to be incorporated into the sperm tail.

No homozygous *Odf2* knockout pups were recovered from 286 pups from heterozygous matings, suggesting that *Odf2* is essential for embryonic development. These findings are in contrast with earlier studies of *Odf2* expression, which identified it as being testis specific (Brohmann *et al.*, 1997; Hoyer-Fender *et al.*, 1998; Schalles *et al.*, 1998; Shao *et al.*, 1997; Turner *et al.*, 1997). *Odf2* was reportedly expressed specifically in male germ cells from the secondary spermatocyte stage onwards, with expression reaching its maximal level in the round spermatid (Horowitz *et al.*, 2005; Hoyer-Fender *et al.*, 1998; Turner *et al.*, 1997). ODF2 is a major component of the outer dense fibers of the sperm tail and consistent with this we found high *Odf2* expression in the testis. However we also found low *Odf2* expression in all adult tissues examined. This finding is consistent with more recent studies that have identified ODF2 as a widespread component of the centrosome (Nakagawa *et al.*, 2001). Furthermore, F9 mouse cells with a homozygous targeted knockout of *Odf2* displayed centrosomes that failed to generate primary cilia, implying that *Odf2* gene function is not restricted to formation of the outer dense fibers of the sperm tail, but rather plays an additional wide-spread role at the centrosome (Ishikawa *et al.*, 2005). The results presented here support this conclusion, showing that mice lacking wild-type ODF2 protein are not viable by the blastocyst stage. Such an essential function of *Odf2* may explain its high sequence conservation. For example, the amino acid sequences of human and mouse ODF2 share greater than 97% homology. Therefore, an essential role for *Odf2* during the early stages of mouse embryogenesis is implied by these findings. However, it is interesting to note that *Odf2* knockout embryos die at stages that precede the formation of primary cilia, as primary cilia defects were the primary phenotype of an *Odf2* knockout F9 cell line (Ishikawa *et al.*, 2005). A detailed analysis of the role of ODF2 during preimplantation stages is now underway following our finding that *Odf2* is expressed in the preimplantation embryo.

Mice carrying a heterozygous insertion in the *Odf2* gene were viable and both male and female heterozygotes transmitted the mutant allele in backcrossing experiments. F₁ generation mice displayed a TRD, transmitting the mutant allele at levels above those predicted by Mendelian ratios. However, in backcrossing experiments, F₃ generation mice transmitted the mutant allele at Mendelian ratios suggesting that the observed TRD seen in the F₁ generation was due to genetic background effects. Neither male nor female heterozygous mice displayed any gross morphological abnormalities and both sexes showed normal fertility. Furthermore, heterozygous males had apparently normal sperm parameters: neither sperm counts nor sperm motility were significantly different between heterozygous and wild-type males. This absence of an obvious sperm phenotype could be due to the redundant function of other major protein components of the outer dense fibers, such as ODF1. An alternative hypothesis is that *Odf2* expression from the wild-type allele is up regulated in heterozygotes to compensate for loss of expression from the mutant allele. This

is supported by our observation that testes from heterozygous mice showed a significant upregulation of wild-type *Odf2* expression. This implies that haploid round spermatids are able to communicate and rescue the deficiency in *Odf2* expression resulting from the mutant allele. However, further studies are required to investigate this phenomenon in more detail.

This study has determined that *Odf2*, a major component of the outer dense fibers of the sperm tail, is also expressed in the preimplantation embryo where it plays a critical role. Although in contrast to early reports showing that *Odf2* expression is testis specific, these findings offer support to recent reports showing wide-spread expression of *Odf2* and may complement the recent finding that *Odf2* has a centrosomal function. We are currently investigating whether *Odf2* has an essential centrosomal role during preimplantation development.

Acknowledgments

Contract grant sponsor: National Institutes of Health, Contract grant number: NICHD U01 HD045871.

Nicholas A. Salmon was supported by a Fellowship from the Searle Foundation for the Advancement of Medical Sciences. The authors thank Jemma Jowett and Kehkooi Kee for their helpful comments.

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FIG. 1. Characterization of *Odf2* knockout allele. **(a)** Long range PCR mapping between exons 8 and 9 of the *Odf2* gene. **(b)** Sequence of *Odf2* exon 9 from the wild-type and mutant allele; italics represent vector sequence and underlining denotes additional sequence at insertion site. **(c)** Schematic showing vector insertion site in exon 9 and location of LacZ probe used for Southern blotting; vector sequence is represented by the dashed line and the β -*geo* gene is represented by the dotted box. **(d)** PCR genotyping strategy differentiates between wild-type (147) bp and mutant (676) bp bands. **(e)** Southern blotting with the LacZ probe identifies 3.1 kb vector band.

FIG. 2. Characterization of *Odf2* mutant mRNA transcript. **(a)** RT-PCR from adult testis RNA. Primers amplified specific regions either upstream of the insertion site (Up) or across the insertion site from wild-type to vector sequence (Vector). **(b)** Sequence of *Odf2* exon 9 showing the cryptic splicing site used in the mutant, relative to the insertion site of the vector. **(c)** Northern blot of *Odf2* mRNA isolated from adult testis, probed with *Odf2*, β -geo, and *Gapdh* probes. **(d)** Quantitative RT-PCR of *Odf2* mRNA isolated from adult testes isolated from wild-type (WT) and heterozygous (Het) mice. Expression is normalized to *Gapdh*. Data is presented as mean $\pm$ SEM; bars with different letters are significantly different ($P > 0.05$).

FIG. 3. *Odf2* mRNA expression in wild-type adult tissues and blastocysts, and ODF2:LACZ expression in the heterozygous testis. **(a)** RT-PCR of *Odf2* and *Gapdh* from total RNA isolated from adult wild-type mice: Br = brain, Ep = epididymis, He = heart, Ki = kidney, Li = liver, Lu = lung, Ov = ovary, Te = testis, St = stomach, and Ut = uterus. **(b)** RT-PCR of *Odf2* and *Gapdh* from total RNA isolated from blastocysts; H_2O was included as a negative control and testis cDNA was included as a positive control. **(c-f)** ODF2:LACZ expression within testis sections from adult mice heterozygous for the *Odf2* insertion: (c) Seminiferous tubules viewed at $\times 100$; (d) Elongating spermatozoa viewed at $\times 1,000$; (e) Spermatozoa in the seminiferous tubule viewed at $\times 1,000$; (f) Caput epididymis viewed at $\times 100$.

FIG. 4. Sperm counts and motility assays. (a) Sperm counts from the entire epididymide of adult wild-type (WT) and *Od2* heterozygous (Het) mice. (b) Sperm motility assays from the caudal epididymide of adult wild-type (WT) and heterozygous (Het) mice. Sperm progressing across the slide were recorded as 'progressing' while 'motile' refers to total sperm showing signs of motility, from repeated twitching to progression.

Table 1**Mouse Breeding and Genotyping Data**

F₁	Het × Het	Backcross (♂ Het)	Backcross (♀ Het)
(a) Progeny resulting from heterozygote crosses and backcrosses using parents from the F ₁ generation			
WT (+/+)	26	6	33
Het (+/-)	113	35	51
Hmz (-/-)	0		
<i>Total</i>	139	41	84
(b) Progeny resulting from heterozygote crosses and backcrosses using parents from the F ₃ generation			
F₃	Het × Het	Backcross (♂ Het)	Backcross (♀ Het)
WT (+/+)	37	20	14
Het (+/-)	110	20	17
Hmz (-/-)	0		
<i>Total</i>	147	40	31
(c) Genotyping of embryos at a range of developmental stages recovered from heterozygous crosses			
Age	WT (+/+)	Het (+/-)	Hmz (-/-)
e3.5	5	15	0
e6.5–8.5	6	16	0