A mouse model of familial oligoasthenoteratozoospermia

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BACKGROUND: Hrb is an HIV-1 Rev-binding/interacting protein and is a cofactor for Rev export pathway. Hrb interacts with Eps15 homology (EH) domain-containing proteins and is a component of EH network and functions in vesicle sorting. Earlier, we reported that Hrb-deficient male mice are infertile and that they show oligozoospermia. Their sperm lack acrosomes and present globozoospermia. The aim of this study was: (i) to investigate the additional defects in spermatogenesis in Hrb-deficient mice; and (ii) to investigate the effect of acrosomelessness on spermatid differentiation in Hrb-deficient mice. METHODS: Hrb–/– testes, epididymides, spermatids and sperm were analyzed by histology and electron microscopy. Centrioles were analyzed in spermatids and sperm by indirect immunofluorescence technique. RESULTS: Hrb–/– male mice exhibited multiple anomalies during meiosis and spermiogenesis that produced developmentally impaired sperm with unshaped or deformed nuclei, loss in cell polarity, intracellular flagellar coiling, multinucleation, supernumerary centrioles and multiflagellation. A total of 13.0% Hrb–/– sperm showed macrocephaly. The Hrb–/– sperm exhibited variation in head size and shape, disarrayed cellular organelles, nuclear and cytoplasmic vacuolization, mitochondrial loss or scattering and no forward motility. CONCLUSIONS: These aberrations, in Hrb–/– mouse spermatids and sperm, are reminiscent of human familial male infertility with oligoasthenoteratozoospermia syndrome. The Hrb-deficient mouse may be useful in understanding familial oligoasthenoteratozoospermia syndrome.

Key words: centrioles/manchette/multiflagellation/oligoasthenoteratozoospermia

Introduction

The HIV-1 Rev-binding/Rev-interacting protein Hrb (also known as RAB or Rip) has been shown to be a cellular cofactor of the HIV-1 Rev protein and plays a role in the Rev export pathway. Hrb interacts with Eps15 homology (EH) domain-containing proteins and is a component of EH network and functions in vesicle sorting (Fritz et al., 1995; Santolini et al., 1999). It displays a zinc-finger region in its N-terminus and contains several FG repeats, which are characteristics of nucleoporins. It also contains four NPF motifs located towards the C-terminus of the protein. In both human and mouse Hrb, deletion or mutation of the four NPFs completely abolishes its binding to the EH-interacting proteins (Santolini et al., 1999). It displays a zinc-finger region in its N-terminus and contains several FG repeats, which are characteristics of nucleoporins. It also contains four NPF motifs located towards the C-terminus of the protein. In both human and mouse Hrb, deletion or mutation of the four NPFs completely abolishes its binding to the EH-interacting proteins (Santolini et al., 1999). We generated Hrb-deficient mice by gene targeting and showed that mutant males are infertile. We reported that Hrb plays an essential role in Golgi-derived proacrosomic vesicles fusion and that Hrb–/– spermatids lack acrosome biosynthesis during spermiogenesis (Kang-Decker et al., 2001). Lack of an acrosome does not prevent the development of the acroplaxome in Hrb–/– spermatids (Kierszenbaum et al., 2004). Myosin-Va-bound proacrosome vesicles tether to acroplaxome and form a pseudoacrosome. As spermiogenesis advances, round-shaped spermatid nuclei display several nucleopodes at the acroplaxome–pseudoacrosome site. The current study was designed to investigate, in detail, the effect of acrosomelessness on spermatid differentiation and on the resulting sperm and additional defects in spermatogenesis due to the presence of supernumerary centrioles in spermatids in Hrb-deficient mice.

Materials and methods

Animals

Hrb-deficient mice were produced by crossing Hrb+/– (heterozygote) male and female. Mice were generated at Mayo Clinic (Kang-Decker et al., 2001). The Hrb targeting vector contained a 12.0 kb Hrb 129 Sv/J genomic DNA fragment in which a unique, 500 bp SpeI–StuI fragment had been replaced by a promoterless IRES-lacZ-neo selection cassette, interrupting the Hrb gene at amino acid 108.

Testis weight, sperm concentration and sperm motility

Testes were excised from mice and weighed. Caudal epididymal sperm were retrieved and capacitated as described earlier (Juneja, 2002). Total caudal epididymal sperm were counted and the motility of 10³ capacitated sperm from each mouse was determined by using a hemocytometer (n = 10 mice in wild-type and in Hrb+/– group).
Figure 1. Spermatid and sperm defects in $Hrb^{−/−}$ mouse. Light microscopy. (A–F) Toluidine blue-stained sections of Spurr-embedded seminiferous tubules at stage VII of spermatogenesis. (A and B) Wild-type spermatids. Spermatids, at step 16 (white arrows) have elongated and condensed nuclei. Spermatids, at step 7 (blue arrowheads), have round nuclei. (C–F) $Hrb^{−/−}$ spermatids. Spermatids, at step 16, show non-elongated and condensed nuclei (yellow arrow, C). At step 7, spermatids show round nuclei (blue arrowheads, C–E) and nuclei with varied shapes (green arrowheads, D). Multinucleated (yellow arrowheads, D and E), macrocephalic (green arrow, E) and multiflagellated (black arrowheads, F) spermatids are present in $Hrb^{−/−}$ seminiferous epithelium. Note the upper row of three cells showing disintegration of intercellular walls (red arrows in insert, C), whereas the lower row of three cells has intact walls. (G and H) Toluidine blue-stained sections of Spurr-embedded cauda epididymis. The wild-type lumen contains a homogeneous mixture of sperm (red arrowheads, G), whereas the $Hrb^{−/−}$ lumen contains a heterogeneous mixture of cells and sperm (green arrowheads, H). Sg, Ps, and Sr indicate spermatogonium, pachytene spermatocyte and Sertoli cell, respectively (B, C and E). Scale bars, 5 µm (A–C); 2 µm (D); 5 µm (E) and 20 µm (G and H).
Spermatogenic defects in the Hrb-deficient mouse

Histological examination and transmission electron microscopy (TEM)
The testes and caudae epididymides, isolated from 3-month-old male mice, were processed and examined as previously described (Bartoov et al., 1990). Biopsies from testis and cauda epididymis were fixed for 4 h in 2.5% glutaraldehyde/0.6% formaldehyde (buffered with sodium cacodylate, pH 7.2), and post-fixed in 2% buffered OsO₄. Samples were dehydrated in graded ethanol and embedded in Spurr. Semi-thin sections (0.5 μm thick) were stained with 0.5% toluidine blue and assessed by light microscopy. Thin sections (0.09 μm thick) were examined using a JEOL 1200 EXII transmission electron microscope.

Scanning electron microscopy (SEM)
The caudal epididymal sperm, isolated from 3-month-old male mice, were processed and examined as previously described (Bartoov et al., 1990). The luminal contents of cauda epididymis were retrieved in M2 medium (Sigma Chemical Co.). The cells in luminal contents were washed with M2 medium and resuspended in the same medium. The cell suspensions were spread over poly-L-lysine-coated slides and incubated at room temperature for 15 min. The cells were fixed in Trumps fixative (4% formaldehyde/1% glutaraldehyde in 10 mM phosphate buffer, pH 7.2) for 1 h and washed twice in 10 mM phosphate buffer for 2 min each time. The cells were dehydrated through increasing concentrations of ethanol (60, 70, 80, 95, 100%) for 10 min in each. Each specimen was loaded in CPD holder and immediately immersed in 100% ethanol, placed in critical point dryer, mounted and coated with Au/Pd. The specimens were viewed in S-4700 scanning electron microscope.

Indirect immunofluorescence and confocal microscopy
Centrin protein is a marker for centriole. Centrioles were localized in spermatids and sperm by indirect immunofluorescence of centrin as described previously (Hart et al., 1999). The testis was excised, the seminiferous tubules removed from testis and the outer capsule was discarded. The tubules were minced gently for 3–4 min in 1 ml M16 medium (Sigma Chemicals Co.) with fine scissors. The minced tissue was treated with 0.05% collagenase/trypsin mixture as described (Gerton and Millette, 1984). The cell suspensions, thus obtained, were washed with M2 medium and resuspended in M2 medium. The caudal epididymal luminal contents were recovered as described (Juneja, 2002) in 0.5 ml M2 medium. Cell suspensions obtained from testis or cauda epididymis were spread over poly-L-lysine-coated glass slides and left at room temperature for 15 min and then fixed in cold methanol (−20°C) for 15 min. The slides were dried to evaporate methanol for 10 min, treated with 0.5% Triton X for 3 min followed by four washings with PBS (0.01 M phosphate buffer containing 2.7 mM KCl and 137 mM NaCl, pH 7.4). The slides were treated with blocking buffer (PBS containing 5% normal goat serum, 1% glycerol, 0.1% bovine serum albumin, and 0.1% fish skin gelatin) for 30 min and treated with anti-centrin mAb 20H5 (1:800 dilution of mouse ascites in blocking buffer; anti-body obtained from J.Salisbury, Mayo Clinic) for 60 min. The slides were washed twice with PBS, treated with DNA binding fluorochrome Hoechst 33258 (10 μg/ml in PBS; Sigma Chemicals Co.) for 5 min and washed twice with PBS for 2 min each. The slides were mounted using antifade mounting medium. The red fluorescence signal from Alexa Fluor 568 was visible using rhodamine filter and the blue fluorescence of Hoechst 33258-bound DNA was recorded using UV light. Sample images were recorded by using a Zeiss LSM 310 scanning laser confocal microscope.

Specific definitions
We used the following definitions for presentation of our data in this paper. The presence of ≥ 2 centrioles in a cell is termed as supernumerary centrioles. A cell with >1 nucleus is termed a multi-nucleated cell. A mature spermatid or sperm with >1 flagellum is termed a multiflagellated cell. A sperm with a head larger than normal sperm heads is defined as macrocephalic. A round-headed sperm (mono- or multiflagellated) is defined as globospermic.

Results
Hrb−/− male mice were infertile while females were fertile. The Hrb−/− mice contained a significantly lower number of sperm in their caudae epididymides and the results were similar to our studies reported earlier (data not shown). The testis size did not differ between wild-type and Hrb−/− mice as reported earlier (Kang-Decker et al., 2001). A total of 4.14 ± 0.61% (± SEM) Hrb−/− caudal epididymal capaci-tated sperm showed feeble lateral motion while 100% sperm presented no forward motility. On the other hand, 66.52 ± 2.24% wild-type capacitated sperm showed hyper-activated forward motility (n = 10 mice in wild type and in Hrb−/− group; motility of 105 sperm/mouse assessed). Light microscopy of seminiferous tubules, at stage VII, showed a number of differences between wild-type and Hrb−/− mice (Figure 1A–F). Wild-type spermatids, at step 16, had

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elongated and condensed nuclei, and were present in groups in the seminiferous epithelium (Figure 1A and B). On the other hand, Hrb–/– spermatids, at step 16, showed condensed and non-elongated nuclei. They were not present in groups (Figure 1C). Hrb–/– spermatids showed multinucleation (Figure 1D and E), macrocephaly (Figure 1E) and multiflagellation (Figure 1F). At step 7, the nuclei in wild-type spermatids presented round shape (Figure 1A and B). On the other hand, the nuclei in Hrb–/– spermatids presented round shape (Figure 1C–E) as well as other varied shapes (Figure 1D). Wild-type cauda epididymis showed the presence of a homogeneous mixture of sperm, whereas Hrb–/– cauda epididymis contained a heterogeneous mixture of cells and sperm in the lumen (Figure 1G and H).

Hrb–/– mouse presents multiple sperm types and shapes

Percent different types and shapes of sperm, retrieved from Hrb–/– cauda epididymides, are plotted in Figure 2. Hrb–/– mice showed two major and one minor sperm types: intracellularly coiled, 45.5%; non-coiled, 52.5%; and headless, 2.0%. The non-coiled sperm further comprised two types: monoflagellated, 31.5% (Figure 3B) and multiflagellated, 21.0% (Figure 3C–E). The non-coiled sperm presented two shapes: globospermic, 39.5% (31.5% globospermic were monoflagellated, Figure 3B; and 8.0% globospermic were multiflagellated, Figure 3C) and macrocephalic, 13.0% (all multiflagellated, Figure 3D and E). The number of flagellae in multiflagellated sperm varied from two (Figure 3C) to 13 (Figure 3E). Wild-type sperm comprised two types: non-coiled, 99.5%; and headless, 0.5%. All the wild-type sperm were monoflagellated and showed one kind of head shape that was elongated and hook-shaped (Figure 3A). Furthermore, in Hrb–/– multiflagellated sperm, the flagella showed either one common origin from sperm head (Figure 3F and G) or multiple origins from the same sperm head (Figure 3H). In the former case, the part of the flagella was delimited by a common plasma membrane which presented partial symplast (Figure 3I and J).

Figure 3. Hrb–/– sperm show multiflagellation. Sperm were retrieved from cauda epididymides and analyzed by SEM (A–E), phase contrast microscopy (F–H) and TEM (I and J). (A) Wild-type sperm shows monoflagellation. (B–E) Hrb–/– sperm show monoflagellation (B) or multiflagellation (C–F). Multiflagellated sperm show smaller heads (C) or macrocephaly (D and E). (F–H) Hrb–/– multiflagellated sperm. Multiple flagellae from one common origin (arrowheads, F and G) and from multiple origins in the sperm head (arrows, H). The triangle may indicate a membranous vesicle or residual cytoplasm (G). (I and J) Hrb–/– multiflagellated sperm showing two flagellae (I) and three flagellae (J). The flagellae are delimited by a common plasma membrane (arrowheads). Scale bars, 10 μm (A–E); 5 μm (F–H) and 0.2 μm (I and J).
Ectopic location of the manchette causes multiple defects in Hrb−/− spermatids

The wild-type spermatids, at step 9 (Figure 4A) and at steps 12 and 13 (Figure 4B), showed the presence of the acrosome and the manchette posterior to acrosome. Spermatids contained an ‘elongating’ and ‘elongated’ nucleus, respectively. The wild-type spermatid, at step 16, had an elongated nucleus with an acrosome (Figure 4C). Hrb−/− spermatid showed the absence of the acrosome and manchette around the nucleus (Figure 4D). The nucleus remained unshaped. This type of Hrb−/− spermatids can be expected to differentiate into mature spermatids with round nucleus (Figure 4K). In another Hrb−/− spermatid aberration, the manchette showed tangential location on the nucleus. The round nuclear surface appears to be flattened at the manchette–nuclear contact area, providing the nucleus an abnormal shape (Figure 4E). Furthermore, Hrb−/− spermatids presented different extent of manchette invagination in the nucleus (Figure 4F–I). Spermatids showed varied jaw-shaped nuclei (Figure 4F and G), indentation of nucleus (Figure 4H), manchette-enforced vacuolization of nucleus (Figure 4I) and dumbbell-shaped nucleus (Figure 4J). In all these Hrb−/− spermatids, the nuclei were condensed, unshaped or deformed, and non-elongated (Figure 4E–K).

Hrb−/− macrocephalic sperm show varied head shapes and disrupted internal structures

Hrb−/− multinucleated spermatids also lacked the acrosome (Figure 4L). Hrb−/− macrocephalic sperm showed large varied and/or irregular-shaped heads with varied degree of multiflagellation (Figure 3D and E) and aberrant internal structures (Figure 4M and N).

Hrb−/− mice present intracellularly coiled sperm

Intracellularly coiled sperm presented the major aberration in Hrb−/− sperm population (Figure 2) which included fully coiled (Figure 5A–C and E) and partly coiled (Figure 5D and F). The coiled sperm provided a varied number of shapes to Hrb−/− sperm. All the wild-type sperm, on the other hand, were non-coiled (Figure 3A). Hrb−/− coiled sperm showed many intracellular aberrations (Figure 5G–J). Flagellar coiling in Hrb−/− sperm is the result of alteration in ‘nucleus–centrioles–axoneme’ axis, which, in turn, is caused by the manchette invagination in the nucleus (Figure 6A and B).
Hrb−/− spermatids and sperm show supernumerary centrioles

A total of 42.0% spermatids (10.50 ± 0.64 spermatids per 25 spermatids per mouse; mean ± SEM; n = 4 mice) presented supernumerary centrioles in Hrb−/− mice (Figure 7C–F), whereas the others showed a pair of centrioles (Figure 7B). The wild-type spermatids showed a pair of centrioles (Figure 7A). The wild-type spermatids, at step 16, show elongated and condensed nucleus with an acrosome. (D) Complete dislocation of the manchette in Hrb−/− spermatid. The acrosome is absent as expected, but the manchette is not visible at its normal expected location (black arrows). (E) Tangential location of the manchette in the Hrb−/− spermatid. The manchette is present on the nuclear surface tangentially and flattens the round nuclear surface at the contact area. (F–I) Manchette invagination in nucleus in Hrb−/− spermatids. The nuclei in spermatids show different extent of manchette invagination (F–I). As a result, nucleus becomes jaw-shaped (F and G), indented (H), manchette-enforced vacuolated (I) and dumbbell-shaped with jaw (J). (K) Hrb−/− spermatid showing unshaped nucleus. (L) Hrb−/− multinucleated spermatid also showing no acrosome. (M and N) Macrocephaly in Hrb−/− sperm. A macrocephalic Hrb−/− sperm in the lumen of seminiferous tubule (M). The head contains cytoplasmic vacuoles (v), two unshaped nuclei without acrosomes and two axonemes each with loosely arranged mitochondrial sheath. Some mitochondria are scattered ectopically in the anterior region of the head. Midpiece regions are engulfed in the head providing the sperm a macrocephalic shape. A releasing biflagellated Hrb−/− sperm (N). The nucleus is rectangular and acrosomeless. The midpiece along with mitochondrial sheath is absent. Scale bars, 1 μm (A–C) and 2 μm (D–N).

**Hrb−/− spermatids and sperm show supernumerary centrioles**

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Both wild-type and Hrb−/− mice showed the presence of centrioles in testicular sperm (Figure 8A–O). Caudal epididymal sperm were used as negative controls where centrioles are degenerated in mouse (Figure 8P and Q). A total of 34.0% Hrb−/− testicular sperm (8.50 ± 1.04 sperm per 25 sperm per mouse; n = 4 mice) presented supernumerary centrioles, whereas the others presented a pair of centrioles. The wild-type testicular sperm showed a pair of centrioles (Figure 8A and B). Hrb−/− headless sperm also presented centrioles at the base of the flagellum (Figure 8N and O).

A total of 15.0% Hrb−/− sperm (3.75 ± 0.25 sperm per 25 sperm per mouse; n = 4 mice) were multinucleated.
Figure 5. *Hrb*<sup>−/−</sup> sperm show intracellular flagellar coiling. Intracellularly coiled sperm retrieved from cauda epididymis from *Hrb*<sup>−/−</sup> mice. (A–F) SEM of coiled sperm. The flagella are fully coiled (A–C and E) and partly coiled (D and F). (G–J) TEM of coiled sperm. A sperm with four intracellular flagellar coils (G). Upper left part of sperm section has four axonemal sections (arrowheads) which make coils with four axonemal sections in the lower right part of the sperm section. Similarly, sperm with two or three (H) and five (I) flagellar coils. A half sperm section with 15 axonemal sections; the sperm appears to be a multiflagellated (with > 1 flagellum) due to a large number of axonemal sections (J). TEM of coiled sperm (G–J) show mitochondrial (mi) scattering, nuclear (nu) and cytoplasmic vacuoles (v), multinucleation, acrosomelessness, aberrant shaped nuclei and possibly multiflagellation. Scale bar, 1 μm (A–F), 0.5 μm (G), 1 μm (H), 0.5 μm (I) and 1 μm (J).
Figure 6. Mechanics of flagellar coiling. (A and B) TEM of testicular sperm in longitudinal section. A wild-type sperm, releasing from seminiferous epithelium, shows an elongated nucleus, an implantation fossa and a centriole apparatus (A). A partly coiled Hrb<sup>−/−</sup> sperm is shown in the lumen of seminiferous tubule (B). Nuclear invagination (inv) of the manchette displaces the implantation fossa from its original location on the nuclear membrane causing a deviation in nucleus–centrioles–axonemal axis to an extent that the flagellum is formed in an altered direction, and becomes intracellularly coiled. The sperm does not present any nucleopodes. The sperm loses its distinct poles. ac and ax indicates acrosome and axoneme, respectively. Arrowheads indicate invaginated nuclear membrane in the insert. Scale bar, 2 μm (A) and 3 μm (B).

Discussion
Infertility is a heterogeneous syndrome in men. Defective development in spermatogenesis can be the end result of a multitude of causes, including genetic defects. Idiopathic infertility exists in the majority of infertile men. We reported earlier that Hrb<sup>−/−</sup> mice are infertile and that their spermatids lack acrosome biogenesis (Kang-Decker et al., 2001). Here we showed defects in spermatids and sperm due to the lack of acrosome biogenesis and additional defects in spermatogenesis due to the presence of supernumerary centrioles in Hrb-deficient mice.

Ectopic manchette causes nuclear deformity in spermatids
Globozoospermia is a human infertility syndrome caused by spermatogenesis defects (Lalonde et al., 1988). In patients with teratozoospermia and familial infertility, all the spermatozoa were globoospermic with no acrosome enzyme activity (Stanislavov and Ganev, 1998). Spermatozoa of Csnk2a2<sup>−/−</sup> mice, in which males are infertile, are globoospermic and present nuclei with detached acrosome (Xu et al., 1999). The globoospermia accompanied acrosomal and nuclear shape defects as well as aberrant mitochondrial arrangement (Battaglia et al., 1997). Familial infertility in brothers with teratozoospermia showed acrosomeless round-headed sperm and aberrant mitochondrial sheath (Nistal et al., 1978). The manchette is a transient structure which provides an elongated shape to the spermatid nucleus at steps 9–11 during spermiogenesis (Russell et al., 1991b). Any disturbance in manchette location may adversely affect the elongation process in nucleus. Ectopic location of the manchette or its dislocation at steps 9–11 caused nuclear shape deformity in az/hazh mice (Meistrich et al., 1990). The current study provided a detailed analysis of the disturbance in manchette location in Hrb<sup>−/−</sup> acrosomeless spermatid. Nuclear elongation of round spermatid was blocked in Tfr2<sup>−/−</sup> -deficient mice which had an abnormal structure of the acrosome with defective spermiogenesis (Zhang et al., 2001). In male sterile hpy mice, acrosomal distortions, abnormal heads and flagellar dysgenesis accompanied infertility (Bryan, 1977). Lis1<sup>GT/GT</sup> mutant spermatids failed to form correct acrosomes, and nuclei appeared distorted in size and shape (Nayernia et al., 2003). Though it is evident from human familial male infertility reports, and from animal studies, that the acrosome defects are linked to sperm shape aberrations and male infertility syndrome, the etiology of this relation is poorly understood. The current study presents the Hrb<sup>−/−</sup> mouse as a crystal clear model in which all the spermatids are completely acrosomeless and a number of nuclear and

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Sperm shape anomalies and flagellar defects are shown. The posterior rim of the acrosome is closely associated with the perinuclear ring of the manchette through nucleus, plasma membrane or ectoplasmic specializations of the Sertoli cell (Russell et al., 1991b). This association between the acrosome and manchette does not exist in the Hrb–/– spermatid, which lacks the whole body of the acrosome. Lack of acrosome bio- genesis in Hrb–/– spermatids presents adverse consequences of the manchette’s function in shaping the spermatid nucleus.

Intracellular coiling of flagellum in Hrb−/− sperm
Manchette invagination has been shown to cause nuclear deformity in azh/azh spermatids (Meistrich et al., 1990). Also in an earlier report on Hrb−/− mice, manchette invagination in the nucleus has been shown to cause nucleopodes in nuclear acroplaxome and loss of spermatid polarity (Kierszenbaum et al., 2004). The current study shows that nuclear deformity in the spermatid depends upon the extent of invagination and ectopic location of the manchette, i.e.
Figure 8. *Hrb*<sup>−/−</sup> sperm show supernumerary centrioles and multinucleation. Sperm, retrieved from testis (A–O) and from cauda epididymis (P and Q), were assessed for the presence of centrioles and nuclei as described in the Figure 7 legend. Fluorescence in nuclei is not shown in some sperm (H–J) in order to view centrioles better. Flagellae (arrows) are visible as low non-specific fluorescence. (A and B) Wild-type sperm. Each of the two wild-type sperm shows a pair of centrioles. The nucleus–centrioles–flagellar axis is similar in both the sperm. (C–O) *Hrb*<sup>−/−</sup> non-coiled sperm. In three mononucleated monoflagellated sperm, each with a pair of centrioles, the nucleus–centrioles–flagellar axis varies among different sperm, indicating deviations in the axis and the loss of original cell polarity (C–E). A binucleated triflagellated sperm with centrioles at three different spots (F). A mononucleated and multiflagellated sperm with three pairs of centrioles (G, note that the sperm is magnified). Two biflagellated sperm, each flagellum with a pair of centrioles (H). Macrocephalic triflagellated sperm with supernumerary centrioles (I and J). (K–M) *Hrb*<sup>−/−</sup> coiled sperm. A mononucleated sperm with a pair of centrioles at its origin (K). A multinucleated multiflagellated sperm with two pairs of centrioles (L). A mononucleated sperm with supernumerary centrioles (M). (N and O) *Hrb*<sup>−/−</sup> headless sperm. A sperm with two pairs of centrioles and two flagella (N). A sperm with one flagellum and a pair of centrioles (O). (P and Q) *Hrb*<sup>−/−</sup> sperm from cauda epididymis. Centrioles are not visible. A mononucleated biflagellated (P) and a trinucleated triflagellated (Q) sperm. Scale bars, 2 μm (A and B) and 1 μm (C–Q).
complete dislocation from the nucleus, tangential location of the manchette on the nucleus or manchette invagination in the nucleus. Also, the loss of polarity or alteration of the nucleus–centrioles–axonemal axis in spermatids depends upon the extent of nuclear deformity, i.e. indentation, vacuolization, nucleopodes formation and resulting jaw size in the nucleus due to manchette invagination. In the case where the deformed nucleus displaces the implantation fossa and causes an alteration of the nucleus–centrioles–axoneme axis, an intracellular coiled sperm results. The study also finds that nucleopodes formation may not be a mandatory component for loss of cell polarity (Figure 6B). Interestingly, a total of 45.5% Hrb−/− sperm were intracellularly coiled, which presents the major aberration in the Hrb−/− sperm population. The deviations in the nucleus–centrioles–flagellar axis are also present in the non-coiled Hrb−/− sperm, but the extent varies and is insufficient to cause the coiling of flagellum around the nucleus. Intracellular flagellar coiling has been shown to be present in sperm in patients with familial male infertility (Nistal et al., 1977).

**Supernumerary centrioles and multinucleation in Hrb−/− spermatids**

Supernumerary centrosomes or centrioles have been described for many cancers. Numerical centrosome aberrations are frequently accompanied by genomic instability and loss of cellular architecture and cell polarity (Doxsey, 2001). Supernumerary centrosomes can arise through four possible models: centrosome overduplication, aborted cell division, cell fusion, and de novo centrosome synthesis (Nigg, 2002). The majority of animals studied, including mammals, have two centrioles in their mature sperm (Krioutchkova and Onishchenko, 1999). Since Hrb protein is abundantly present in spermatocytes undergoing meiosis and in spermatids (Kang-Decker et al., 2001), the current study shows that the lack of Hrb during meiosis and early spermiogenesis causes the appearance of supernumerary centrioles; however, the mechanism needs to be investigated in future studies. Our results suggest that supernumerary centrioles, in Hrb−/− spermatids, derive from three possible routes: (i) overduplication of the centrosome during meiosis (Figure 7D) in which the spermatids may differentiate into mononucleated, multicentric and monoflagellated (Figure 8M) or multiflagellated (Figure 8G) sperm (note that our interpretation on the formation of multiflagellae from overduplicated centrioles within mononucleated spermatids is based on centrin detection only; further studies may be needed for additional demonstration, i.e. double or triple labelling of different known proteins in centriole and transmission electron microscopy); (ii) by meiotic division deficiency (see yellow arrowheads in Figure 1D and Figure 7E; centrosomes acquisition); or (iii) by spermatids fusion (see neighboring spermatids showing disintegrating intercellular walls, red arrowheads in Figures 1C and 7C; centrosomes accumulation), and these spermatids may differentiate into multinucleated, multicentric and multiflagellated sperm (Figure 8F and L). A genetic disorder was shown in two brothers with meiotic division deficiency phenotype (Escalier, 2002). Multinucleated giant cells present in human old age have been shown to be formed due to the confluence of membranes in neighboring germ cells (Miething, 1993).

**Hrb−/− sperm present human male infertility syndrome**

We showed that the sperm from Hrb−/− mice present multinucleation, supernumerary centrioles, multiflagellation and macrocephaly. The first example of multiflagellate sperm in the animal kingdom was reported in the termite, *Mastotermes darwiniensis*. The spermid with two centrioles undergoes centrosome amplification and transforms into multiflagellate sperm (up to 100 flagellae) with one centriole per flagellum. Interestingly, the termite sperm are acrosomeless and feebly mobile (Baccetti and Dallai, 1977). A familial case of asthenoteratozoospermia showed a high percentage of irregular large heads and up to four tails (Nistal et al., 1977). Patients with familial history of male infertility had multiflagellation, macrocephaly and acrosomal malformation in sperm (Escalier, 1983). A patient with disturbed meiosis had an absence of the acrosome in 65% of spermatozoa, large head shape in 32% and two to four tails in 61% of spermatozoa (Pieters et al., 1998). In a family with a history of male infertility, half of the male members showed 68% megalophecal sperm with multi-tails (Kahraman et al., 1999). A patient with oligoasthenoteratozoospermia showed meiotic anomaly with a high percentage of polyplody. Most of the sperm were morphologically abnormal, with irregularly large and/or binucleated heads, and were multiflagellated (up to eight tails) (Wegner et al., 2001). Another report in a patient with oligoasthenoteratozoospermia syndrome, with a familial history that revealed multiple cases of infertility, multiple defects were observed in sperm with polyplody (2 to ≥4), macrocephaly, multiflagellation (2–5 flagellae), nuclear and cytoplasmic vacuolization, disorganization of mitochondrial helix sheath, acrosomal abnormality and chromatin packaging defects (Benzacken et al., 2001). Polyplody was found in macrocephalic, large-headed sperm with multiflagellae in human infertility cases (Devillard et al., 2002). Assessment of oligoasthenoteratozoospermia in three patients linked sperm chromosomal and sperm multinucleation defects to acrosomal defects (72–80% sperm population), multi-tail deformities (49–76%) and amorphous large-headed sperm (54–76%) (Lewis-Jones et al., 2003). Several genes in

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**Figure 9.** A bar chart presents supernumerary centrioles in different sperm types in Hrb−/− mice. Sperm were retrieved from testis. A total of 100 sperm of each sperm type were identified randomly (25 sperm from each of four mice) and were evaluated for supernumerary centrioles. The data were pooled. No wild-type sperm showed supernumerary centrioles.
Figure 10. A diagrammatic summary showing sperm defects in Hrb-deficient mice. Wild-type primary spermatocyte (non-polar cell) undergo two consecutive meiotic divisions (left). Centrosome duplication takes place during meiosis, followed by cytokinesis to produce round spermatids (polar cells) each with one centrosome or a pair of centrioles. The newly formed spermatid undergoes acrosome biogenesis followed by the appearance of the manchette, nuclear elongation and condensation, and differentiates into normal mature spermatids (a or b). The wild-type mature spermatid is released into seminiferous lumen as normal polar sperm (A or B with acrosomal pole and flagellar pole) after shedding its extra cytoplasmic components including manchette into a cytoplasmic droplet. Hrb−/− primary spermatocytes (right) show several anomalies (shaded rectangles only) during its journey to sperm formation. Supernumerary centrioles results from one or more of these three steps: (i) through centrosome overduplication during meiosis, (ii) by meiotic division deficiency (centrosomes acquisition), or (iii) by spermatids fusion (centrosomes accumulation). As a result, spermatids are produced with normal or supernumerary centrioles and, with mononucleus or multinuclei. Lack of acrosome biogenesis in Hrb−/− spermatids causes: (i) complete dislocation of manchette from the nuclear region which prevents the nucleus from elongating, and differentiates into mature spermatid with round nucleus (c), (ii) tangential location of manchette on nucleus: the manchette dislocates from its original location from nuclear region and contacts the round nuclear surface tangentially which becomes flat at the manchette–nuclear contact area and differentiates into mature spermatid with aberrant-shaped nucleus (e), and (iii) manchette invagination in nucleus causes the displacement of implantation fossa on nuclear membrane which, in turn, deviates the nucleus–centrioles–axoneme axis, thus causing a loss of sperm cell polarity and altering the direction of flagellar formation, resulting in intracellular flagellar coiling in many mature spermatids (f). Hrb−/− mature spermatids (c–f), thus formed, differentiate into non-coiled abnormal sperm with partial deviations in cell polarity (C–E) or intracellular coiled abnormal sperm with complete loss of polarity (F). Note that our interpretation on the formation of multiflagella from overduplicated centrioles within mononucleated spermatids (see spermatid ‘d’) is based on centrin detection only; further studies may be needed for additional demonstration.
mouse have been shown to affect spermatogenesis process. Male fertility is impaired in mgcl-1-null mice. Lack of nuclear lamina organization leads to abnormal acrosome structure and gigantic sperm with multiple heads, flagella and nuclei embedded with the manchette (Kimura et al., 2003). In Hrb<sup>−/−</sup> mice, sperm show all those defects which are present in familial male infertility cases of oligoasthenoteratozoospermia syndrome.

Multiflagellated sperm show partial symplast in Hrb<sup>−/−</sup> mice. In a familial asthenozoospermia and teratospermia showing multi-tails, all the tails of a sperm show a common plasma membrane until the midpiece where they separate into individual tails (Nistal et al., 1977). Formation of symplasts has been shown in sys/sys mutants, in which intercellular bridges that connect round spermatids widens (Russell et al., 1991a).

In conclusion, the aberrations in Hrb<sup>−/−</sup> mouse spermatids and sperm are reminiscent of human familial male infertility with oligoasthenoteratozoospermia syndrome. The Hrb-deficient mouse may be useful in understanding familial oligoasthenoteratozoospermia syndrome.

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### References


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Spermatogenic defects in the Hrb-deficient mouse