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外层致密纤维在Akap4基因缺陷致小鼠精子尾部多种形态异常中的作用

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[摘要] 目的：探讨外层致密纤维(outer dense fobres，ODF)在Akap4基因缺陷引起小鼠精子尾部多种形态异常中的作用。**方法：**通过基因编辑技术构建Akap4基因缺陷小鼠模型。实验分为2组：成年Akap4基因缺陷雄鼠为实验组(n=7)，成年野生型雄鼠为对照组(n=7)，比较2组小鼠的体重和睾丸重量；采用计算机辅助精液分析(computer aided sperm analysis，CASA)检测精子活动力，改良巴氏染色(modified pap staining)检测精子形态学，扫描及透射电镜观察精子超微结构，免疫荧光检测精子尾部蛋白表达及定位，睾丸切片HE及PAS染色检测睾丸生精功能，透射电镜观察曲精细管超微结构。**结果：** Akap4基因缺陷小鼠精子总活动力为8.81%，明显低于野生型小鼠(P<0.01)，且无正常形态精子，尾部缩短与尾部卷曲比例达91.18%，明显高于野生型小鼠(P<0.01)，睾丸重量、睾丸生精功能、精子数量2组比较差异无统计学意义(P>0.01)； Akap4基因缺陷精子的纤维鞘纵柱缺失，横肋柱结构部分残留，主段的3和8号ODF排列紊乱，与ODF2蛋白定位结果相符；睾丸中精子纤维鞘发育障碍，无正常纤维鞘结构形成，但未见异常膨大的精子尾部。**结论：**Akap4基因缺陷使纤维鞘横肋发育不良，纵肋的缺失引起3和8号ODF分离，“9+2”微管结构紊乱，使主段管腔异常膨大，导致小鼠精子尾部多种形态异常。

[关键词] 外层致密纤维；精子尾部多种形态异常； Akap4； 雄性不育； 纤维鞘

Role of outer dense fiber in multiple morphological abnormalities of the sperm flagella in Akap4 gene defect mice

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ABSTRACT

Objective: To determine the role of outer dense fiber (ODF) in multiple morphological abnormalities of the sperm flagella in Akap4 gene defect mice.

Methods: Akap4 knock-out (KO) mouse model was established by using gene editing technology. Akap4-KO male mice were identified by genotype. Seven sexually mature male Akap4-KO mice

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served as an experimental group, and 7 sexually mature wild-type (WT) male mice served as a control group. The changes in body weight and testicular weight were measured. Computer aided sperm analysis (CASA) was used to detect sperm motility. Sperm morphology was detected by modified Periodic Acid-Schif (PAS) staining. The ultra-structure of sperm was observed under the scanning and transmission electron microscope. Sperm flagella associated protein expression and localization were detected by immunofluorescence. Spermatogenesis function of testis was evaluated by HE and PAS staining. Ultra-structure of seminiferous tubules was observed under the transmission electron microscope.

Results: Akap4-KO mice had no natural fertility. The sperm motility of Akap4-KO male mice was lower than that of WT male mice (8.81% vs 46.02%, $P<0.01$). In Akap4-KO male mice the percentage of sperm, with shortened tail and coiled tail was 91.18% which was higher than that of WT male mice ($P<0.01$). There was no statistically significance in the testicular weight, spermatogensis function, and sperm count between the 2 groups ($P>0.01$). The longitudinal column of fibrous sheath in Akap4-KO male mice was absent, and the residues of transverse rib remained, which was consistent with the immunofluorescence localization of AKAP3 protein. No. 3 and No. 8 ODF in the principal piece were disordered, which was in consistent with ectopic localization of ODF2 protein.

Conclusion: Multiple morphological abnormalities of the sperm flagella in mice are resulted from disorder of "9+2" microtubules and the abnormally expanded lumen at the proximal of the principal piece via causing dysplasia of the transverse rib due to Akap4 gene defect, and separation of the ODF of No. 3 and No. 8 via loss of longitudinal column.

KEY WORDS outer dense fibre; multiple morphological abnormalities of the sperm flagella; Akap4; male infertility; fibrous sheath

进入现代社会以来，由于人们生活节奏加快、环境污染等因素的影响，人类的精子质量有下降趋势[1]。精子受精需经历生成、运输与存储、获能、释放、与卵子结合等过程，因此具备一定活动力的精子是受精过程的关键。精子通过尾部鞭毛等一系列附属结构的作用实现尾部鞭毛的摆动，主要结构包括线粒体鞘(mitochondrial sheath，MS)、纤维鞘(fibrous sheath，FS)、外层致密纤维(outer dense fiber，ODF)、"9+2"微管结构。精子尾部的附属结构之间的协调作用非常复杂，精子多种形态异常(multiple morphological abnormalities of the sperm flagella，MMAF)是造成大部分男性不育的主要原因[2]。MMAF的精子几乎都伴有精子尾部附属结构的紊乱，附属结构任何缺陷都会导致精子形态异常，表现为短的、卷曲的、缺失的和/或不规则的鞭毛，最终导致精子尾部运动异常[3]，因此需要对男性异常精子尾部形态进行深入的研究。

ODF是哺乳动物精子尾部特有的细胞骨架结构，它们由9个纤维组成。ODF在颈部与副中心体紧密接触，与9个二联微管偶联并向后延伸至主段，并且长度不一。人类ODF由10种主要蛋白质和至少15种次要蛋白质组成，主要蛋白包括ODF1，ODF2，ODF3等[4-9]。ODF2是中心体支架的组成部分，并且与母中心粒的附属结构相互作用[10-11]；免疫共沉淀实验证实了ODF2与微管蛋白的相互作用[12]；然而在精子尾部FS与ODF的相互作用关系仍缺乏相关研究。

A型激酶锚定蛋白4(AKAP4)是FS成分中最丰富的蛋白质，定位于FS的横肋柱及纵柱，参与一系列的能量代谢过程，为精子尾部鞭毛的摆动提供动力[13]。AKAP4基因是唯一位于X染色体上的基因[14]，AKAP4基因缺陷会导致男性精子FS结构缺陷，导致男性不育[15]。酵母双杂交实验证实了AKAP4与AKAP3蛋白有物理结合作用[5]。免疫共沉淀结果证实敲除QRICH2基因的小鼠睾丸AKAP3与QRICH2蛋白存在相互作用，并且AKAP3与ODF2蛋白的表达明显下调[16]。因此，本研究选择形成FS最活跃的Akap4基因，构建缺陷小鼠模型，旨在探讨FS对ODF在精子尾部的作用，阐述精子尾部附属结构的相互关系，为MMAF的研究提供新的思路，为临床诊断及治疗男性不育提供新的方向。

1 材料与方法

1.1 动物

SPF级雄性性成熟C57BL/6J小鼠(10~12周龄)购自上海南方模式生物有限公司。

1.2 仪器和试剂

透射电子显微镜(H-7500)、扫描电子显微镜(S-3000N)均产自日本日立公司；激光共聚焦显微镜(SP-8)产自德国莱卡公司；计算机辅助精液分析(Computer Aided Sperm Analyse，CASA)系统产自日本Olympus公司；凝胶电泳仪、PCR仪产自美国Bio-Rad公司；DNA提取试剂盒购自德国Qiagen公司；ODF2和AKAP3抗体均购自美国Proteintech公司；AKAP4定制于美国Genscript公司；α/β Tubulin抗体购自英国Abcam公司；Alexa Fluor 488兔抗鼠二抗和Alexa Fluor 568羊抗兔二抗购自美国Invitrogen公司；DAPI，BSA，Triton-100及BSA粉剂购自美国Sigma公司；苏木精-伊红染色(hematoxylin-eosin staining，HE)和PAS染色(Periodic Acid-Schiff stain)试剂盒、抗荧光淬灭剂购自上海碧云天生物技术有限公司；25%的戊二醛溶液购自日本Nacalai公司。

1.3 方法

1.3.1 Akap4基因缺陷小鼠的构建

根据靶序列Akap4信息设计针对X染色体该基因位点的guide-RNA，在外显子4和5及外显子6和7之间的非编码区设计敲除(knock-out，KO) 位点(图1A)。经活性检测后，将具有活性的guide-RNA和Cas9体外转录成RNA。通过显微注射将guide-RNA，Cas9 mRNA注射到受精卵中，引导Cas9对靶序列进行敲除；基因编辑技术敲除Akap4基因的5，6号外显子，使移码突变，导致蛋白功能缺失(图1A)；从后代中筛选获得阳性雄鼠和杂合雌鼠，最后挑选性成熟杂合雌鼠进行扩大繁殖。

1.3.2 Akap4基因缺陷雄性小鼠基因型鉴定

剪取待鉴定的小鼠尾(约4 mm)，置于1.5 mm离心管中，按Qiagen DNA 提取试剂盒说明书提取DNA，将提取的DNA模板用P1/P2，P3/P4引物对进行PCR反应。PCR引物如下：P1引物，5'-CCAGATCAG-TGATCCTAACTAA-3'；P2引物，5'-GACCTCAGTACCTAGACCCTA-3'，P3引物，5'-TTACATATACATACATATAATAGC-3'；P4引物，5'-GGCATACTTCTGGAGATCATTGAG-3'。对于野生型(wild-type，WT)小鼠，P1/P2引物对能产生4.4 kb产物，P3/P4引物对能产生500 bp产物；对于纯合突变雄性小鼠，P1/P2引物对能产生1.1 kb产物，P3/P4引物无产物。

1.3.3 小鼠精子活动力检测

用颈椎脱臼法处死小鼠，剪除腹部毛发，剪开皮肤、腹腔，分离并游离附睾，摘取双侧附睾并放入37 ℃盛有约1 mL 0.3% BSA的spermrinse精子培养液的平皿中，钝性剥离附睾尾，于37 ℃孵育箱中孵育5 min，吸取上清液，制得精子悬液。吸取10 μL精子悬液置于MAKLER精子计数板，在CASA系统下测量小鼠精子活力。

1.3.4 改良巴氏染色法检测精子形态

将精子涂片风干后，立即经95%乙醇固定至少15 min后进行染色。经梯度乙醇处理后，以苏木精染3 min，待流水回蓝后用梯度乙醇处理，再以橘黄G染色液染1~3 min，95%乙醇洗2次，持续10~20 s；EA-50染色2~5 min，95%乙醇洗2次，持续10~20 s；无水乙醇洗2次，持续15 s；二甲苯透明，封片胶封固，干燥后于显微镜下观察。

1.3.5 免疫荧光检测

以新配制的4%多聚甲醛于室温固定精子涂片10 min，PBS缓冲液漂洗15 min，用0.5% Triton X-100于室温穿孔15 min。再用PBS缓冲液漂洗15 min，1% BSA室温封闭30 min。加入1% BSA稀释的一抗，于湿盒中4 ℃孵育过夜。过夜后用PBS缓冲液漂洗15 min，加入1% BSA稀释的二抗，于37 ℃孵育1 h。以PBS缓冲液漂洗15 min，5 μg/mL DAPI染色5~10 min，PBS缓冲液漂洗15 min。用抗淬灭剂封片，在激光共聚焦显微镜下拍照。

1.3.6 HE和PAS染色

用改良的Davidson固定液固定睾丸，于4 ℃过夜。在脱水、透明、浸蜡、包埋后切片，采用HE和PAS染色，用甘油封片后，于显微镜下观察。

1.3.7 电镜包埋块的制作

将附睾尾精子离心成团后去上清，加入2.5%电镜专用戊二醛500 μL固定，切勿吹悬，置室温1 h。以PBS漂洗去除戊二醛，切割已固定精子团，经过锇酸固定、脱水漂洗、渗透、包埋、超薄切片后使用3%醋酸铀-枸橼酸铅进行双染色，用透射电镜观察，拍片。

1.3.8 扫描电镜样品制备

吸取适量的精子悬液于EP管中，并滴加适量2.5%的戊二醛溶液固定，滴于金属托盘上，于4 ℃过夜。经锇酸固定、脱水漂洗、纯醋酸异戊酯处理样品60 min，临界点干燥、镀金并离子溅射仪镀金、镀膜后，于扫描电镜下观察。

1.4 统计学处理

实验所得数据采用统计软件GraphPad Prism 8.0进行统计学分析及作图，计量资料的比较采用*t*检验，

结果采用均数±标准差($\bar{x}\pm s$)表示，以P<0.01为差异有统计学意义。

2 结 果

2.1 Akap4基因缺陷小鼠精子活力

提取敲除小鼠的DNA，利用PCR技术对小鼠进行基因型鉴定(图1B)。与WT小鼠对比，Akap4-KO小鼠精子活动力低下(图1C)。WT与Akap4-KO小鼠精子比较，在A级快速前向运动、B级慢速前向运动、C级非前向运动、D级极慢或不动的比例方面，差异具有统计学意义(P<0.01，图1D)，在曲线速度、直线速度、平均速度的比较中差异亦具有统计学意义(P<0.01)，在睾丸重量、精子总量、线性指数、直线指数、振动指数的比较中差异无统计学意义(P>0.01，表1)。

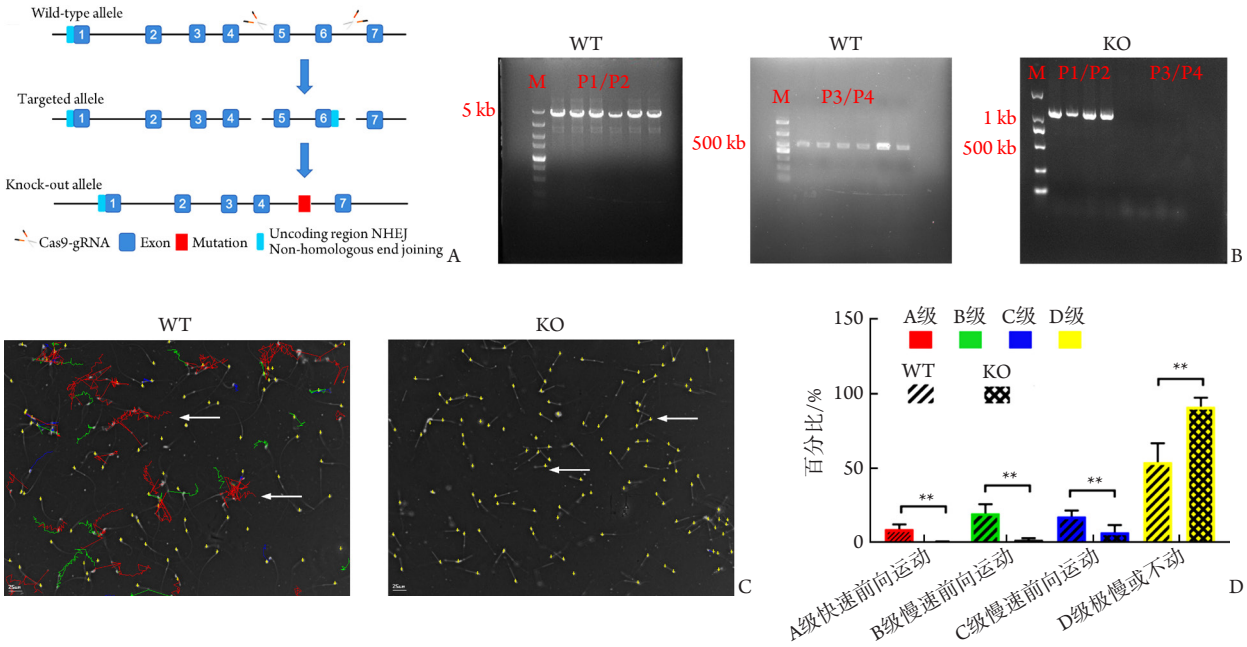

图1 Akap4基因缺失使小鼠精子活动力低下

Figure 1 Deletion of the Akap4 gene causes abnormal sperm motility

A: Schematic illustration of the targeting strategy for generating Akap4-knock-out (KO) mice. The frameshift mutation was generated by deleting exon 1 and exon 2 of Akap4 gene. B: Genotypes of wild-type (WT) mice and Akap4-KO male mice were carried out by PCR with P1/P2 and P3/P4 primers which generated 1.1 kb sequences in Akap4-KO male mice. C: Morphology and motility by CASA. Compared with WT mice, sperms in Akap4-KO mice were almost at D-level motility (white arrow). Red path represents A-level motility sperm, the green path represents B-level motility sperm, the blue path represents C-level motility sperm, and the yellow path represents D-level motility sperm. D: Different motility rates of WT and Akap4-KO sperm. M: DNA Marker, WT vs KO. **P<0.01, n=7

2.2 小鼠精子形态学分析

与WT小鼠对比，Akap4-KO小鼠精子尾部缩短、卷曲，主段结构紊乱，微管从精子尾部脱离(图2A)。Akap4-KO小鼠无正常精子，尾部缩短与卷曲的比例达到91.18%(P<0.01，图2B)。二者的精子尾部缺失和成角的比例差异无统计学意义(P>0.01)。与WT小鼠精子扫描电镜结果比较，Akap4-KO小鼠精子尾部主段缩短、膨大、无正常FS结构(图2C)；Akap4-KO小鼠精子主段FS结构紊乱，但仍可见一些FS结构残留(白色箭头)，膨大的主段内部充满残余细胞质(图2D)。Akap4-KO小鼠精子无AKAP4蛋白表达(图2E)。

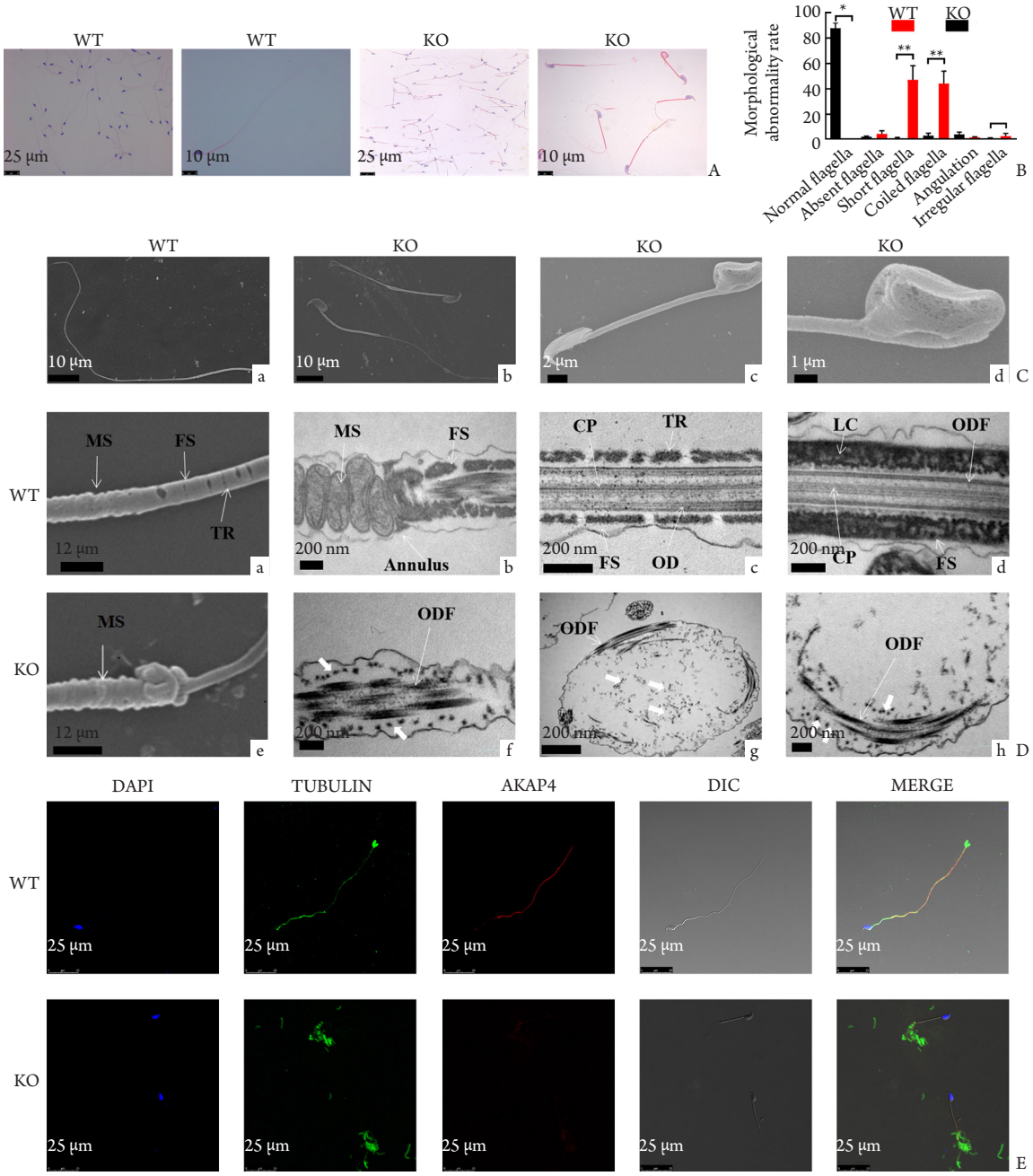

图2　Akap4基因缺失使小鼠尾部异常

Figure 2　Akap4 deletion leads to abnormal sperm flagella in male mice

A: Compared with WT male mice sperm under the light microscope, Akap4-KO mice shows MMAF, such as absent and short flagella, flagella of angulation, and irregular caliber. B: Morphological abnormality rate of Akap4-KO sperm. Most sperms in Akap4-KO mice present with short and coiled flagella. $^{**}P<0.01$, $n=7$. C: Most sperms in Akap4-KO mice present with short or coiled flagella under the scanning electron microscope (SEM). a: Spermatozoon with a normal flagellum under SEM. b and c: Most sperms in KO mice with short, or coiled flagella under SEM. d: Local enlargement of figure c. D: Ultra-structures of sperm in WT and Akap4-KO mice. Abnormal principal piece (PP) of sperm flagella in Akap4-KO mice under the SEM and transmission electron microscope (TEM). a and e: Fibrous sheath dysplasia in the connection of middle piece and PP from WT and Akap4-KO mice under the SEM. b: Normal annulus by longitudinal section of WT mice in the connection of middle piece and PP under the TEM. c and d: Transverse rib (TR) and longitudinal column (LC) by longitudinal section in WT mice under the TEM. f and g: Disordered distribution of ODF and microtubules in KO mice under the TEM. LC disappeared abnormally with the ectopic distribution of residual TR (white arrow) and coiled abnormally ODF in Akap4-KO mice. h: Local enlargement of figure g. ODF is circled by the ectopic distribution of residual TR (solid white arrow) in Akap4-KO mice. E: Absent AKAP4 protein in Akap4-KO mice by immunofluorescence. DAPI: Head (Blue); Tubulin: Microtubules (Green); AKAP4: Principal piece (Red)

表1 小鼠精子活动力分析(n=7)

Table 1 Analysis of sperm motility in WT and KO male mice by CASA (n=7)

组别	体重/g	左侧睾丸/g	右侧睾丸/g	精子总量/(10^6·mL^{-1})	总活动力/%
WT*	29.6(27.9~32.3)	0.100(0.083~0.111)	0.103(0.095~0.111)	57.1(41.1~87.4)	46.0(25.5~62.42)
Akap4-KO*	29.9(26.6~36.3)	0.098(0.088~0.108)	0.099(0.084~0.113)	41.3(22.1~69.1)	8.8(3.63~19.35)††

组别	曲线速度/(μm·s^{-1})	直线速度/(μm·s^{-1})	速度/(μm·s^{-1})	线性指数/%	直线指数/%	振动指数/%
WT*	130.1(114.9-147.7)	42.4(39.6~47.2)	61.4(57.0~63.5)	32.8(28.3~36.7)	69.0(65.7~74.3)	47.4(42.8~49.9)
Akap4-KO*	77.0(59.6~107.7)††	21.1(15.7~37.2)††	32.7(24.8~50.2)††	27.1(21.5~34.5)	63.7(56.8~74.0)	42.3(37.8~47.0)

*括号内为最小值与最大值。与WT比较，††P<0.01

2.3 小鼠睾丸超微结构分析

WT小鼠与Akap4-KO小鼠曲精细管第VIII时期的HE染色结果提示两者生精功能无明显差异(图3A)，PAS染色也未见明显差异(图3B)。与WT小鼠睾丸比较，Akap4-KO小鼠睾丸精子未见FS发育(图3C)，而ODF无明显异常。从中段到主段末端的精子横截面观察精子尾部附属结构(图4A)，可见正常小鼠精子中段MS与主段FS环绕着与ODF偶联的“9+2”微管结构，FS由近端到远端逐渐变圆，可见9个ODF长度不等，从精子尾部近端往末端延伸的过程中，在主段近端3和8号ODF与纵柱融合消失(图4Ab~c)，在主段远端2，4，7，9号ODF逐渐消失，1，5，6号ODF一直延续到主段末端才最终消失。Akap4-KO小鼠精子主段全长FS结构缺陷，主段近端3和8号ODF脱离“9+2”微管结构，可见残留部分FS结构(白色箭头)。Akap4-KO小鼠精子表达横肋柱成分AKAP3蛋白(图4B)；对ODF2蛋白进行定位，证实3和8号ODF在Akap4-KO小鼠精子主段脱离“9+2”微管结构(图4C)。

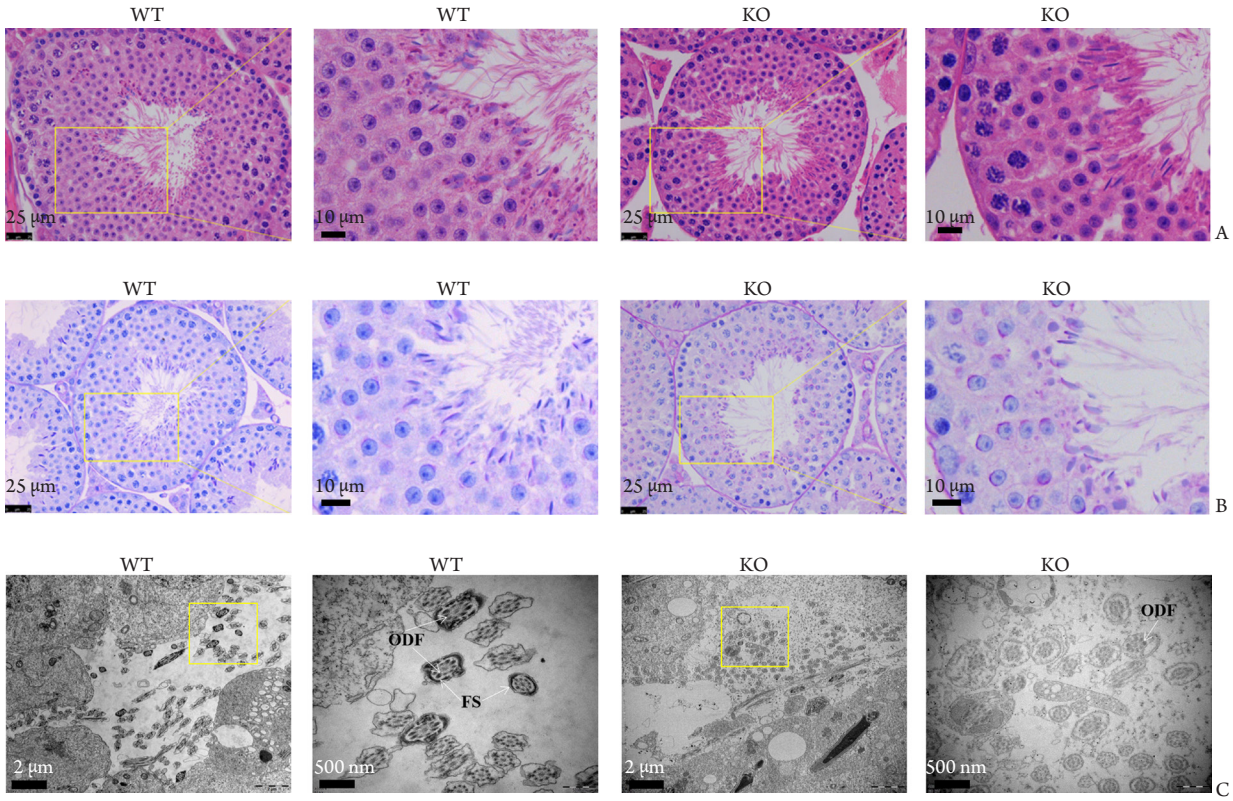

图3 Akap4-KO雄性小鼠睾丸超微结构异常

Figure 3 Ultra-structures of testis in Akap4-KO mice

A: VIII stage of seminiferous tubule in WT and KO mice shows no significant morphological abnormalities by HE staining. B: VIII stage of seminiferous tubule in WT and KO mice shows no significant morphological abnormalities by PAS staining. C: Abnormal development of PP in KO mice under the TEM

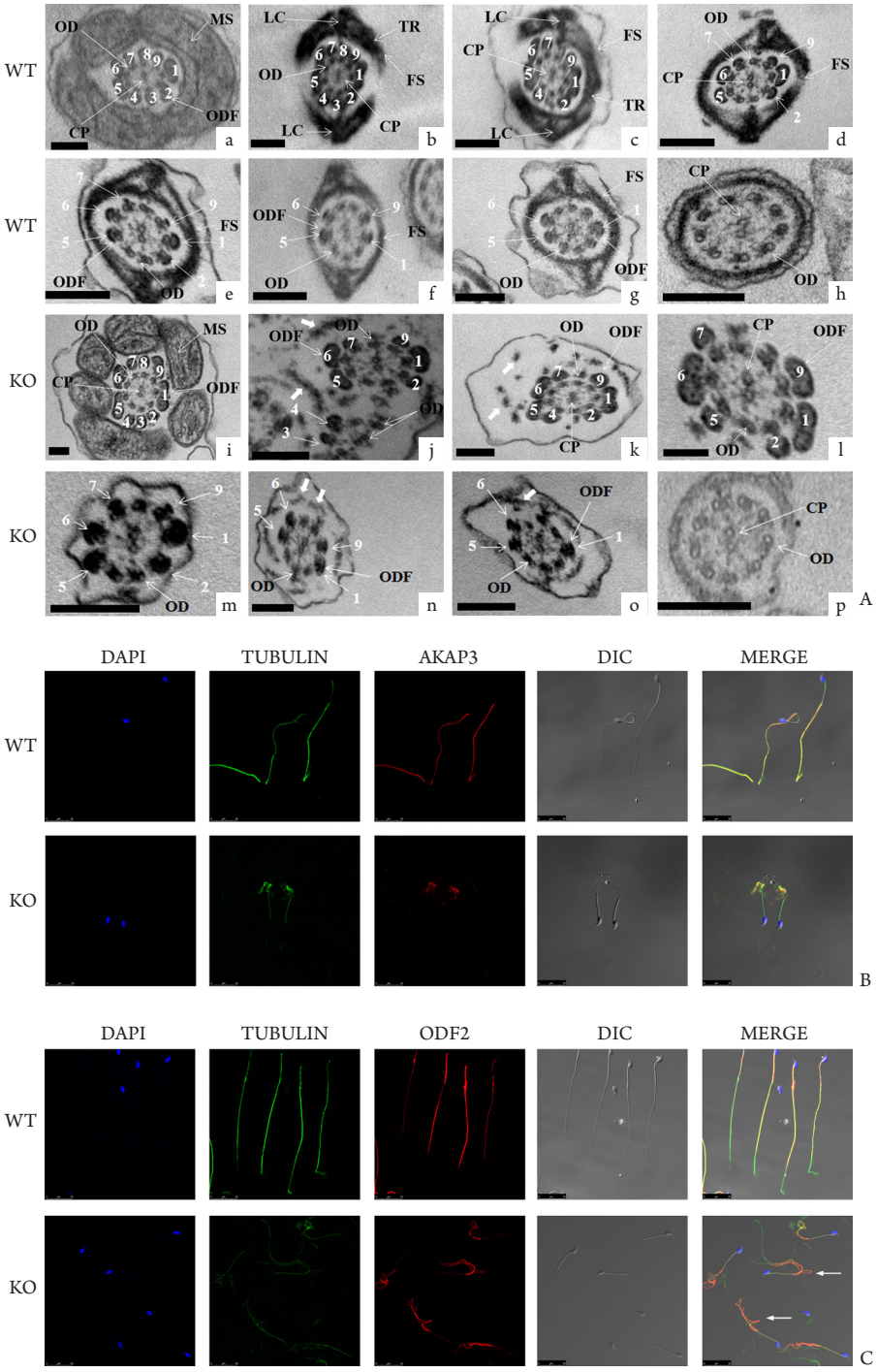

图4　Akap4-KO雄性小鼠精子超微结构异常

Figure 4　Ultra-structures of sperm in Akap4-KO mice

A: Cross sections of the sperm in WT and Akap4-KO mice show no significant development abnormalities of ODF and central microtube (CP), but the dysplasia of fibrous sheath (FS) is seen in Akap4-KO mice under the TEM. a–h: Normal distribution of ODF in WT mice. a and i: Mitochondrial sheath (MS) of middle piece shows no significant ultra-structural abnormalities in WT and KO mice. j: Longitudinal column from KO mice disappeared in PP leads to the disordered distribution of No. 3 and 8 ODF and the instability of outer double microtubules. j, n, and o: Transverse ribs disordered distribution along the PP (solid white arrow). a–p: Bar=200 nm. B–C: Existence of AKAP3 and the disordered distribution (solid white arrow) of ODF2 in Akap4-KO mice by immunofluorescence. DAPI: Head (Blue); Tubulin: Microtubules (Green); AKAP3: Principal piece (Red); ODF2: Outer dense fiber (Red). Bar=25 μm

3 讨 论

目前已知导致人类精子MMAF的基因有AKAP4，DNAH1，CFAP43，CFAP44，CCDC39，CFAP69，QRICH2，WDR66，FSIP2，AMRC2，TTC21A[16-25]，这些基因缺陷影响了FS及微管的发育，但未见有ODF相关基因突变导致人类致病的报道。据最新研究[26]结果，在Odf2杂合突变型小鼠模型中，由于Odf2基因杂合缺失引起的单倍剂量不足效应，导致雄性Odf2杂合突变体小鼠不育，产生出头颈分离不活动但活着的精子。研究人员分析了55份人类弱精症精子样本，发现都存在不同程度的ODF相关蛋白表达水平下调的现象[27]，以上研究都提示ODF在精子尾部具有重要作用。

本研究通过透射电镜检测Akap4基因缺失雄性小鼠成熟精子尾部超微结构，发现ODF发育无明显异常，但3，8号ODF发生紊乱，而3，8号之外的ODF并未受到明显影响，为了更进一步证实该结果，通过ODF2蛋白的荧光定位证实了3，8号ODF的异常。笔者认为AKAP4蛋白的缺失不会影响ODF发育，但会影响3，8号ODF与FS的锚定。通过分析小鼠精子活力和形态学证实Akap4基因缺陷导致小鼠精子活动力低下和MMAF。AKAP3蛋白定位于FS的横肋[13]，笔者在透射电镜下发现Akap4基因缺陷小鼠精子尾部主段存在电子致密物影，进一步通过免疫荧光技术证实了AKAP3蛋白的表达；但与WT小鼠对比，荧光信号强度偏弱；CASA结果显示Akap4基因缺陷精子存在一部分活动力，提示Akap4基因缺陷精子仍可在附睾尾获能。在睾丸透射电镜结果中并未见到主段膨大的精子，笔者推测Akap4基因缺陷精子尾部主段在睾丸早期发育过程中并未膨大，精子在附睾尾游出过程中，由于3，8号ODF脱离“9+2”微管结构，才导致主段出现膨大，最终呈现MMAF。笔者认为Akap4基因的缺失不会影响FS相关基因的表达，但会下调FS相关基因的表达量，精子尾部附属结构的发育涉及不同的基因调控网络，而这些基因调控网络之间也存在相互作用，共同维护精子的结构与功能；并且推测ODF2，AKAP3蛋白缺陷能够导致小鼠MMAF，甚至可以作为人类MMAF的潜在基因[28-29]，其具体作用机制仍需要进一步研究。

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