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·*Original Article*·

Intramanchette transport during primate spermiogenesis: expression of dynein, myosin Va, motor recruiter myosin Va, VIIa-Rab27a/b interacting protein, and Rab27b in the manchette during human and monkey spermiogenesis

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Abstract

Aim: To show whether molecular motor dynein on a microtubule track, molecular motor myosin Va, motor recruiter myosin Va, VIIa-Rab27a/b interacting protein (MyRIP), and vesicle receptor Rab27b on an F-actin track were present during human and monkey spermiogenesis involving intramanchette transport (IMT). **Methods:** Spermiogenic cells were obtained from three men with obstructive azoospermia and normal adult cynomolgus monkey (*Macaca fascicularis*). Immunocytochemical detection and reverse transcription-polymerase chain reaction (RT-PCR) analysis of the proteins were carried out. Samples were analyzed by light microscope. **Results:** Using RT-PCR, we found that dynein, myosin Va, MyRIP and Rab27b were expressed in monkey testis. These proteins were localized to the manchette, as shown by immunofluorescence, particularly during human and monkey spermiogenesis. **Conclusion:** We speculate that during primate spermiogenesis, those proteins that compose microtubule-based and actin-based vesicle transport systems are actually present in the manchette and might possibly be involved in intramanchette transport. *(Asian J Androl 2008 Jul; 10: 561–568)*

Keywords: intramanchette transport; manchette; spermiogenesis

1 Introduction

Teratozoospermia and maturation arrest during spermiogenesis are two forms of male infertility for which the causes remain unknown and there is currently no cure. Understanding the developmental mechanisms by which round spermatids evolve into elongated spermatids during normal spermiogenesis will help to treat such male infertility patients.

Immature round spermatids undergo dynamic morphological changes, acrosome formation, nuclear condensation, and elongation in the sperm head, and sperm tail

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formation during spermiogenesis. The manchette, a bundle of microtubules, is thought to play a role in those changes [1, 2]. The manchette, which is transiently formed in the distolateral region of the cytoplasm, radiates from the perinuclear ring of human Sb2 spermatids and monkey step 8 spermatids. The manchette appears at the time when the spermatid nucleus initiates elongation and disappears when elongation and condensation approach completion. The appearance and disappearance of the manchette are likely related to the dynamic morphological changes in the spermatids during spermiogenesis.

Sperm cells obtained from the *azh* mutant mouse have an abnormal head shape as a result of an abnormal shaping of the nucleus [3]. The microtubules of the manchette in this mouse display ectopic positioning, perhaps related to the abnormal head shape. Spermatids and sperm from the *azh* mutant mouse also have a lasso-like coiled tail and a high frequency of head dislocation and decapitation [4, 5].

The intramanchette transport (IMT) mechanism has been proposed to deliver molecules to the centrosome and the developing sperm tail during spermiogenesis [6–10]. The Golgi generates two types of vesicles, proacrosomal and non-acrosomal. Proacrosomal vesicles are transported to the acroplaxome, where they fuse and organize the acrosome [11]. Non-acrosomal vesicles are transported by the IMT mechanism. IMT might have two transport systems, microtubule-based and actin-based vesicle transport systems [12]. The former is analogous to intraflagellar transport, relying on the microtubule-based motor proteins kinesin/dynein and microtubules for transporting cargo proteins [13]. The latter uses the molecular motor proteins myosin Va and VIIa [14, 15], the motor recruiter myosin Va, VIIa-Rab27a/b interacting protein (MyRIP) [16, 17], and the vesicle receptor Rab27a/b [18, 19]. These proteins have primarily been studied in melanosome transport. A number of models have attempted to explain why two transport systems are necessary in developing spermatids and how they interact with each other. Some researchers have postulated that fast and long-range transport of molecules is mediated by the microtubule-based transport system, whereas short-range local transport is mediated by the actin-based system [20]. Intramanchette cargos might switch from a microtubule track to an actin track by exchanging a microtubule-based molecular motor, such as kinesin or dynein, for the actin-based molecular motor myosins Va/VIIa [21]. This process might involve the motor recruiter MyRIP/melanophilin to determine a cargo's final destination, and the vesicle receptor (Rab27a/b) might facilitate binding of a motor recruiter to enable microtubule-to-actin track switch of the cargo vesicle on the microtubule track.

The role of the molecular motors kinesin/dynein, as part of a microtubule-based vesicle transport system in IMT in primates, has not been investigated. Actin is present in the acroplaxome and along microtubule bundles of the manchette [21], and detected in immunoblotting of fractionated manchette [4, 22]. With the exception of the molecular motor myosin Va in rats [21], no evidence has shown that the molecular motors myosin Va/VIIa, the motor recruiter MyRIP, and the vesicle receptor Rab27a/b are involved in vesicular transport by way of F-actin (track) along microtubules of the manchette during spermiogenesis. In this report, we show that dynein, myosin Va, MyRIP, and Rab27b are localized to the manchette during primate spermiogenesis, speculating that these factors might possibly be involved in IMT.

2 Materials and methods

2.1 Collection of human spermatogenic cells

Sample collection and procedures were approved by the Ethics Committee of Tohoku University School of Medicine (Miyagi, Japan) and Suzuki Memorial Hospital (Miyagi, Japan). Informed consent was obtained from the subjects. Samples were obtained from three men with obstructive azoospermia in whom testicular sperm aspiration was carried out for diagnostic purposes. Normal spermatogenesis was confirmed in the specimen prior to use for these experiments. Evaluation of spermatogenic ability involved histologic examination of the testicular sperm aspiration sample; a Johnson score [23] of nine or greater was judged as normal spermatogenesis. The mean age of the patients was 36 years. The mean follicle-stimulating hormone (FSH) level in the patients was 2.8 mIU/mL.

2.2 Animals

The right testis and epididymis of a 17-year-old normal adult cynomolgus monkey (*Macaca fascicularis*) was obtained from the Tsukuba Primate Research Center, National Institute of Biomedical Innovation (Tsukuba, Japan). The animal, who weighed 5.8 kg, was fed fruits and a commercial monkey diet (type AS; Oriental Yeast,

Tokyo, Japan). All experiments were carried out according to the guidelines for animal experimentation of the National Institute of Biomedical Innovation.

2.3 Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from the testis of the monkey using an RNeasy mini kit (Qiagen, Tokyo, Japan). Total RNA (1 μg) was used as the template for first-strand cDNA synthesis using Superscript III reverse transcriptase (Gibco BRL, Eggenstein, Germany). One microliter of each cDNA was used as the template for PCR reactions with the following primers: dynein (GenBank accession number XM_001092103) forward (F), CCGTATTTGGGTCTATGA and reverse (R), TGAGCTCTAGGACACAAAGTT; myosin Va (GenBank accession number XM_001084476) F, AGGTGTTGAATCTGTATACTCC, and R, AGAGTCTTTCCTGTCTCGTA; MyRIP (GenBank accession number XM_001115628) F, CTCCAAGGCTCCTCAACAAAC, and R, TTGGGTCAAGGCACTGTCG; and Rab27b (GenBank accession number XM_001083017) F, GGGAACTGGCTGACAAAT, and R, CCACCATTGACAGTATCG (Nihon Gene Research Laboratories, Sendai, Japan). The reaction was cycled for 33 cycles, each of which consisted of denaturation at 95ºC for 30 s, annealing at 61ºC for MyRIP and 53ºC for dynein, Myosin Va, and Rab27b for 30 s, and extension at 72ºC for 30 s, followed by a 7-min extension at 72ºC.

2.4 Indirect immunofluorescence

We analyzed testes from obstructive azoospermic patients and a wild-type monkey. Isolated seminiferous tubules in modified human tubal fluid (HTF containing HEPES buffer; Irvine Scientific, Santa Ana, CA, USA) with 10% serum substitute supplement (Irvine Scientific, Santa Ana, CA, USA) were minced with two fine forceps. After filtering minced tissues through 70 μm mesh to remove tissue debris, cell suspensions were centrifuged for 5 min at 400 × *g*. The pellets were resuspended in an appropriate amount of modified HTF (approximately 2–3 mL) to achieve the proper cell density. After a second centrifugation, cell suspensions were allowed to stand at 37ºC for 30 min to allow the spermatogenic cells to recover. Spermatogenic cells were allowed to adhere to coverslips coated in poly-L-lysine (Sigma, St. Louis, MO, USA) and fixed with 2% formaldehyde in microtubule-stabilizing buffer (50 mmol/L piperazine-1,4-bis(2-ethanesulfonic acid [PIPES], 5 mmol/L O,O'-bis(2-aminoethyl)ethyleneglycol-N,N,N',N'-tetraacetic acid (EGTA), and 5 mmol/L $MgSO_4$) for 1 h [24]. After rinsing the coverslips in phosphate-buffered saline, cells were permeabilized for 1 h with 1% Triton X-100 in phosphate-buffered saline (Sigma, St. Louis, MO, USA). Nonspecific antibody binding was prevented by incubation for 1 h with normal goat serum at 37ºC. Cells were incubated with monoclonal antibodies against β-tubulin (T5293, diluted 1:100; Sigma), dynein (heavy chain) (D1667, diluted 1:50; Sigma), or polyclonal antibodies (sc9104, diluted 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) to detect microtubules, myosin Va (M4812, diluted 1 : 100; Sigma), MyRIP (ab10149, diluted 1:50; Abcam, Cambridge, MA, USA), or Rab27b (18973, diluted 1:100; IBL, Takasaki, Japan). Pre-immune mouse immunoglobulin (Ig)G1 antibody (diluted 1:20; Chemicon, Temecula, CA, USA) and rabbit IgG (diluted 1:100; Santa Cruz Biotechnology) were used for control experiments. Primary antibodies were detected with fluorescein-isothiocyanate-conjugated goat anti-mouse (Zymed Laboratories; San Francisco, CA, USA) and tetramethyl rhodamine isothiocyanate-conjugated antirabbit (Sigma) antibodies (both IgG, diluted 1:40). DNA was detected by labeling with Hoechst 33342 dye (Hoechst, Kumamoto, Japan). Coverslips were mounted in a drop of VectaShield mounting medium (Vector Laboratories, Burlingame, CA, USA).

2.5 Characteristics of spermatogenic cells and cell imaging

We compared the morphologic characteristics of fixed human spermiogenic cells with those of previously identified fixed cell types [25, 26] as described by Johnson *et al.* [27, 28]. We referred to descriptions of monkeys by Clermont and Leblond [29, 30] and Clermont [31] for this comparison. Coverslips were examined on a Leica DMRXA/HC epifluorescence microscope (Leica Microsystems, Heidelberg, Germany). Images were pseudocolored using Adobe Photoshop software (Adobe Systems, Mountain View, CA, USA) and printed on a color laser printer (Oki Microline 5300; Oki Data, Tokyo, Japan).

3 Results

3.1 RNA expression in monkey testis

RT-PCR was used to detect transcripts of the molecular motor myosin Va, the motor recruiter MyRIP,

and the vesicle receptor Rab27b in monkey testis. In addition to dynein, whose localization to sperm tails has been characterized, transcripts encoding myosin Va, MyRIP, and Rab27b were present in monkey testis (Figure 1). The RT-PCR results were not quantitative as co-amplification with a housekeeping gene was not included. To our knowledge, this result is the first report examining non-human primate testis, although Rab27b was detected in human testis [32] and myosin Va, MyRIP, and Rab27b had been previously seen in mouse testis [10, 21].

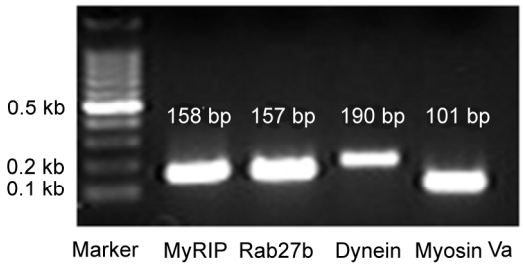

Figure 1. Gene expression of the molecular motor myosin Va, the motor recruiter MyRIP, and the vesicle receptor Rab27b and the molecular motor dynein in monkey testis were determined by RT-PCR. The numbers at the left indicate molecular marker size in kilobases (kb). The base pair (bp) size of each transcript is indicated.

3.2 Immunological localization of dynein on microtubule tracks in human and monkey spermatids

The immunolocalization of dynein was examined by immunofluorescence during human (Figure 2) and monkey (Figure 3) spermiogenesis. In elongating spermatids Sb2 and Sc (Figure 2A–C), and stage 8–10 spermatids (Figure 3A–C), the nuclei became elongated and condensed. The manchette, a bundle of microtubules that extends from the equatorial region of the nucleus toward the developing tail, began to materialize. More

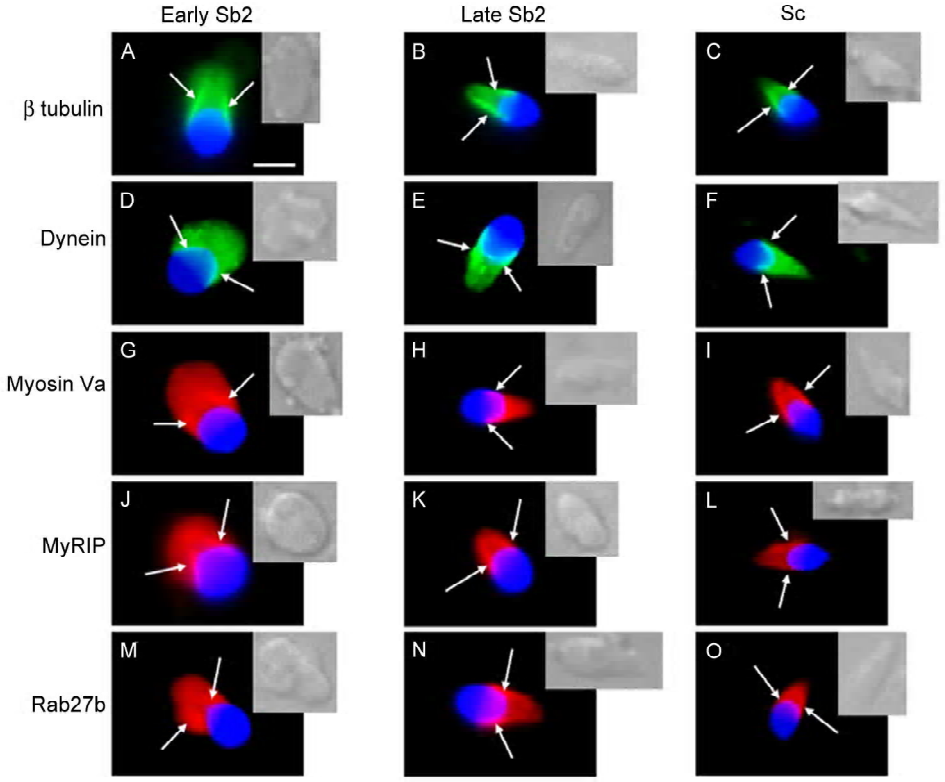

Figure 2. Immunolocalization of dynein, myosin Va, MyRIP, Rab27b and β tubulin in human spermatids with the corresponding phase contrast microsocopic images (insets). Green: β tubulin, dynein; Red: myosin Va, MyRIP, Rab27b; Blue: DNA. In elongating spermatids, Sb2 and Sc spermatids, the nuclei have become elongated; the manchette (A–C), a bundle of microtubules that extends from the equatorial region of the nucleus to the developing tail, is accompanied by strong dynein, myosin Va, MyRIP, and Rab27b staining (D–F, G–I, J–L, M–O, arrow). Scale bar = 10 μm.

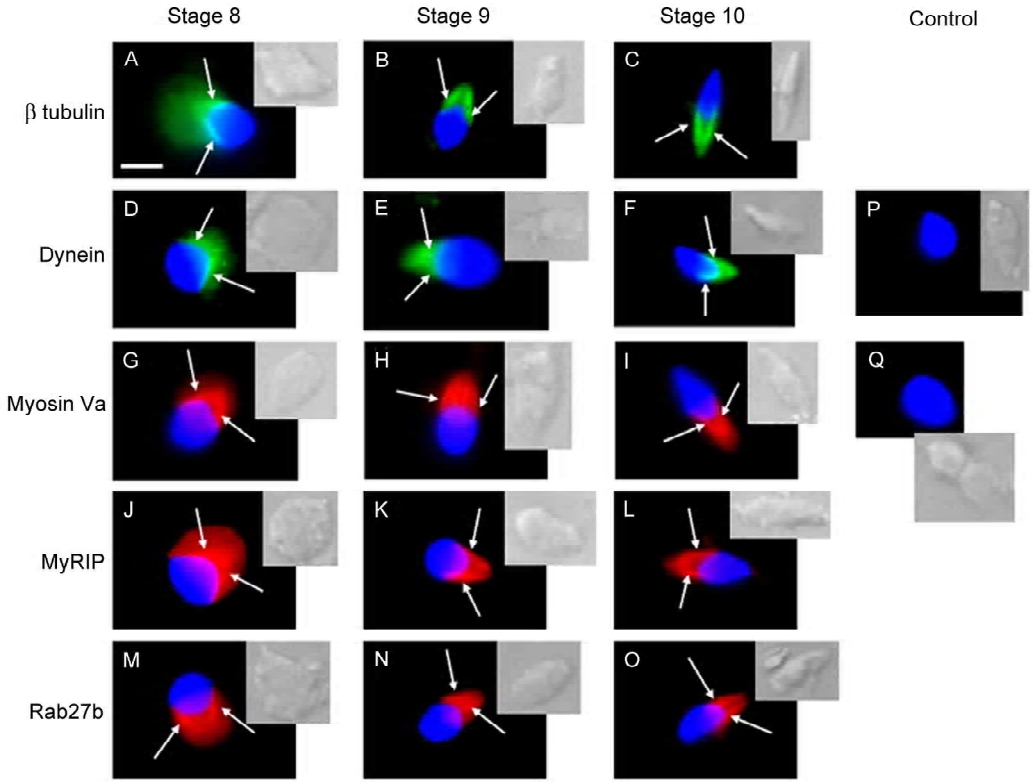

Figure 3. Immunolocalization of dynein, myosin Va, MyRIP, Rab27b and β tubulin in monkey spermatids with the corresponding phase contrast microsocopic images (insets). Green, β tubulin, dynein; Red, myosin Va, MyRIP, Rab27b; blue; DNA. In elongating spermatids; stage 8-10 spermatids, the nuclei have become elongated; the manchette (A–C), a bundle of microtubules that extends from the equatorial region of the nucleus to the developing tail, is accompanied by strong dynein, myosin Va, MyRIP, and Rab27b staining (D–F, G–I, J–L, M–O, arrow). Negative control (P, Q). Bar = 10 μm.

intense dynein immunoreactivity was localized to the manchette than cytoplasm (Figures 2D–F and 3D–F). When mouse IgG1 was applied as monoclonal primary antibody in place of antidynein antibodies, dynein could not be detected in the manchettes of stage 9 spermatids (Figure 3P). These results indicate that dynein colocalizes with microtubules that constitute the manchette during spermiogenesis.

3.3 Immunological localization of myosin Va, MyRIP, and Rab27b on F-actin tracks in human and monkey spermatids

In addition to dynein, the immunolocalization of myosin Va, MyRIP, and Rab27b were examined during human (Figure 2) and monkey (Figure 3) spermiogenesis. In elongating spermatids Sb2 and Sc (Figure 2A–C) and stage 8–10 spermatids (Figure 3A–C), nuclei become elongated and condensed. The manchette, a bundle of microtubules that extends from the equatorial region of the nucleus toward the developing tail, began to develop at this stage. Staining for myosin Va, MyRIP, and Rab27b was more intense in the manchette (Figures 2G–I, J–L, M–O and 3G–I, J–L, M–O) than that in the cytoplasm. When rabbit IgG was applied as primary antibody in place of antimyosin Va, antiMyRIP, and antiRab27b antibodies, no specific staining could be detected in the manchettes of stage 9 spermatids (Figure 3Q). We repeated each experiment at least three times.

4 Discussion

The timing of the appearance and disappearance of the microtubular manchette suggested a function in the dynamic morphological changes in spermatids throughout spermatogenesis. Therefore, the IMT mechanism, which contributes to vesicular transport events, is needed

for the dynamic morphological changes of spermatids. Understanding this process and identifying the molecular factors involved in IMT have been interesting targets for research. There are a number of reports examining IMT and these two vesicle transport systems in conditions other than spermiogenesis. Molecular motors on the microtubule track, such as kinesin [33, 34] and cytoplasmic dynein [35], are found in testis and in the manchettes of other species. The expression of kinesin-II was confirmed in rat spermatid tail [33]. Kinesin, a heterotetramer, consists of two heavy chains and two light chains (KLCs). The heavy chains contain a catalytic domain necessary for ATP hydrolysis and microtubule binding. KLCs might function in cargo binding or in the regulation of kinesin activity. Mice encode three KLC isoforms, KLC1, KLC2, and KLC3. KLC3, which is expressed in round and elongating spermatids, is observed in sperm tails, suggesting a specialized function in this location [34]. Cytoplasmic dynein is reported to be associated with manchette microtubules spermiogenesis in rat [35]. The signal for cytoplasmic dynein in rats corresponded well with the expression pattern of the manchette in step 7–10 spermatids in humans and monkeys. In intraflagellar transport, which uses the molecular motors kinesin and dynein and the same kind of microtubule-based vesicle transport system as IMT, simultaneous genetic knockout of the two kinesin-II motor subunits completely abrogated the formation of cilia in *Tetrahymena* [36]. Removal of the gene encoding one of the subunits of kinesin-II, *KIF3A*, by CreloxP mutagenesis from mouse photoreceptor cells resulted in extensive apoptotic death of photoreceptor cells [37]. Vesicles in these cells, such as those containing opsin, accumulated within the inner segment, suggesting that those materials could not be transported to the outer segment along the connecting cilium.

Myosin Va, the molecular motor on the F-actin track, is associated with the manchette and manchette-associated vesicles within rat spermatids [21]. To our knowledge, however, there is little additional evidence that actin-based vesicular transport participates in IMT. Actin-based vesicular transport has primarily been studied through melanosome transport. Rab GTPases regulate melanosome vesicle formation, docking, tethering, and fusion [18, 19]. Rab27a, a melanosomal membrane protein, recruits myosin Va to the melanosome surface through a rabphilin-like effector protein, melanophilin [15]. In melanosomes, myosin Va binds indirectly to Rab27a through Slac2-a/melanophilin, a synaptotagmin-like protein homolog lacking the C2 domain-a: [14]. Slac2-c, a homolog of Slac2-a, interacts with Rab27a/b and myosin Va/VIIa; this protein is highly expressed in the brain, lung, and testis [17]. MyRIP, which has structural similarities to Slac2-a/melanophilin, interacts with both Rab27a and myosin VIIa and is associated with melanosomes [16]. Griscelli syndrome, a human genetic disease, and the corresponding mouse model, *ashen* [38], result from a defect in the Rab27a gene [18]. Patients have partial albinism of hair and skin resulting from the failure of melanosome transport to keratinocytes.

Which cargo proteins are transported by IMT during spermiogenesis? There are several possibilities. 1) Cargo proteins might be transported to the developing sperm tail by IMT for tail formation. This idea is supported by a number of reports detailing that keratins, including *Sak57* [6], Odf1 [39, 40], and Odf2 [41], keratin-associated proteins, such as Spag4 [42] and Spag5 [43], the 26S proteasome [4, 7], *N*-arginine convertase [44], an RNA-binding protein [45], and type 4 cAMP-specific phosphodiesterase [46] are transiently stored in the manchette. *Sak57*, Odf1, Odf2, and the 26S proteasome are sorted to the outer dense fibers of the tail, whereas *N*-arginine convertase is sorted to the axoneme. 2) Cargo proteins necessary for spermatid nuclear condensation are transported by IMT. This idea is supported by a report describing the presence of Ran, a Ras-related GTPase, in the cytoplasm and nucleus of round spermatids and in the manchettes of elongating spermatids. Ran GTPase is thought to control the trafficking of nuclear proteins during the spermatid nuclear condensation [47]. The manchette might play a role in trimming the residual spermatid cytoplasm. By pulling the cytoplasm down to the distal side, residual cytoplasm can be discarded during spermiation [48]. Unnecessary materials in the cytoplasm could be transported to the distal side through the microtubule or F-actin tracks in IMT.

In conclusion, this study suggested that, during primate spermiogenesis, the manchette contains the molecular motor dynein on a microtubule track, and the molecular motor myosin Va, the motor recruiter MyRIP, and the vesicle receptor Rab27b on an F-actin track. We speculated that these factors that compose microtubule-based and actin-based vesicle transport systems might actually be involved in IMT. In particular, this is the first report regarding expression of these factors in the

manchette during primate spermiogenesis.

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