Pathol. Oncol. Res. (2010) 16:427–433
DOI 10.1007/s12253-009-9232-3

Immunohistological Profile of the Ras Homologous B Protein (RhoB) in Human Testes Showing Normal Spermatogenesis, Spermatogenic Arrest and Sertoli Cell Only Syndrome

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Received: 10 June 2009 / Accepted: 16 November 2009 / Published online: 8 December 2009

Abstract Ras homologous B protein (RhoB) belongs to the Ras homologous subfamily which consists of low molecular weight (21 kDa) GTP-binding proteins. Rho proteins are regulatory molecules associated with various kinases and as such they mediate changes in cell shape, contractility, motility and gene expression. To date, no data are available about the expression pattern of RhoB protein in the human testis showing normal and abnormal spermatogenesis. The present study addresses these issues. Human testicular biopsy specimens were obtained from patients suffering from post-testicular infertility (testis showing normal spermatogenesis, 10 cases) and testicular infertility (testis showing Sertoli cell only syndrome and spermatogenic arrest, 10 patients each). The expression of RhoB was examined using in situ immunofluorescent staining methods. In testes showing normal spermatogenesis, RhoB had a strong expression in the seminiferous epithelium (cytoplasm of Sertoli-cells, spermatogonia and spermatocytes) and in the interstitium (Leydig cells). RhoB expression was weak in the myofibroblasts and absent in the spermatids and sperms. In the testes showing abnormal spermatogenesis, RhoB expression was moderate in the seminiferous epithelium (cytoplasm of Sertoli cells, spermatogonia and spermatocytes) and was completely absent in the Leydig cells, myofibroblasts, spermatids and sperms. To the best of our knowledge, this study provides the first morphological indication that RhoB protein is expressed in human testis and its expression undergoes testicular infertility associated changes. These findings suggest the involvement of RhoB in the process of spermatogenesis in human and their possible therapeutic ramifications in testicular infertility are open for further investigations.

Keywords RhoB · Human testis · Fertility · Infertility · Ras

Abbreviations

CT	Connective tissue
RhoB	Ras homologous B protein
IR	Immunoreactivity
TSA	Tyramide signal amplification

Introduction

Ras homologous B protein (RhoB) protein represents a family of small GTP binding proteins that are implicated in many important cellular functions including cell proliferation, migration and cytoskeletal reorganization [1]. RhoB proteins are regulatory molecules that link surface receptors to the organization of the actin cytoskeleton and thus mediate changes in cell shape, contractility, motility and gene expression [2, 3]. Rho protein is activated by GTP binding and is inactivated by hydrolyzing GTP to GDP. This process is under the influence of several physiological and pathological conditions including growth factors,

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vasoactive substances, smoking and cellular injury. Rho proteins are associated with various kinases [1]. Rho GTPases participate in the regulation of apoptosis [4], endocytosis, vesicle trafficking and regulated secretion [5], endothelial cell functions, cytokine expression, genotoxic stress-induced signalling [6], membrane remodelling events, cell–cell contacts, angiogenesis, cell cycle progression and neoplastic transformation. Mutations or amplification of the members of the Ras gene family have been found in a variety of human tumors [7, 8]. Several studies suggest that targeting Rho protein per se or its downstream effector molecules such as Rho associated kinases (LIM kinase) [9] may have several therapeutic ramifications in pathologies such as hypertension, angina, myocardiac infarction, atherosclerosis and tumor metastasis [1].

The members of the RhoBTB subfamily of Rho GTPases are present in all vertebrates [10, 11]. RHOBTB1 is highly expressed in the stomach, skeletal muscle, placenta, kidney and testis. RhoBTB genes are detected in the fetal tissues and are upregulated in some cancer cell lines [10]. Several studies indicated the implication of RhoB in the testicular biology especially in the regulation of the junctional dynamics between the cells of the seminiferous epithelium [9, 12–15]. GTPase activating proteins (p45-GAP, n-chimaerin) were found in the tissue extract from the rat testes (Manser, 1992 #62). Up-regulation of Rho family small G-proteins was shown to be implicated as important modulators of tight junctional dynamics of the testis following the specific ligand activation of L-selectin (Lui, 2003 #24). Interestingly, normal testis development is disrupted by the deletion of LIMK2 (RhoB associated kinase) [9]. The inhibition of Rho-Associated coiled-coil-containing protein kinase (an activator of Focal adhesion, kinase-related non-kinase can suppress cancer-associated gene (is a novel cancer/testis antigen)-promoted motility) [16]. Moreover, Kamai and his colleagues addressed the role of Rho small GTP-binding protein (Rho) in the progression of testicular germ cell tumours. They examined the expression levels of mRNAs of Rho genes in paired tumorous and non-tumorous tissues from 45 specimens of testicular germ cell tumours using reverse transcription-polymerase chain reaction. The mRNA levels of RhoA was found in both neoplastic and normal testicular tissues whereas the mRNAs of RhoB and RhoC were not detected in either tissue [17].

Taken together, although RhoB protein seems to play critical role in the testicular biology, no data are available about the topic of expression pattern of RhoB protein in the human testis showing normal and abnormal spermatogenesis. To fill this existing gap in literature, we carried out this study using immunofluorescent staining methods in testicular tissue specimens from patients with testicular and post-testicular infertility. We addressed two questions: what is the expression pattern of RhoB in testis showing normal spermatogenesis? and whether this expression pattern displays any testicular infertility-related changes (abnormal spermatogenesis). To the best of our knowledge, this investigation is the first to address these issues.

Materials and Methods

Testis tissue samples Thirty testicular biopsies were obtained after informed consent from patients suffering from different forms of infertility; 10 patients were suffering from post-testicular infertility, 10 patients were suffering from testicular infertility (spermatogenesis arrest and 10 patients were suffering from testicular infertility) (Sertoli cell only-related infertility).

The study was approved by the institutional Ethical Committee. Samples were frozen abruptly in liquid nitrogen, preserved and stored in cryomedium at −80°C until they were used for cryosectioning. 8 μm-thick cryosections were prepared and dried at room temperature for about 30 min. Sections were, then, fixed in cold acetone (−20°C) for 10 min., re-dried at room temperature for 60 min and preserved by (−20°C).

Immunohistochemistry Cryosections of human testis samples from different patients with different testicular and post-testicular infertility were immunostained using rabbit polyclonal IgG anti-human RhoB (Santa Cruz Biotechnology, USA). To visualise the antigen-antibody complex; the highly sensitive immunofluorescent tyramide signal amplification (TSA) labelling technique (PerkinElmer Life Science, Boston, MA) was performed. For this purpose, cryosections were pre-washed in Tris-acid-Tween buffer (TNT, pH 7.5), followed by washing in 3% hydrogen peroxide (H_2O_2). Sections were, then, incubated with lower concentrations of primary antibodies diluted in Tris-acid-blocking buffer (TNB, pH 7.2, 1:1000) overnight at 4°C. Next, sections were washed in TNT and incubated with Cy3-conjugated F(ab)2 fragments of goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in TNB (1:200) for 30 min at room temperature. Thereafter, sections were incubated with streptavidin horseradish peroxidase (1:50 in TNB) for 30 min at room temperature. Finally, tetramethylrhodamine-isothiocyanate-tyramide amplification reagent was administrated (1:50 in amplification diluent provided with the kit) for 30 min at room temperature, followed by counterstaining with 4′,6′-diamidino-2-phenylindole (DAPI) and mounting in levamisole (DAKO Corporation; Carpenteria, CA, USA). The TSA signals were visualised under a fluorescence microscope (Carl Zeiss, Jena, Germany). Negative controls were obtained by omission of primary antibody and positive

control was obtained from human blood vessel immunostained with RhoB.

Results

Strong expression of RhoB protein in human testis showing normal spermatogenesis Immunohistochemical staining of the human testis showing normal spermatogenesis revealed a prominent expression of RhoB in both the interstitium and the seminiferous tubules (Figs. 1a–b and 2a). In the seminiferous tubules, a strong RhoB immunoreactivity (IR) was detected in the cytoplasm of Sertoli cells (Figs. 1a–d and 2a–b) and in the germinal epithelium, preferentially primordial germ cells (Figs. 1a–d and 2a–b), spermatogonia (Figs. 1a–d and 2a, b), primary spermatocytes (Figs. 1a–d and 2a–b) and secondary spermatocytes (Figs.1a–d and 2b). A negative RhoB expression was detected in the spermatids (Figs. 1a, c, d and 2a, b). RhoB immunostaining was not seen in the spermatozoa (Figs. 1a–d and 2a–b).

No RhoB expression was detected in the connective tissue sheath of the seminiferous tubules (Figs. 1a–d and 2a–b). In the interstitium, a very strong RhoB immunoreactivity was found in the cytoplasm of Leydig cells (Figs. 1a–b and 2a, b) and blood vessels. A moderate RhoB immunoreactivity was seen in some myofibroblasts and other connective tissue cells (Figs. 1a and 2a). The immunostaining of RhoB was contrasted by the positive and negative controls (Fig. 3a and b, respectively).

Weak expression of RhoB protein in the seminiferous epithelium and Leydig cells of the testis showing abnormal spermatogenesis (spermatogenic arrest) The testes showing abnormal spermatogenesis had atrophic seminiferous tubules with thick connective tissue sheath and a larger interstitium. The cell types surveyed within seminiferous tubules were Sertoli cells, primordial germ cells, spermatogonia, primary spermatocytes and some secondary spermatocytes. In contrast to the normal functional testis, testes with spermatogenic arrest showed a decreased expression of RhoB, particularly in the seminiferous

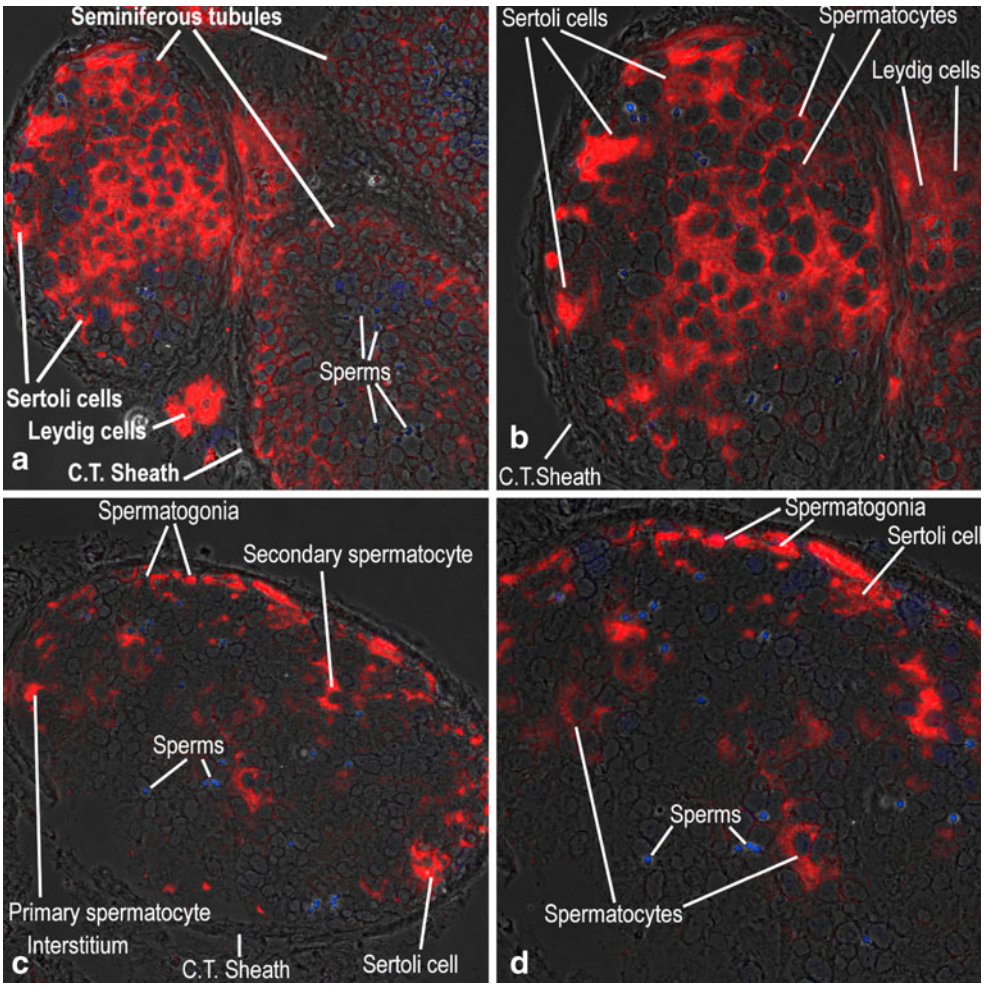

Fig. 1 Expression pattern of RhoB in normal fertile human testis. Specimens are obtained from patients suffering from posttesticular infertility. **a** 34 Y (200×), **b** magnification of **a** (320×), **c** 43 Y (200×), **d** magnification of **c** (320×). Note the sperms which appear as *small dots* of strong bright blue colour

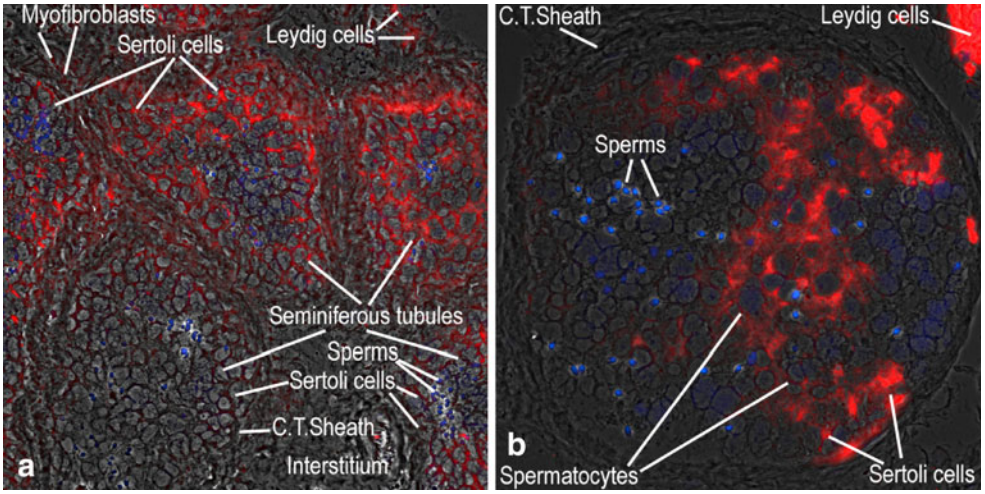

Fig. 2 Expression pattern of RhoB in normal fertile human testis. Specimens are obtained from patients of different ages suffering from posttesticular infertility. **a** 51 Y (200×), **b** 54 Y (320×)

tubules (Fig. 4a, b). Within the seminiferous tubules, the immunoreactivity of RhoB was prominent only in the Sertoli cells, primordial germ cells and spermatogonia, whereas it was weak in primary and secondary spermatocytes compared to the testis showing normal spermatogenesis (Fig. 4a–b). No RhoB immunoreactivity was observed in the seminiferous tubule connective tissue sheathes (Fig. 4a–b). In the interstitium, RhoB immunoreactivity was completely absent from the cytoplasm of Leydig cells and myofibroblasts (Fig. 4a, b).

Decreased expression of RhoB protein in the Sertoli and Leydig cells in the testis showing Sertoli cell only syndrome In testis samples obtained from patients suffering from Sertoli cell only syndrome, the seminiferous tubules had the thickest connective tissue sheath and largest interstitium (Fig. 4c–d). A decreased expression of RhoB protein in the seminiferous tubules and interstitium (Fig. 4c–d) was observed. The staining intensity of RhoB was moderate in the cytoplasm of the Sertoli cells (Fig. 4c, d). In the interstitium, RhoB immunoreactivity was completely absent from Leydig cells and the myofibroblasts (Fig. 4c, d).

Discussion

The human testis is composed of convoluted seminiferous tubules residing in a stroma containing Leydig cells. The seminiferous tubules are lined by multilayered epithelium with most mature cells towards lumina. They have a basal lamina and contain Sertoli cells, spermatogonia, spermatocytes, spermatids and spermatozoa; all except spermatozoa are held together by narrow cytoplasmic bridges. The Sertoli cells rest on a basement membrane, surround germ cell elements and are joined by tight junctions with cytoplasmic extensions that form blood-testis barrier [12, 18–20]. During spermatogenesis, dynamic cellular rearrangements involving the actin cytoskeleton are required for both Sertoli and germ cells. The small GTPase Rho; functions as a molecular switch that

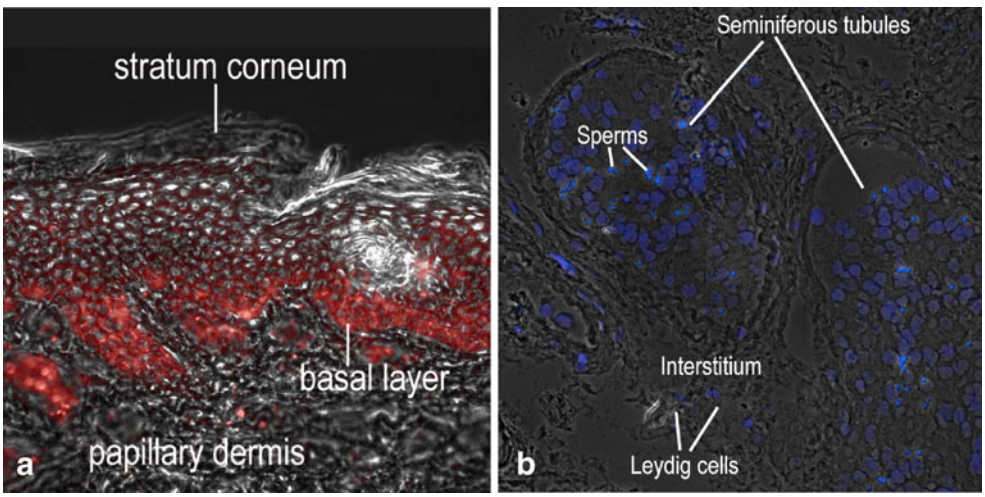

Fig. 3 **a** and **b** are positive and negative controls, respectively, for the immunostaning. **a** human skin, **b** normal human testis (200×)

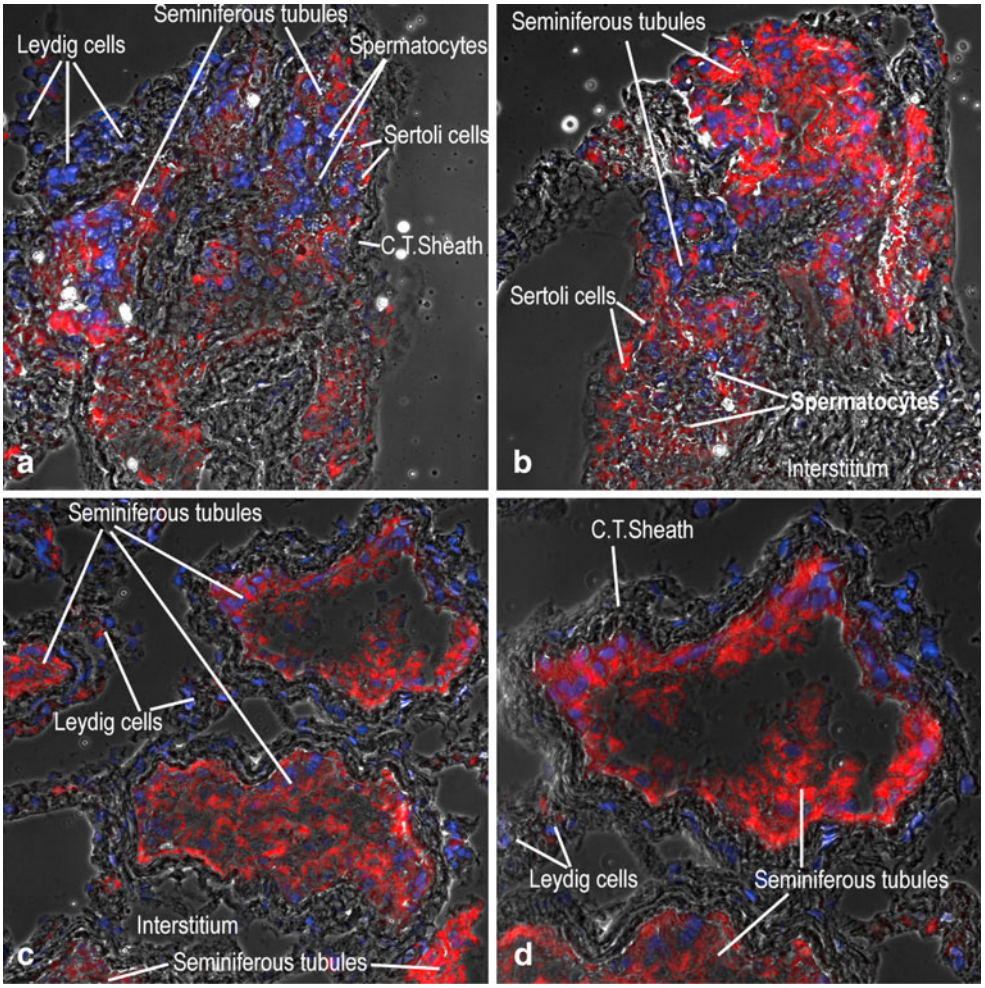

Fig. 4 Expression pattern of RhoB in abnormal non-fertile human testes representing two types of testicular infertility. **a** and **b** from patients suffering from spermatogenesis arrest (200×). **c** and **d** from patients suffering from Sertoli cell only infertility (200× and 320×)

regulates various cellular processes such as cell adhesion, motility, gene expression and cytokinesis. Rho family small G proteins have been implicated in the regulation of cellular rearrangements through the control of the actin cytoskeleton in several cell types in vertebrates [21, 22]. To date, the distribution of RhoB protein in the human testis is unknown. This investigation tries to address this issue and clearly demonstrates two observations. RhoB protein has a cell type-specific expression pattern in human testis showing normal spermatogenesis. Also, the expression of this protein is altered in testis with abnormal spermatogenesis.

RhoB protein is expressed in cell-type specific pattern in human testis showing normal spermatogenesis In our series, the expression of RhoB in testis showing normal spermatogenesis concurs with previous studies (rat model). Studies by Lui and his colleagues indicated that the localization of RhoB in the seminiferous epithelium is stage specific, being lowest in stages VII–VIII prior to spermiation, and displays cell-specific association during the epithelial cycle. During spermatogenesis the developing germ cells must migrate progressively from the basal to the adluminal compartment but remain attached to the seminiferous epithelium. The cell–cell actin-based adherens junctions between Sertoli and germ cells undergo extensive restructuring in the seminiferous epithelium to facilitate this germ cell movement across the epithelium [13–15]. Rho GTPases is critically involved in the regulation of these events (particularly cytokinesis) in other epithelia. This contention is supported by several experimental findings. RhoA have been detected at both the mRNA and protein levels in both the Sertoli and germ cells. Inactivation of Sertoli cell Rho family proteins yields disruption of the actin cytoskeleton [22]. In the testis, several investigations indicated that the in vitro assembly of the Sertoli-germ cell adherens junctions is associated with a transient but significant induction of RhoB. The disruption of adherens junctions in Sertoli-germ cell co-cultures induced RhoB expression. Treatment of adult rate with chemicals that perturb Sertoli-germ cell adherens junctions in vivo markedly induced RhoB expression in the testis, but not in other organs (kidney and brain) [13].

Nakamura et al examined the tissue distribution and cellular localization of rhophilin, a 71 kDa Rho-binding

protein in mice. Rhophilin mRNA was highly expressed in adult testis, but was absent in the testis of W/WV mice deficient in germ cells. Immunofluorescence analysis revealed two lines of striated staining running in parallel in the principal piece of the sperm tail. These results suggest that rhophilin is expressed in germ cells and is localized in the fibrous sheath of the sperm tail [23]. Takahashi et al indicated that LIM-Kinase (LIMK, including LIMK1 and LIMK2) can phosphorylate and inactivate cofilin, an actin-depolymerizing factor regulating actin reorganization. The same research group generated Limk2 gene-deficient mice in which three LIMK2 isoforms were disrupted in a Cre-mediated fashion resulting in impaired cofilin phosphorylation. Rho-dependent LIMK activation did not occur. The testes of Limk2-/- mice were smaller in size with partial degeneration and increased apoptosis of spermatogenic cells in the seminiferous tubules. Moreover, the viability of Limk2-/- spermatogenic cells, when cultured under stressed conditions was diminished. Furthermore, the potential for germ cells to differentiate in a regenerative state was severely impaired in Limk2-/- testis. The authors proposed that LIMK2, especially the testis-specific isoform tLIMK2, plays an important role in proper progression of spermatogenesis by regulation of cofilin activity and/or localization in germ cells [24]. Moreover, during spermatogenesis, the first morphological indication of spermatogonia differentiation is incomplete cytokinesis, followed by the assembly of stable intercellular cytoplasmic communications. Citron kinase is a myotonin-related protein acting downstream of the GTPase Rho in the control of cytokinesis. Citron kinase knockout mice display a dramatic testicular impairment, with embryonic and postnatal loss of undifferentiated germ cells and complete absence of mature spermatocytes [25]. Taken collectively, these observations highlights the role of Rho GTPases, and in particular RhoB in the process of spermatogenesis [13–15].

Altered expression of RhoB protein in human testis showing abnormal spermatogenesis The altered expression of RhoB protein in testis showing abnormal spermatogenesis may be due to impairment of generation or dysregulaton of upstream signaling events or altered expression of GDP dissociation inhibitors. The Rho GDI (GDP dissociation inhibitor) family includes Rho GDIalpha, -beta, and -gamma. It is a regulator that keeps the Rho family members in the cytosol as the GDP-bound inactive form. The Rho GDI also translocates the GDP-bound form from the membranes to the cytosol after the GTP-bound form accomplishes their functions. Rho GDIalpha is ubiquitously expressed in mouse tissues and shows GDI activity on all the Rho family members in vitro [26]. Togawa et al. generated mice lacking Rho GDIalpha. Rho GDIalpha -/- male mice were infertile and showed impaired spermatogenesis with vacuolar degeneration of seminiferous tubules in their testes [26].

The cell type-specific expression of RhoB protein (i.e. strong reactivity in Sertoli cells, Leydig cells, spermatogonia and spermatocytes versus absent expression in the spermatids and sperms) suggests that RhoB expression may be regulated during the cycle of the seminiferous epithelium. A hypothesis to be tested is that the RhoB protein is primarily needed during initial steps of spermatogenesis in which there is an active migration (cytokinesis) of the germ cells to the luminal side of the tubules. This also indicates that RhoB protein expression may correlate both with the level of differentiation of the spermatogenic cells and the histological onset of maturation to the stage of spermatozoa.

In summary, from our observations, we would like to raise a novel hypothesis that RhoB protein may play a role in spermatogenesis in human. To our knowledge, the present study is the first demonstration of the expression of this molecule in the human testis. It also provides the first morphological indication that RhoB protein expression is altered in testis showing abnormal spermatogenesis.

Acknowledgment § The kind help of Prof. W. Schultze, Dept. Of Andrology, University Hospital of Hamburg-Eppendorf, Hamburg University, Germany, is gratefully acknowledged.

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