NC1-peptide derived from collagen α3 (IV) chain is a blood-tissue barrier regulator: lesson from the testis

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Collagen α3 (IV) chains are one of the major constituent components of the basement membrane in the mammalian testis. Studies have shown that biologically active fragments, such as noncollagenase domain (NC1)-peptide, can be released from the C-terminal region of collagen α3 (IV) chains, possibly through the proteolytic action of metalloproteinase 9 (MMP9). NC1-peptide was shown to promote blood–testis barrier (BTB) remodeling and fully developed spermatid (e.g., sperm) release from the seminiferous epithelium because this bioactive peptide was capable of perturbing the organization of both actin- and microtubule (MT)-based cytoskeletons at the Sertoli cell–cell and also Sertoli–spermatid interface. The ultrastructure known as the basal ectoplasmic specialization (ES) and apical ES, respectively. More importantly, recent studies have shown that this NC1-peptide-induced effects on cytoskeletal organization in the testis are mediated through an activation of mammalian target of rapamycin complex 1/ribosomal protein S6/transforming retrovirus Akt1/2 protein (mTORC1/ rpS6/Akt1/2) signaling cascade, involving an activation of cell division control protein 42 homolog (Cdc42) GTPase, but not Ras homolog family member A GTPase (RhoA), and the participation of end-binding protein 1 (EB1), a microtubule plus (+) end tracking protein (+TIP), downstream. Herein, we critically evaluate these findings, providing a critical discussion by which the basement membrane modulates spermatogenesis through one of its locally generated regulatory peptides in the testis.

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INTRODUCTION

In the seminiferous tubules of mammalian testes, the basement membrane (BM) appears as a sheet-like homogenous substance of approximately 0.15 μm in thickness that lays at the base of the seminiferous epithelium, in direct contact with the Sertoli cells and spermatogonia, which is a modified form of extracellular matrix (ECM). Nonetheless, the BM that lays adjacent to the base of the seminiferous epithelium is similar to the BM that appears as a thin but dense sheet-like ECM that underlies all epithelia and endothelia in multicellular animals. However, the BM has remarkably diverse functions tailored to individual tissues and organs, including seminiferous tubules, blood vessels, and lymphatic vessels, through tightly regulated spatial and temporal expression of proteins and glycoproteins that modify its structure and composition. In the testis, the BM is also an integrated component of the endothelium of testicular blood vessels/capillaries, and also lymphatic vessels located just underneath the tunica albuginea. The BM in rodent testes, including seminiferous tubules, is constituted mostly by Type IV collagen chains (e.g., collagen α3 [IV]) and laminin chains (e.g., laminin-α1 and laminin-α2), which serve as the BM backbone and supported by entactin, heparin sulfate proteoglycans, fibronectin, fibronectin, and fibrinogen.

In review, we focus exclusively on the BM of seminiferous tubules for discussion unless otherwise specified. Because studies in recent years have shown that besides serving as the structural component of the seminiferous tubules, the BM in seminiferous tubules is crucial to support spermatogenesis. An earlier report has noted that passive transfer of antiserum against the seminiferous tubule basement membrane induced orchitis, which was associated with focal sloughing of the seminiferous epithelium in rats. In addition, passive immunization with anti-laminin (another constituent component of the basement membrane) IgG in guinea pigs was found to induce seminiferous epithelial damage that led to spermatogenic arrest. Furthermore, the use of an anti-collagen (Type IV) antibody was shown to perturb the Sertoli cell tight junction–permeability barrier function. Collectively, these studies have shown that any approaches that would impede BM function, such as through the use of antibodies to perturb the structural components of the BM, would lead to a disruption of spermatogenic function through focal and structural epithelial damage across the epithelium in the testis. However, the molecular mechanism(s) underlying these earlier observations was (were) not known.

Studies to date have shown that there are 29 subtypes of collagen in the mammalian tissues from I to XXIX, and genetic mutations/variations on many of the collagen genes lead to multiple genetic disorders in...
NC1-peptide, similar to the use of its purified recombinant protein, pCI-neo which was used for its overexpression in Sertoli cells. NC1-peptide was then cloned into the mammalian expression vector pCI-neo which was then used to produce recombinant NC1-peptide either in vitro or in vivo. Because the BTB in the testis in vivo could be perturbed by NC1-peptide, its overexpression in the testis was also accompanied by epithelial damage, wherein germ cell exfoliation was notably detected. However, since the disrupted BTB integrity induced by the overexpression of NC1 was transient, it could be “resealed,” thus the NC1-peptide-induced defects in spermatogenesis were also reversible. Furthermore, NC1-peptide was found to exert its regulatory effects by inducing cytoskeletal disorganization of the F-actin and microtubule (MT) networks. For instance, the F-actin network prominently expressed at the ectoplasmic specialization (ES) structures, namely the apical ES at the Sertoli–spermatic interface, and the basal ES at the BTB, were grossly disrupted across the seminiferous epithelium, where F-actin was extensively truncated across the epithelium when compared to control testes. MT-based tracks that are used to support proper endocytic vesicle-mediated protein trafficking (e.g., residual bodies, lysosomes) and spermatid transport were found to be extensively truncated, thereby failing to support these crucial cellular events that led to defects in spermatogenesis. As noted in control testes, MT-based tracks are aligned perpendicular to the basement membrane that stretch across the entire seminiferous epithelium, however, following overexpression of NC1-peptide, these tracks are extensively truncated and misaligned because some short stretches of MT-tracks are aligned in parallel to the basement membrane. In addition, many cell adhesion protein complexes (e.g., occludin/zona occludens 1 [ZO-1] and N-cadherin/β-catenin) that utilize F-actin for attachments became misdistributed, this thus failed to support germ cell adhesion, thereby leading to germ cell exfoliation across the entire epithelium, perturbing spermatogenesis. Nonetheless, the underlying mechanism, or signaling/partner proteins that mediate the NC1-peptide effects in the testis, remains unknown.

NC1-PEPTIDE AND SERTOLI CELL FUNCTION – ROLE OF mTORC/RPS6/MTORC1/2 SIGNALING COMPLEX AND CDC42 GTPASE

Studies have shown that the mammalian target of rapamycin (mTOR), a Ser/Thr protein kinase and a member of the phosphatidylinositol 3-kinase-related kinase family, is a known regulator of cell growth, cell proliferation, cell motility, cell survival, protein synthesis, cell energy status, autophagy, protein transcription, spermatogenesis, and tumorigenesis, found in virtually all mammalian cells. When mTOR binds to its adaptor protein called regulatory-associated protein of mTOR (Raptor) or rapamycin-insensitive companion of mTOR (Rictor), it creates the mammalian target of rapamycin complex 1 (mTORC1) or mTORC2, respectively, which has diversified cellular functions due to the differences in its downstream signaling cascades. Studies have shown that mTORC1 is a crucial modulator of BTB function and spermatogenesis, which exerts its effects downstream via rpS6 and Akt1/2 by inducing BTB remodeling, thereby facilitating the transport of preleptotene spermatocytes across the BTB at stage VIII–IX of the epithelial cycle in the rat testis. Because ribosomal protein S6 (rpS6) is a phosphorylation-inducible protein translation regulator, mTORC1 regulates a wide range of cellular events in mammalian cells, including Sertoli cells, such as remodeling of the Sertoli cell BTB in the testis during the epithelial cycle of spermatogenesis. In fact, a quadruple phosphomimetic mutant of rpS6 (i.e., p-rpS6-mutant) by mutating p-rpS6 at the four phosphorylatable (i.e., activated) sites at S235, S236, S240, and S244 (S, Ser) to S235E, S236E, S240E, and S244E (E, Glu) by site-directed mutagenesis to make this mutant constitutively active has been prepared. This p-rpS6-mutant is a potent BTB remodeling inducer based on several humans. Among the 29 collagen subtypes, Type IV collagen is a network-forming collagen, which is not only the predominant structural component of the BM in the mammalian testis, but also the glomerular basement membrane in the kidney. Similar to other collagens, Type IV collagen is comprised of three α chains, which, in turn, create a triple helical structure, which serves as the monomer (Figure 1) and the building block of the collagen network. Six genetically distinct α chains are known to date, namely α1–α6, with collagen α3 (IV) being the most predominant collagen chain in the BM of the testis. Each monomer has a noncollagenous 7S domain (approximately 15 amino acid residues from the N-terminus), a long middle collag enous domain of approximately 1400 amino acid residues of the Gly-Xaa-Yaa repeats, and a C-terminal noncollagenous (NC1) domain of approximately 230 amino acid residues of about 28 kDa (Figure 1). These monomers can dimerize at the C-terminal region to form dimers by utilizing the 7S noncollagenous domain, which is an important structural cross-linking domain to generate collagen IV networks, or dimerize at the N-terminal 7S domain region to create spider-like tetramers, which in turn create the superstructure, becoming a crucial structural component to sustain the basement membrane.

COLLAGEN FRAGMENTS AND BIOLOGICAL ACTIVITIES – STUDIES FROM OTHER EPITHELIA AND THE TESTIS

There are numerous reports in the literature based on studies in multiple epithelia, endothelia, tissues, and organs regarding the biologically active fragments derived from different collagen chains in the mammalian body that affect various cellular events including cell adhesion, junction permeability, cell differentiation, cell survival, pathogenesis of diseases, and tumorigenesis. Among the 29 collagen subtypes, Type IV collagen is a basement membrane component to sustain the basement membrane. In addition, extracellular matrix enzymes (e.g., FNase) produce the small biologically active collagen fragments by degrading the larger collagen molecules. Type IV collagen is comprised of three α chains, which, in turn, create a triple helical structure, which serves as the monomer (Figure 1). The monomer has a noncollagenous 7S domain (approximately 15 amino acid residues from the N-terminus), a long middle collagenous domain of approximately 1400 amino acid residues of the Gly-Xaa-Yaa repeats, and a C-terminal noncollagenous (NC1) domain of approximately 230 amino acid residues of about 28 kDa (Figure 1). These monomers can dimerize at the C-terminal region to form dimers by utilizing the 7S noncollagenous domain, which is an important structural cross-linking domain to generate collagen IV networks, or dimerize at the N-terminal 7S domain region to create spider-like tetramers, which in turn create the superstructure, becoming a crucial structural component to sustain the basement membrane.

STUDIES FROM OTHER EPITHELIA AND THE TESTIS

Regulation of spermatogenesis by NC1-peptide

SW Liu et al
In this context, it is of interest to note that besides germ cell exfoliation, many elongated spermatids displayed defects in their polarity, wherein the heads of these spermatids no longer pointed toward the basement membrane as noted in control testes, but deviated by 90°–180° from the intended orientation. This observation is of interest because it suggests that the action of NC1-peptide on the testis may correlate with cell polarity proteins. In the testes, similar to other organs, cells in the seminiferous epithelium, namely germ cells and Sertoli cells, robustly express the three cell polarity protein complexes found in C. elegans and Drosophila. These include the Par-, the Crumbs-, and the Scribble-based polarity protein complexes including their partner proteins, which are crucial to provide apico-basal polarity during spermatogenesis. As such, the maximal number of developing spermatids can be packed (and organized) in the limited space of the seminiferous tubules in the testis to sustain the production of 30–40 × 10⁶ sperm per male (or 300 × 10⁶ sperm per man) on a daily basis. Furthermore, studies have shown that the testes (and also Sertoli cells and germ cells per se) also express planar cell polarity (PCP) proteins (e.g., Van Gogh-like 2 [Vangl2] and disheveled 3 [Dvl3]) to maintain PCP across the seminiferous epithelium. This orderly alignment of polarized developing spermatids, mostly stage 17–19 spermatids in stages V–VIII tubules, conferred by cell polarity proteins and PCP proteins, is crucial to support the last maturation phase of spermatids and their transition to spermatozoa such that nourishments and/or chemical signals can be properly transmitted from Sertoli cells to spermatids to support this series of events.

**Figure 2:** Signaling cascade of NC1-peptide that regulates spermatogenesis. The signaling cascade involves mammalian target of rapamycin complex 1 (mTORC1), phosphorylated (activated)-ribosomal protein S6 (p-rpS6), phosphorylated (activated)-transforming retrovirus Akt1/2 protein (p-Akt1/2), activated cell division control protein 42 (activated Cdc42), end-binding protein 1 (EB1; a microtubule plus [+] end tracking protein (+TIP)), and epidermal growth factor receptor pathway substrate 8 (Eps8, an actin barbed end capping and bundling protein). NC1: noncollagenous domain 1.

**NC1-PEPTIDE AND SPERMATOGENESIS – ROLE OF CELL POLARITY PROTEINS**

When NC1-peptide was overexpressed in the testis in vivo, it was noted that besides germ cell exfoliation, many elongated spermatids displayed...
Furthermore, studies have shown that the cell polarity proteins, such as the crumbs homolog-3 (Crb3)/protein associated with Lin-7 1 (Pals1)/Pals1-associated tight junction protein (PatJ) complex and the Scribble/discs large 1 (Dlg1)/fetal giant larvae (Lgl2) complex, that confer apico-basal polarity, support spermatid head and tail polarity as noted in the tests. On the other hand, PCP proteins, such as Vangl2 and Dvl3 also confer and support directional alignment of polarized spermatids across the plane of seminiferous epithelium. Interestingly, both sets of proteins exert their effects to confer their corresponding polarity function via the actin- and MT-based cytoskeletons. This conclusion was reached based on findings that a knockdown or an overexpression of any of these proteins in Sertoli cell epithelium in vitro that mimicked the Sertoli cell BTB in vivo or in testes in vivo was found to perturb spermatid polarity (and adhesion) or spermatid PCP through changes in the organization of F-actin and/or MT.

We thus examined the role of these polarity proteins in mediating NC1-peptide-induced changes in spermatogenesis in the testes. Interestingly, during NC1-peptide induced defects in spermatogenesis in the testis, a general trend of downregulation on the expression of the Crb3-based polarity protein complex including Crb3, Pals1, and PatJ, the Par-based polarity complex including partitioning defective protein 6 (Par6), Cdc42 and atypical protein kinase C (aPKC), but not the Scribble-based complex including Scribble, Lgl2 and Dlg1, was noted. The downregulation was shown to begin at approximately 6–12 h following NC1-peptide overexpression even before obvious phenotypic changes were detected across the seminiferous epithelium, and considerably downregulation was noted by day 3 and day 7 after NC1-peptide overexpression. On the other hand, both PCP proteins Prickle 1 and Dvl3 also displayed a downregulation trend during NC1-peptide mediated defects in BTB function and spermatogenesis as early as 12 h following its overexpression. These findings are important because they illustrate that cell polarity and PCP proteins are possibly involved in NC1-peptide-mediated defects in spermatogenesis.

**NC1-PEPTIDE REGULATES CYTOSKELETAL ORGANIZATION – ROLE OF CYTOSKELETAL REGULATORY PROTEINS**

Cytoskeletal function across the seminiferous epithelium in the mammalian testis is known to be regulated mostly by the actin- and MT-based cytoskeletons. Interestingly, these two cytoskeletons are localized adjacent to one another to confer the testis-specific adherens junction known as the ES. For instance, the BTB created by adjacent Sertoli cells in the testis is constituted by coexisting TJ, basal ES, and gap junction, which are actin-based cell junctions, wherein the adhesion protein complexes all utilize F-actin for their attachment. Studies by electron microscopy have shown that MTs are laying adjacent to the actin microfilaments, illustrating that these two cytoskeletons are working in concert to support structural and scaffolding function, and other cellular functions such as endocytic vesicle-mediated protein trafficking.

Following overexpression of NC1-peptide in the testis in vivo, the organization of F-actin and MT networks across the seminiferous epithelium is grossly disrupted, wherein F-actin no longer restrictively expresses at the apical and basal ES to support spermatid adhesion and BTB integrity, instead it becomes diffusely localized at these sites. Furthermore, MT-conferred tracks that lay perpendicular against the basement membrane and stretch across the entire seminiferous epithelium as noted in control tests become extensively truncated, broken into shorter track-like fragments, and some even lay parallel to the basement membrane, making them incapable of transporting cell organelles (e.g., residual bodies and phagosomes) to their desired sites across the epithelium.

These changes are contributed by disruptive changes on the spatial expression of actin- and MT-based regulatory proteins such as Arp3 and Eps8 for F-actin and MARK4, EB1, and dynein 1 for MT. These changes thus perturb cell adhesion function of germ cells and Sertoli cells via changes in the distribution of TJ- and basal ES-adhesion proteins, thereby failing to support adhesion protein complexes that lead to germ cell exfoliation and BTB disruption (i.e., making the barrier “leaky”). More importantly, the distribution of Dvl3 across the seminiferous epithelium, which co-localizes with MTs, is also considerably disrupted by becoming extensively truncated. Because Dvl3 is necessary to confer proper organization of actin- and MT-based cytoskeletons, the misdistribution of Dvl3 following NC1-peptide overexpression in the testes as recently reported appears to contribute to the disorganization of MTs and F-actin across the seminiferous epithelium. Collectively, these findings indicate that NC1-peptide is working in concert with the cell polarity and PCP proteins to modulate cytoskeletal function to maintain seminiferous epithelial homeostasis to support spermatogenesis.

**ROLE OF EB1, A MICROTUBULE +TIP, IN NC1-PEPTIDE-MEDIATED EFFECTS ON SPERMATOGENESIS**

EB1, end-binding protein 1, also called microtubule-associated protein RB/E8 family member 1 (MAPRE1) in humans, is a +TIP that binds to the rapidly growing end of MTs, which together with EB2 and EB3 are known to stabilize MT protofilaments by reducing the risks of MTs to switch from a rapidly growing state to undergoing catastrophe. Studies have shown that EB1 is highly expressed by Sertoli and germ cells in the rat testes, which co-localizes with MTs (visualized by α- or β-tubulin staining, wherein α-/β-tubulin oligomers serve as the building blocks of MTs and appear as short “dot-like” structures along the MTs). EB1 is crucial to support Sertoli cell function because its knockdown (KD) by RNAi using specific EB1 siRNA duplexes by approximately 80% perturbed the Sertoli cell TJ-permeability barrier function through changes in the distribution of TJ- (e.g., coxsackievirus and adenovirus receptor [CAR], ZO-1), an basal ES- (e.g., N-cadherin, β-catenin) proteins at the Sertoli cortical zone. As anticipated, KD of EB1 perturbs MT organization across the Sertoli cell cytosol, wherein MTs no longer stretch across the cell cytosol but retract from cell peripheries due to a considerable reduction in microtubule polymerization. Importantly, EB1 KD also perturbs the organization of F-actin across Sertoli cell cytosol wherein actin filaments no longer stretch across the Sertoli cell cytosol as noted in controls. Instead, actin filaments become extensively truncated and randomly aligned across the cell cytosol. These changes are the result of mis-localization of the actin regulatory proteins such as actin-related protein 3 (Arp3, which together with Arp2 creates the Arp2/3 complex is known to induce branched actin nucleation) and epidermal growth factor receptor pathway substrate 8 (Eps8, an actin barbed end capping and bundling protein). Furthermore, there is a considerable increase in the association of Arp3 with neuronal Wiskott–Aldrich syndrome protein (N-WASP) following EB1 KD in Sertoli cell epithelium because N-WASP is known to activate the Arp2/3 complex to induce branched actin polymerization. Thus, an increase in N-WASP and Arp3 association favors the actin network in Sertoli cells across the seminiferous epithelium to assume a branched configuration, thereby destabilizing actin cytoskeleton. As such, the ability of the EB1-silenced Sertoli cells to bundle actin filaments considerably declines when compared to that of control cells. Taken collectively, these findings have unequivocally demonstrated the physiological significance of this +TIP to maintain the homeostasis of
the actin- and MT-based cytoskeletons to support Sertoli cell function and spermatogenesis. Interestingly, overexpression of NC1-peptide in the testes in vivo (but also Sertoli cells cultured in vitro) with an established functional TJ-barrier that mimicked the BTB in vivo was found to associate with a considerable decline in EB1 expression.20 This observation is consistent with an earlier report using primary Sertoli cells cultured in vitro where the overexpression of NC1-peptide indeed induced a considerable downregulation of EB1 expression.21 Collectively, these findings support the notion that EB1 is crucial in mediating the regulatory effects of NC1-peptide in the testis (Figure 2). To confirm this possibility, the full-length cDNA encoding EB1 was overexpressed in Sertoli cells cultured in vitro with an established TJ-barrier, which was found to block the NC1-peptide-mediated Sertoli cell TJ-barrier perturbation by restoring the ability of these cells to induce MT and actin polymerization, rescuing the NC1-peptide-mediated disorganization of MT and F-actin cytoskeletons.22 Taken collectively, these findings have shown that EB1 is a crucial putative downstream regulatory protein of the NC1-peptide (Figure 2).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Emerging evidence has indicated that the basement membrane in the adult rat testis is producing a regulatory biomolecule called NC1-peptide. NC1-peptide is generated locally in the testis from the collagen α3 (IV) chain which is a major constituent component of the basement membrane, likely via the proteolytic action of MMP-9. Furthermore, NC1-peptide exerts its regulatory effects to support spermatogenesis via the mTORC1/p-rp56/p-Akt1/2 pathway26–28 and activated Arp329 involving the actin regulatory protein Ep8 and the MT regulatory protein EB1 downstream.20 However, many questions remain unanswered. Which is (are) the upstream regulatory protein(s) that governs the proteolytic cleavage of collagen α3 (IV) chains in the basement membrane during the epithelial cycle of spermatogenesis? What is the identity of the integrin receptor that binds onto the NC1-peptide ligand to induce integrin signaling? What are the details of the signaling cascades utilized by NC1-peptide besides mTORC1-based signaling proteins noted in Figure 2? It is expected that many of the questions will be answered in the coming years, which will provide a better understanding on spermatogenesis.

AUTHOR CONTRIBUTIONS

CYC conceived the project and wrote the paper; SWL, HTL and CYC researched on the topics and searched for relevant literature at PubMed, which were evaluated and discussed in this review; HTL and CYC prepared the figures; and SWL, HTL, RSG and CYC discussed the concepts evaluated in this review. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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Regulation of spermatogenesis by NC1-peptide

SW Liu et al

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