Asian Journal of Andrology (2021) 23, 123–128
www.asiaandro.com; www.ajandrology.com

Open Access

REVIEW

Male Health

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

Shi-Wen Liu[1,2], Hui-Tao Li[1,2], Ren-Shan Ge[1], C Yan Cheng[1,2]

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.

Asian Journal of Andrology (2021) **23**, 123–128; doi: 10.4103/aja.aja_44_20; published online: 08 September 2020

Keywords: collagen α3 (IV) chain; F-actin; microtubules; noncollagenase domain (NC1)-peptide; spermatogenesis; testis

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).[1,2] 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.[3–5] 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.[4,6–8] In the testis, besides the seminiferous tubules, 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 fibrulins.[1,2,9,10] In this 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.[11] 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.[12] Furthermore, the use of an anti-collagen (Type IV) antibody was shown to perturb the Sertoli cell tight junction–permeability barrier function.[13] 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

[1]The Second Affiliated Hospital and Yuying Children's Hospital, Wenzhou Medical University, Wenzhou 325027, China; [2]The Mary M. Wohlford Laboratory for Male Contraceptive Research, Center for Biomedical Research, Population Council, 1230 York Ave, New York, NY 10065, USA.
Correspondence: Dr. CY Cheng (yancheng01@aol.com)
Received: 04 February 2020; Accepted: 26 May 2020

humans.[14,15] 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.[1,16,17] 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.[18,19] 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 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,[20] which is an important structural cross-linking domain to generate collagen IV networks,[21,22] or dimerize at the N-terminal 7S domain region to create spider-like tetramers,,[23,24] which in turn create the suprastructure,[19,25] which becomes 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,[24,26–29] which also involve an activation of integrin-based receptors.[30] For instance, NC1 domain from collagen IV is capable of stimulating branching morphogenesis of submandibular gland through β1-integrin and phosphatidylinositol-3-kinase/transforming retrovirus Akt (PI3K/Akt) signaling.[31] NC1 domain from different collagen subtypes has been shown to serve as an endogenous inhibitor of angiogenesis.[32,33] Collectively, these findings in other epithelia thus support the notion that biologically active collagen fragments can be generated in the basement membrane in the testis to modulate cellular functions across the seminiferous epithelium to support spermatogenesis. It was first reported that the use of an anti-collagen IV antibody was capable of perturbing the Sertoli cell tight junction (TJ)-permeability barrier function *in vitro*.[13] Findings from this study suggested that the release of NC1 domain (or other peptides) from collagen α3 (IV) chains involved an activation of matrix metalloproteinase-9 (MMP-9) mediated by tumor necrosis factor-α (TNFα) produced locally at the site released from Sertoli cells, wherein MMP-9 cleaved NC1-peptide from the collagen chain through proteolytic degradation, thereby generating a bioactive NC1-peptide endogenously in the testis during the epithelial cycle.[13] The NC1-peptide cDNA was subsequently obtained by polymerase chain reaction (PCR) and cloned into different expression vectors which were then used to produce recombinant NC1-peptide either in *E. coli* or human embryonic kidney cell line Lenti-X 293T cells. This recombinant NC1-peptide was then used to test for its biological activity in the Sertoli cell culture system, illustrating that NC1-peptide was capable of perturbing Sertoli cell TJ-barrier function reversibly.[34] NC1-peptide was then cloned into the mammalian expression vector pCI-neo which was used for its overexpression in Sertoli cells cultured *in vitro* but also testes *in vivo*.[35] It was noted that the overexpression of NC1-peptide, similar to the use of its purified recombinant protein, was able to induce reversible blood–testis barrier (BTB) disruption both *in vitro* and *in vivo*.[35] 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.[35] 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.[35] Furthermore, NC1-peptide was found to exert its regulatory effects by inducing cytoskeletal disorganization of the F-actin and microtubule (MT) networks.[35] For instance, the F-actin network prominently expressed at the ectoplasmic specialization (ES) structures, namely the apical ES at the Sertoli–spermatid 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.[35] 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.[35] As noted in control testes, MT-based tracks are aligned perpendicular to the basement membrane that stretch across the entire seminiferous epithelium,[35–38] 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/zonula 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,[35] perturbing spermatogenesis. Nonetheless, the underlying mechanism, or signaling/partnership proteins that mediate the NC1-peptide effects in the testis, remains unknown.

NC1-PEPTIDE AND SERTOLI CELL FUNCTION – ROLE OF MTORC/RPS6/AKT1/2 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.[39–43] 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.[39–43] 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,[44–46] 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,[47] including Sertoli cells, such as remodeling of the Sertoli cell BTB in the testis during the epithelial cycle of spermatogenesis.[43] 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[46] to make this mutant constitutively active has been prepared. This p-rpS6-mutant is a potent BTB remodeling inducer based on several

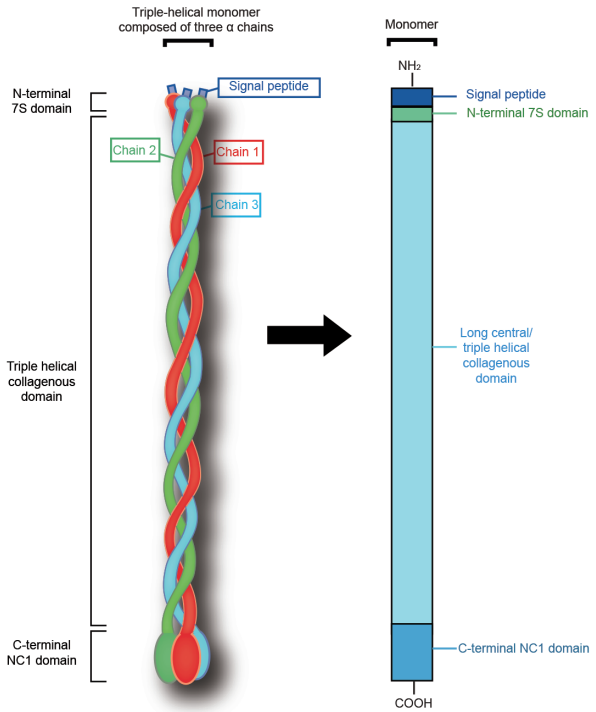

Figure 1: Structure and functional domains of collagen α3 (IV) chain. Collagen α3 (IV) chains are one of the major constituent components of the basement membrane which is located at the base of the seminiferous epithelium in the mammalian testis. Each collagen monomer of collagen α (IV) is comprised of three collagen chains which are associated with one another as a triple helical structure (left panel). The resulting monomer contains a short N-terminal 7S domain of approximately 15 amino acid residues, with a signal peptide of 28 amino acids at the N-terminus, followed by a long collagenous domain of approximately 1400 residues of Gly-Xaa-Yaa repeats and a C-terminal noncollagenous domain (NC1) of approximately 230 residues (right panel).

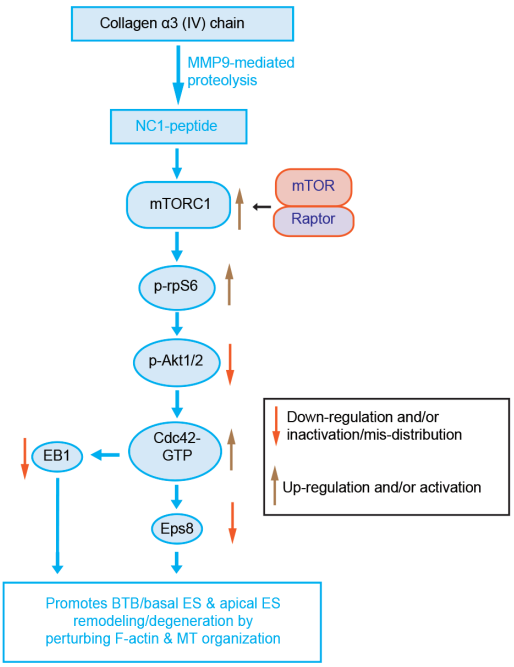

Figure 2: Signaling cascade of NC1-peptide that regulates spermatogenesis. The signaling cascade involves mammalian target of rapamycin complex 1 (mTORC1), phosphorylated (activated)-ribosomal protein S6 (p-rpS6), phosphorylated (activated)-transforming retrovirus Akt1/2 protein (p-Akt1/2), activated 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.

recent studies in the testis *in vivo*.[48–51] For instance, overexpression of this p-rpS6-mutant in the testis *in vivo* that induces BTB remodeling transiently can enhance drug transport (or permeability) across the BTB using the nonhormonal male contraceptive adjudin as a candidate drug.[48–50] Interestingly, NC1-peptide that induces BTB remodeling transiently,[35] is recently shown to activate the mTORC1/rpS6/Akt1/2 signaling pathway,[52] also involving an activation of Cdc42, but not RhoA, downstream[52] (**Figure 2**). In this context, it is of interest to note that Cdc42, a small GTPase, is a known regulator of actin[53,54] and MT[55] cytoskeletons, exerting its regulating effects through cytoskeleton regulatory proteins. Our findings are also consistent with this concept because the activated Cdc42 induced by NC1-peptide overexpression in Sertoli cell epithelium indeed was associated with a considerable downregulation on the expression of both actin barbed end capping and bundling protein epidermal growth factor receptor pathway substrate 8 (Eps8) and end-binding protein 1 (EB1) (a microtubule plus [+] end tracking protein [+TIP])[52] (**Figure 2**). In brief, it is now established that the mTORC1/rpS6/Akt1/2 and activated Cdc42 is the putative signaling pathway utilized by NC1-peptide to exert its effects to regulate spermatogenesis (**Figure 2**).

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 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.[35] 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 testis, 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,[56–58] 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[59,60] to sustain the production of 30×10^6– 40×10^6 sperm per male rodent (or 300×10^6 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][61,62] and disheveled 3 [Dvl3][63]) to maintain PCP across the seminiferous epithelium. In brief, PCP refers to the orderly alignment of directional polarized spermatids, wherein the spermatid heads point toward the basement membrane and their tails toward the tubule lumen, across the plane of the seminiferous epithelium.[64,65] This orderly alignment of polarized developing spermatids, mostly step 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.[66,67]

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)/lethal giant larvae (Lgl2) complex[68,69] that confer apico-basal polarity, support spermatid head and tail polarity as noted in the testis. On the other hand, PCP proteins, such as Vangl2 and Dvl3[61–63] 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[61–63,68,69]

We thus examined the role of these polarity proteins in mediating NC1-peptide-induced changes in spermatogenesis in the testis. 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.[70] 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.[70] 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.[70] 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.[71–74] Interestingly, these two cytoskeletons are localized adjacent to one another to confer the testis-specific adherens junction known as the ES.[66,67,73,75] 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.[67,76,77] 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.[67,71–73] 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.[35] Furthermore, MT-conferred tracks that lay perpendicular against the basement membrane and stretch across the entire seminiferous epithelium as noted in control testes[36,38,78] 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.[35] 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").[35] More importantly, the distribution of Dvl3 across the seminiferous epithelium, which co-localizes with MTs, is also considerably disrupted by becoming extensively truncated.[70] Because Dvl3 is necessary to confer proper organization of actin- and MT-based cytoskeletons,[63] the misdistribution of Dvl3 following NC1-peptide overexpression in the testis as recently reported[70] 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/EB family member 1 (MAPRE1) in humans, is a +TIP that binds to the rapid growing end of MTs,[79,80] 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.[79–82] 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).[36,83] 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.[36] 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.[36] 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.[36] Instead, actin filaments become extensively truncated and randomly aligned across the cell cytosol.[36] 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[84]) and epidermal growth factor receptor pathway substrate 8 (Eps8, an actin barbed end capping and bundling protein[85,86]).[36] 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[36] because N-WASP is known to activate the Arp2/3 complex to induce branched actin polymerization.[87] 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.[36] As such, the ability of the EB1-silenced Sertoli cells to bundle actin filaments considerably declines when compared to that of control cells.[36] 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 testis *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.[70] This observation is consistent with an earlier report using primary Sertoli cells cultured *in vitro,* wherein the overexpression of NC1-peptide indeed induced a considerable downregulation of EB1 expression.[52] 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.[70] 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-rpS6/p-Akt1/2 pathway[88–90] and activated Arp3[52] involving the actin regulatory protein Eps8 and the MT regulatory protein EB1 downstream.[70] 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.

AURHOR 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.

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Institutes of Health (NICHD, R01 HD056034 to CYC) and the National Natural Science Foundation of China (NSFC; No. 81730042 to RSG).

REFERENCES

1 Siu MK, Cheng CY. Extracellular matrix: recent advances on its role in junction dynamics in the seminiferous epithelium during spermatogenesis. *Biol Reprod* 2004; 71: 375–91.
2 Dym M. Basement membrane regulation of Sertoli cells. *Endocr Rev* 1994; 15: 102–15.
3 Kelley LC, Lohmer LL, Hagedorn EJ, Sherwood DR. Traversing the basement membrane *in vivo*: a diversity of strategies. *J Cell Biol* 2014; 204: 291–302.
4 Morrissey MA, Sherwood DR. An active role for basement membrane assembly and modification in tissue sculpting. *J Cell Sci* 2015; 128: 1661–8.
5 Kruegel J, Miosge N. Basement membrane components are key players in specialized extracellular matrices. *Cell Mol Life Sci* 2010; 67: 2879–95.
6 Sekiguchi R, Yamada KM. Basement membranes in development and disease. *Curr Top Dev Biol* 2018; 130: 143–91.
7 Breslin JW, Yang Y, Scallan JP, Sweat RS, Adderley SP, *et al*. Lymphatic vessel network structure and physiology. *Comprehensive Physiol* 2018; 9: 207–99.
8 Thomsen MS, Routhe LJ, Moos T. The vascular basement membrane in the healthy and pathological brain. *J Cereb Blood Flow Metab* 2017; 37: 3300–17.
9 Häger M, Gawlik K, Nyström A, Sasaki T, Durbeej M. Laminin α1 chain corrects male infertility caused by absence of laminin α2 chain. *Am J Pathol* 2005; 167: 823–33.
10 Sasaki T, Fassler R, Hohenester E. Laminin: the crux of basement membrane assembly. *J Cell Biol* 2004; 164: 959–63.
11 Lustig L, Denduchis B, Gonzalez NN, Puig RP. Experimental orchitis induced in rats by passive transfer of an antiserum to seminiferous tubule basement membrane. *Arch Androl* 1978; 1: 333–43.
12 Lustig L, Denduchis B, Ponzio R, Lauzon MP, Pelletier RW. Passive immunization with anti-laminin immunoglobulin G modifies the integrity of the seminiferous epithelium and induces arrest of spermatogenesis in the guinea pig. *Biol Reprod* 2000; 62: 1505–14.
13 Siu MK, Lee WM, Cheng CY. The interplay of collagen IV, tumor necrosis factor-a, gelatinase B (matrix metalloprotease-9), and tissue inhibitor of metalloprotease-1 in the basal lamina regulates Sertoli cell-tight junction dynamics in the rat testis. *Endocrinology* 2003; 144: 371–87.
14 Ricard-Blum S. The collagen family. *Cold Spring Harb Perspect Biol* 2011; 3: a004978.
15 Bateman JF, Boot-Handford RP, Lamande SR. Genetic diseases of connective tissues: cellular and extracellular effects of ECM mutations. *Nat Rev Genet* 2009; 10: 173–83.
16 Prockop DJ, Kivirikko KI. Collagens: molecular biology, diseases, and potentials for therapy. *Annu Rev Biochem* 1995; 64: 403–34.
17 Miner JH. The glomerular basement membrane. *Exp Cell Res* 2012; 318: 973–8.
18 Timpl R. Macromolecular oganization of basement membranes. *Curr Opin Cell Biol* 1996; 8: 618–24.
19 Timpl R, Wiedemann H, van Delden V, Furthmayr H, Kühn K. A network model for the organization of type IV collagen molecules in basement membranes. *Eur J Biochem* 1981; 120: 203–11.
20 Anazco C, Lopez-Jimenez AJ, Rafi M, Vega-Montoto L, Zhang MZ, *et al*. Lysyl oxidase-like-2 cross-links collagen IV of glomerular basement membrane. *J Biol Chem* 2016; 291: 25999–6012.
21 Risteli J, Bachinger HP, Engel J, Furthmayr H, Timpl R. 7-S collagen: characterization of an unusual basement membrane structure. *Eur J Biochem* 1980; 108: 239–50.
22 Glanville RW, Qian RG, Siebold B, Risteli J, Kuhn K. Amino acid sequence of the N-terminal aggregation and cross-linking region (7S domain) of the α1(IV) chain of human bhasement membrane collagen. *Eur J Biochem* 1985; 152: 213–9.
23 Hudson BG, Reers ST, Tryggvason K. Type IV collagen: structure, gene organization, and role in human diseases. Molecular basis of Goodpasture and Alport syndromes and diffuse leiomyomatosis. *J Biol Chem* 1993; 268: 26033–6.
24 Ortega N, Werb Z. New functional roles for noncollagenous domains of basement membrane collagens. *J Cell Sci* 2002; 115: 4201–14.
25 Timpl R, Brown J. Supramolecular assembly of basement membranes. *BioEssays* 1996; 18: 123–32.
26 Oudart JB, Monboisse JC, Maquart FX, Brassart B, Brassart-Pasco S, *et al*. Type XIX collagen: a new partner in the interactions between tumor cells and their microenvironment. *Matrix Biol* 2017; 57-58: 169–77.
27 Cosgrove D, Liu S. Collagen IV diseases: A focus on the glomerular basement membrane in Alport syndrome. *Matrix Biol* 2017; 57-58: 45–54.
28 Okada M, Yamawaki H. A current perspective of canstatin, a fragment of type IV collagen alpha 2 chain. *J Pharmacol Sci* 2019; 139: 59–64.
29 Assadian S, Teodoro JG. Regulation of collagen-derived antiangiogenic factors by p53. *Expert Opin Biol Ther* 2008; 8: 941–50.
30 Barczyk M, Carracedo S, Gullberg D. Integrins. *Cell Tissue Res* 2010; 339: 269–80.
31 Rebustini IT, Myers C, Lassiter KS, Surmak A, Szabova L, *et al*. MT2-MMP-dependent release of collagen IV NC1 domains regulates submandibular gland branching morphogenesis. *Dev Cell* 2009; 17: 482–93.
32 Hamano Y, Kalluri R. Tumstatin, the NC1 domain of α3 chain of type IV collagen, is an endogenous inhibitor of pathological angiogenesis and suppresses tumor growth. *Biochem Biophys Res Commun* 2005; 333: 292–8.
33 Li T, Kang G, Wang T, Huang H. Tumor angiogenesis and anti-angiogenic gene therapy for cancer. *Oncol Lett* 2018; 16: 687–702.
34 Wong EW, Cheng CY. NC1 domain of collagen alpha3(IV) derived from the basement membrane regulates Sertoli cell blood-testis barrier dynamics. *Spermatogenesis* 2013; 3: e25465.
35 Chen H, Mruk DD, Lee WM, Cheng CY. Regulation of spermatogenesis by a local functional axis in the testis: role of the basement membrane-derived noncollagenous 1 domain peptide. *FASEB J* 2017; 31: 3587–607.

36 Tang EI, Mok KW, Lee WM, Cheng CY. EB1 regulates tubulin and actin cytoskeletal networks at the Sertoli cell blood-testis barrier in male rats – an *in vitro* study. *Endocrinology* 2015; 156: 680–93.
37 Li L, Tang EI, Chen H, Lian Q, Ge R, Silvestrini B, *et al*. Sperm release at spermiation is regulated by changes in the organization of actin- and microtubule-based cytoskeletons at the apical ectoplasmic specialization – a study using the adjudin model. *Endocrinology* 2017; 158: 4300–16.
38 Vogl AW, Weis M, Pfeiffer DC. The perinuclear centriole-containing centrosome is not the major microtubule organizing center in Sertoli cells. *Eur J Cell Biol* 1995; 66: 165–79.
39 Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell* 2012; 149: 274–93.
40 Lipton JO, Sahin M. The neurology of mTOR. *Neuron* 2014; 84: 275–91.
41 Hay N, Sonenberg N. Upstream and downstream of mTOR. *Genes Dev* 2004; 18: 1926–45.
42 Bockaert J, Marin P. mTOR in brain physiology and pathologies. *Physiol Rev* 2015; 95: 1157–87.
43 Mok KW, Mruk DD, Cheng CY. Regulation of blood-testis barrier (BTB) dynamics during spermatogenesis via the "Yin" and "Yang" effects of mammalian target of rapamycin complex 1 (mTORC1) and mTORC2. *Int Rev Cell Mol Biol* 2013; 301: 291–358.
44 Mok KW, Mruk DD, Silvestrini B, Cheng CY. rpS6 regulates blood-testis barrier dynamics by affecting F-actin organization and protein recruitment. *Endocrinology* 2012; 153: 5036–48.
45 Mok KW, Chen H, Lee WM, Cheng CY. rpS6 regulates blood-testis barrier dynamics through Arp3-mediated actin microfilament organization in rat Sertoli cells. An *in vitro* study. *Endocrinology* 2015; 156: 1900–13.
46 Mok KW, Mruk DD, Cheng CY. rpS6 regulates blood-testis barrier dynamics through Akt-mediated effects on MMP-9. *J Cell Sci* 2014; 127: 4870–82.
47 Meyuhas O. Ribosomal protein S6 phosphorylation: four decades of research. *Int Rev Cell Mol Biol* 2015; 320: 41–73.
48 Mao B, Li L, Yan M, Wong CK, Silvestrini B, *et al*. F5-Peptide and mTORC1/rpS6 effectively enhance BTB transport function in the testis-Lesson from the adjudin model. *Endocrinology* 2019; 160: 1832–53.
49 Mao BP, Li L, Yan M, Ge R, Lian Q, *et al*. Regulation of BTB dynamics in spermatogenesis – insights from the adjudin toxicant model. *Toxicol Sci* 2019; 172: 75–88.
50 Yan M, Li L, Mao BP, Li H, Li SY, *et al*. mTORC1/rpS6 signaling complex modifies BTB transport function – an *in vivo* study using the adjudin model. *Am J Physiol Endocrinol Metab* 2019; 317: E-121-E38.
51 Li SY, Yan M, Chen H, Jesus TT, Lee WM, *et al*. mTORC1/rpS6 regulates blood-testis barrier (BTB) dynamics and spermatogenetic function in the testis *in vivo*. *Am J Physiol Endocrinol Metab* 2018; 314: E174–90.
52 Su WH, Cheng CY. Cdc42 is involved in NC1-peptide-regulated BTB dynamics through actin and microtubule cytoskeletal reorganization. *FASEB J* 2019; 33: 14461–78.
53 Pichaud F, Walther RF, Nunes de Almeida F. Regulation of Cdc42 and its effectors in epithelial morphogenesis. *J Cell Sci* 2019; 132: jcs217869.
54 Uehara S, Udagawa N, Kobayashi Y. Non-canonical Wnt signals regulate cytoskeletal remodeling in osteoclasts. *Cell Mol Life Sci* 2018; 75: 3683–92.
55 Wojnacki J, Quassollo G, Marzolo MP, Caceres A. Rho GTPases at the crossroad of singaling networks in mammals: impact of Rho-GTPases on microtubule organizaiton and dynamics. *Small GTPases* 2014; 5: e28430.
56 Assemat E, Bazellieres E, Pallesi-Pocachard E, Le Bivic A, Massey-Harroche D. Polarity complex proteins. *Biochim Biophys Acta* 2008; 1778: 614–30.
57 Bonello TT, Peifer M. Scribble: a master scaffold in polarity, adhesion, synaptogenesis, and proliferation. *J Cell Biol* 2019; 218: 742–56.
58 Wong EW, Cheng CY. Polarity proteins and cell-cell interactions in the testis. *Int Rev Cell Mol Biol* 2009; 278: 309–53.
59 Wen Q, Mruk D, Tang EI, Wong CK, Lui WY, *et al*. Cell polarity and cytoskeletons-Lesson from the testis. *Semin Cell Dev Biol* 2018; 81: 21–32.
60 Li L, Gao Y, Chen H, Jesus T, Tang E, *et al*. Cell polarity, cell adhesion, and spermatogenesis: role of cytoskeletons. *F1000Research* 2017; 6: 1565.
61 Chen H, Mruk DD, Lee WM, Cheng CY. Planar cell polarity (PCP) protein Vangl2 regulates ectoplasmic specialization dynamics via its effects on actin microfilaments in the testes of male rats. *Endocrinology* 2016; 157: 2140–59.
62 Chen H, Xiao X, Lui WY, Lee WM, Cheng CY. Vangl2 regulates spermatid planar cell polarity through microtubule (MT)-based cytoskeleton in the rat testis. *Cell Death Dis* 2018; 9: 340.
63 Li L, Mao B, Yan M, Wu S, Ge R, *et al*. Planar cell polarity protein Dishevelled 3 (Dvl3) regulates ectoplasmic specialization (ES) dynamics in the testis through changes in cytoskeletal organization. *Cell Death Dis* 2019; 10: 194.
64 Chen H, Mruk DD, Lui WY, Wong CK, Lee WM, *et al*. Cell polarity and planar cell polarity (PCP) in spermatogenesis. *Semin Cell Dev Biol* 2018; 81: 71–7.
65 Li L, Mao B, Wu S, Lian Q, Ge RS, *et al*. Regulation of spermatid polarity by the actin- and microtubule (MT)-based cytoskeletons. *Semin Cell Dev Biol* 2018; 81: 88–96.
66 Vogl A, Pfeiffer D, Mulholland D, Kimel G, Guttman J. Unique and multifunctional adhesion junctions in the testis: ectoplasmic specializations. *Arch Histol Cytol* 2000; 63: 1–15.
67 Vogl AW, Vaid KS, Guttman JA. The Sertoli cell cytoskeleton. *Adv Exp Med Biol* 2008; 636: 186–211.
68 Gao Y, Lui WY, Lee WM, Cheng CY. Polarity protein Crumbs homolog-3 (CRB3) regulates ectoplasmic specialization dynamics through its action on F-actin organization in Sertoli cells. *Sci Rep* 2016; 6: 28589.
69 Su WH, Wong EW, Mruk DD, Cheng CY. The Scribble/Lgl/Dlg polarity protein complex is a regulator of blood-testis barrier dynamics and spermatid polarity during spermatogenesis. *Endocrinology* 2012; 153: 6041–53.
70 Liu S, Li H, Wu S, Li L, Ge R, *et al*. NC1-peptide regulates spermatogenesis through changes in cytoskeletal organization mediated by EB1. *FASEB J* 2020; 34: 3105–28.
71 O'Donnell L, O'Bryan MK. Microtubules and spermatogenesis. *Semin Cell Dev Biol* 2014; 30: 45–54.
72 O'Donnell L. Mechanisms of spermiogenesis and spermiation and how they are disturbed. *Spermatogenesis* 2014; 4: e979623.
73 Tang EI, Mruk DD, Cheng CY. Regulation of microtubule (MT)-based cytoskeleton in the seminiferous epithelium during spermatogenesis. *Semin Cell Dev Biol* 2016; 59: 35–45.
74 Lie PP, Mruk DD, Lee WM, Cheng CY. Cytoskeletal dynamics and spermatogenesis. *Philos Trans R Soc Lond B Biol Sci* 2010; 365: 1581–92.
75 Mruk DD, Cheng CY. Cell-cell interactions at the ectoplasmic specialization in the testis. *Trends Endocrinol Metab* 2004; 15: 439–47.
76 Li MW, Mruk DD, Cheng CY. Gap junctions and blood-tissue barriers. *Adv Exp Med Biol* 2012; 763: 260–80.
77 Enders G. Sertoli-Sertoli and Sertoli-germ cell communications. In: Russell L, Griswold M, editors. The Sertoli Cell. Clearwater: Cache River Press; 1993. p447–60.
78 Tang EI, Lee WM, Cheng CY. Coordination of actin- and microtubule-based cytoskeletons supports transport of spermatids and residual bodies/phagosomes during spermatogenesis in the rat testis. *Endocrinology* 2016; 157: 1644–59.
79 Akhmanova A, Steinmetz MO. Control of microtubule organization and dynamics: two ends in the limelight. *Nat Rev Mol Cell Biol* 2015; 16: 711–26.
80 Akhmanova A, Steinmetz MO. Microtubule +TIPs at a glance. *J Cell Sci* 2010; 123: 3415–9.
81 Galjart N. Plus-end-tracking proteins and their interactions at microtubule ends. *Curr Biol* 2010; 20: R528–37.
82 Nehlig A, Molina A, Rodrigues-Ferreira S, Honore S, Nahmias C. Regulation of end-binding protein EB1 in the control of microtubule dynamics. *Cell Mol Life Sci* 2017; 74: 2381–93.
83 Mao BP, Li L, Ge RS, Li C, Wong CK, *et al*. CAMSAP2 is a microtubule minus-end targeting protein that regulates BTB dynamics through cytoskeletal organization. *Endocrinology* 2019; 160: 1448–67.
84 Romero S, Le Clainche C, Gautreau AM. Actin polymerization downstream of integrins: signaling pathways and mechanotransduction. *Biochem J* 2020; 477: 1–21.
85 Di Fiore PP, Scita G. Eps8 in the midst of GTPases. *Int J Biochem Cell Biol* 2002; 34: 1178–83.
86 Cheng CY, Mruk DD. Regulation of spermiogenesis, spermiation and blood-testis barrier dynamics: novel insights from studies on Eps8 and Arp3. *Biochem J* 2011; 435: 553–62.
87 Burianek LE, Soderling SH. Under lock and key: spatiotemporal regulation of WASP family proteins coordinates separate dynamic cellular processes. *Semin Cell Dev Biol* 2013; 24: 258–66.
88 Moreira B, Oliveira PF, Alves MG. Molecular mechanisms controlled by mTOR in male reproductive system. *Int J Mol Sci* 2019; 20: 1633.
89 Li N, Cheng CY. Mammalian target of rapamycin complex (mTOR) pathway modulates blood-testis barrier (BTB) function through F-actin organization and gap junction. *Histol Histopathol* 2016; 31: 961–8.
90 Wu S, Yan M, Ge R, Cheng CY. Crosstalk between Sertoli and germ cells in male fertility. *Trends Mol Med* 2020; 26: 215–31.