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Review

A laminin-based local regulatory network in the testis that supports spermatogenesis☆

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ABSTRACT

In adult rat testes, the basement membrane is structurally constituted by laminin and collagen chains that lay adjacent to the blood-testis barrier (BTB). It plays a crucial scaffolding role to support spermatogenesis. On the other hand, laminin-333 comprised of laminin-α3/ß3/γ3 at the apical ES (ectoplasmic specialization, a testis-specific cell-cell adherens junction at the Sertoli cell-step 8–19 spermatid interface) expressed by spermatids serves as a unique cell adhesion protein that forms an adhesion complex with α6ß1-integrin expressed by Sertoli cells to support spermiogenesis. Emerging evidence has shown that biologically active fragments are derived from basement membrane and apical ES laminin chains through proteolytic cleavage mediated by matrix metalloproteinase 9 (MMP9) and MMP2, respectively. Two of these laminin bioactive fragments: one from the basement membrane laminin-α2 chain called LG3/4/5-peptide, and one from the apical ES laminin-γ3 chain known as F5-peptide, are potent regulators that modify cell adhesion function at the Sertoli-spermatid interface (i.e., apical ES) but also at the Sertoli cell-cell interface designated basal ES at the blood-testis barrier (BTB) with contrasting effects. These findings not only highlight the physiological significance of these bioactive peptides that create a local regulatory network to support spermatogenesis, they also open a unique area of research. For instance, it is likely that several other bioactive peptides remain to be identified. These bioactive peptides including their downstream signaling proteins and cascades should be studied collectively in future investigations to elucidate the underlying mechanism(s) by which they coordinate with each other to maintain spermatogenesis. This is the goal of this review.

1. Introduction

In rodent and human testes, the base of the epithelium in each seminiferous tubule is surrounded by the tunica propria (Fig. 1). As noted in any cross sections of the seminiferous tubules, tunica propria is composed of the acellular zones of basement membrane (BM) and type I collagen layer, to be followed by the cellular zones of peritubular myoid cells, lymphatic endothelial cells and some fibroblasts (see the enlarged schematic drawing on the *right* penal in Fig. 1) [1,2]. BM, a modified form of extracellular matrix (ECM) in the mammalian testis [1,2], is in physical contact with the base of Sertoli cells, but also spermatogonia and spermatogonal stem cells [3,4] (Fig. 1). Thus, it is conceivable that there is crosstalk between cells in the seminiferous epithelium and the non-cellular BM components (e.g., laminin chains) to support spermatogenesis through mechanism(s) that yet to be investigated. This crosstalk likely involves integrin-based signaling based on studies in

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other epithelial cell-ECM interactions, most notably during tumorigenesis [5–8]. Studies in the testis have shown that modifications of the BM function by passive transfer of antibodies raised against the seminiferous tubule BM (STBM) [9] or noncollagenous fraction of STBMs [10] are capable of inducing focal sloughing of the seminiferous epithelium. Such epithelial injury in turn induces germ cell exfoliation from the epithelium, leading to infertility. Morphologically, the BM is a thin homogeneous sheet-like structure of 0.15 μm in thickness. The BM is constituted by ECM proteins, most notably type IV collagens (e.g., collagen α3 (IV) chains), laminins (e.g., laminin-α2 chains), heparin sulfate proteoglycans and entactin (also known as nidogen) in rodent testes [1,2,11,12]. Many of these BM constituent proteins are secretory products of Sertoli cells, but peritubular myoid cells are also known to produce some of these proteins (Fig. 1) [13–16]. Furthermore, basement membrane laminins are crucial to support cell homeostasis, differentiation, cell polarity, morphogenesis, organogenesis, vascular function, and tumorigenesis in multiple epithelia and/or endothelia [17–19]. Emerging evidence has unequivocally demonstrated that proteolytic fragments derived from some structural proteins in the basement membrane, in particular laminins, through matrix metalloproteinases (MMPs, e.g., MMP9) via proteolysis can generate biologically active peptides. These bioactive peptides, in turn, are capable of modifying cell adhesion, primordial germ cell (PMC) migration during embryogenesis in organs including the testis to support cellular/tissue homeostasis [20–22]. Furthermore, deletion of laminin chains in rodents in genetic models (Table 1) and/or mutations or genetic variations in laminin chains in

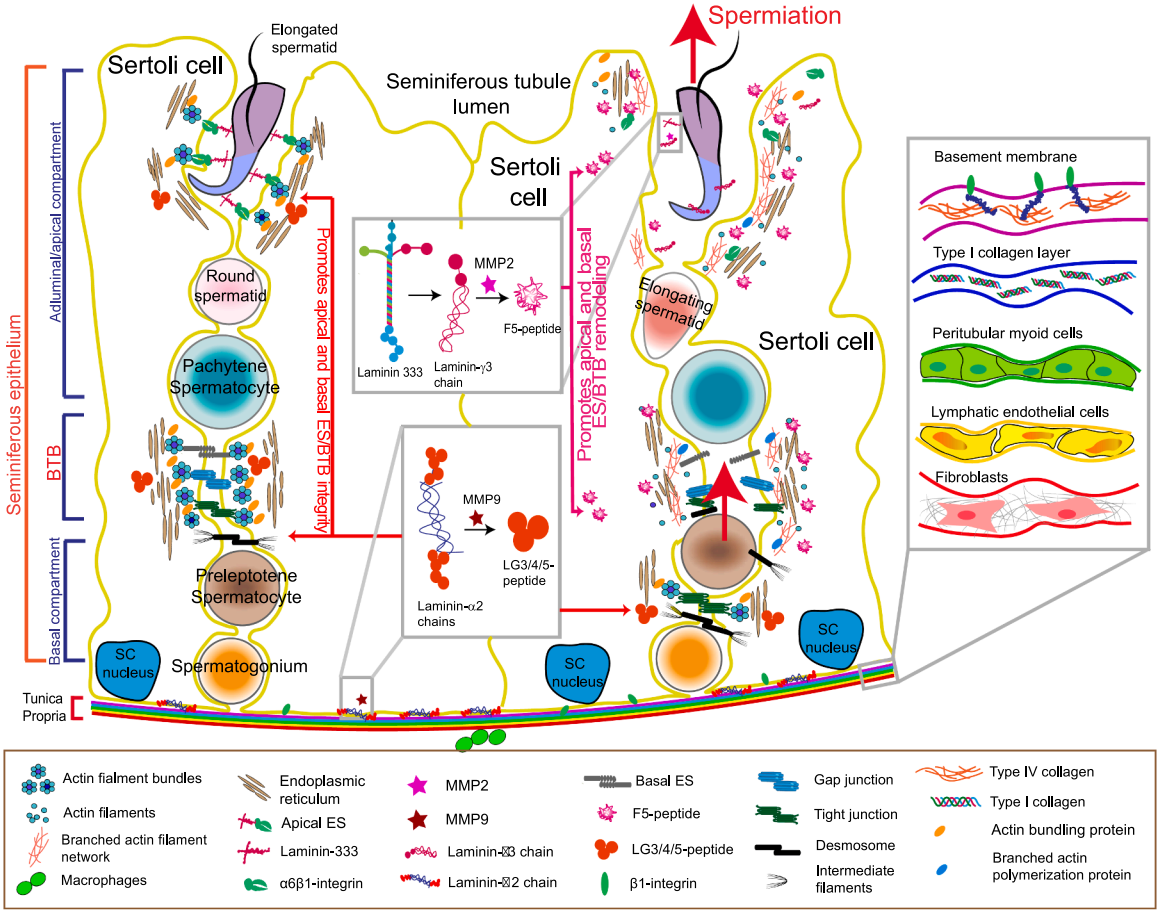


Fig. 1. Schematic drawing that depicts the role of laminin chain-derived F5- and LG3/4/5-peptides in supporting BTB function and spermatogenesis during the epithelial cycle. This is a schematic drawing illustrating a late stage VII tubule (*left* panel) undergoing transition to a stage VIII tubule (*right* panel) to facilitate the release of fully matured spermatids (i.e., spermatozoa) at spermiation. At stage VII of the epithelial cycle, apical ES and basal ES/BTB integrity is supported by actin filaments arranged in bundles, conferred by actin bundling proteins, such as Eps8 and palladin. Both the apical ES and basal ES share similar structural features in which an array of actin filament bundles are sandwiched in-between the ER (endoplasmic reticulum) and the apposing spermatid-Sertoli and Sertoli-Sertoli plasma membranes, respectively. The best studied apical ES adhesion protein complex is the spermatid expressed laminin-333 (comprised of laminin-α3/ß3/γ3 chains) and the Sertoli cell expressed α6ß1-integrin (see text for details). At the basal ES/BTB, the adhesion proteins are comprised of the TJ (tight junction) proteins (e.g., occludin-ZO1, JAM-A-ZO1) and basal ES proteins (e.g., N-cadherin/ß1-catenin; nectin/afadin), which are in turn supported and functionally coordinated by the gap junction proteins (e.g., connexin 43). At stage VII of the epithelial cycle, the apical ES and basal ES/BTB integrity are maintained by LG3/4/5-peptide released from the basement membrane laminin-α2 chain via proteolytic cleavage through the action of MMP9 which was shown to be highly expressed by Sertoli cells and possibly regulated by TNFα which is also a product of Sertoli cells [109]. However, at stage VIII, F5-peptide released from laminin-γ3 chain through the action of MMP2 [21] which promotes remodeling of the basal ES/BTB to facilitate the transport of preleptotene spermatocytes across the immunological barrier, but also the release of mature spermatids (i.e., spermatozoa) through degeneration of the apical ES. Studies have also shown that macrophages residing in the interstitium and along the seminiferous tubules, close to the basement membrane at the tunica propria, that contribute to the maintenance of spermatogonial stem cell niche and testis function (e.g., immunological environment) [110–112] between seminiferous tubules in adult mouse testes may also be the source of MMPs. It is of interest to note that this notion of bioactive peptides generated locally in the testis to support spermatid transport across the seminiferous epithelium during spermatogenesis as depicted here is supported by earlier studies. For instance, the migration of PGCs (primordial germ cells) during embryonic development in the mouse embryo involve interactions between migrating germ cells and extracellular matrix glycoproteins, most notably laminin chains [20] (see text for details). Abbreviations are noted in the boxed key panel, including the composition of the tunica propria.

Table 1
Phenotypes of genetic models in mice following deletion of the corresponding laminin gene.

Gene	Study model	Phenotypes
Lama1	Conditional *Lama1* knockout mice in the epiblast lineage	Mice are viable, displaying behavioral disorders due to impaired formation of the cerebellum [113]
Lama2	*Laminin-α2 chain deficient-dy^{3K}/dy^{3K}* mice	Growth retardation and severe muscular dystrophic symptoms and mice died by 5 weeks of age [114]; congenital muscular dystrophy [115]
Lama3	*Lama3$^{-/-}$*	Arrest in glomerular endothelial cells adjacent to basement membrane in kidneys [116], subepidermal blisters [117]
Lama4	*Lama4$^{-/-}$*	Pre- and postsynaptic modifications at the neuromuscular junction [118], chronic kidney disease [119], enhanced energy expenditure and increased beige subcutaneous adipose tissue [120]
Lama5	*Lama5$^{-/-}$*	Early embryonic lethality: exencephaly, syndactyly, and placentopathy [121], hair follicle regression [122]
Lamb1	*Lamb1$^{-/-}$*	Embryonic fatality by E5.5 [123]
Lamb2	*Lamb2$^{-/-}$*	Pierson syndrome [124], a mitochondria disease due to deletion in mitochondrial DNA (with exocrine pancreas dysfunction and sideroblastic anemia)
Lamb3	Specific conditional *Lamb3* KO mice in liver epithelial cells	No notable phenotype [125]
Lamc1	*Lamc1$^{-/-}$*	Mice died within a day of implantation [123]
Lamc2	*Lamc2$^{-/-}$*	Blistering phenotype on days 1–2 and died within 5 days of birth [126]
Lamc3	*Lamc3$^{-/-}$*	Mice are viable, with retinal dysplasia due to an increase in capillary branching in outer retina [18]

Abbreviations: *Lama*, laminin-α; *Lamb*, laminin-ß; *Lamc*, laminin-γ.

Table 2
Pathological conditions in humans due to mutations or genetic variations in corresponding laminin chains.

Gene (s)	Affected tissue (s)/ organ (s)	Major pathological condition (s)/disease (s)
LAMA1	Testis , brain	Poretti-Boltshauser Syndrome [127], cerebellar cysts and dysplasias [128]
LAMA2	Muscle, placenta	Congenital muscular dystrophy [129]
LAMA3	Skin, dermal–epidermal junction	Junctional epidermolysis bullosa [130]
LAMA4	Heart	Dilated cardiomyopathy [131]
LAMA5	Kidney, eye	LAMA5 multisystem syndrome [132], pediatric nephrotic syndrome [133], defects in kidney, craniofacial and limb development [134]
LAMB1	Brain	Leukoencephalopathy [135,136], bilateral cerebellar cysts and cerebral white matter lesions with cortical dysgenesis [137], cobblestone brain malformation [138]
LAMB2	Kidney, eyes	Pierson syndrome [133]
LAMB3	Skin, dermal–epidermal junction	Junctional epidermolysis bullosa [130]
LAMC1	Endometrium, ovary, brain	Autosomal dominant Dandy-Walker malformation and occipital cephaloceles (ADDWOC) [139], serous tubal intraepithelial carcinoma [140]
LAMC2	Skin, dermal–epidermal junction	Junctional epidermolysis bullosa [130]
LAMC3	Testis, brain, eyes	Complex bilateral occipital cortical gyration abnormalities [141], structural and functional changes in visual attention networks [142]

Abbreviations: *LAMA*, *laminin-α*; *LAMB*, *laminin-β*; *LAMC*, *laminin-γ*.

humans (Table 2), as well as collagen chains, fibronectin, elastin and others also lead to multiple pathological conditions [23–26]. In this review, we mainly evaluate recent data regarding biologically active fragments derived from laminin chains at the basement membrane of the adult rat testes. However, we also focus on the laminin chains at the spermatid/Sertoli cell interface in the seminiferous epithelium, which is a testis-specific *aytpical* anchoring junction type called apical ectoplasmic specialization (ES) [27,28]. Most importantly, we highlight the importance of these bioactive fragments in spermatogenesis in rodent testes (Fig. 1). These findings thus support the emerging concept of a laminin-based local regulatory network that coordinates cellular events across the seminiferous epithelium. Based on these findings, we also propose a hypothetic model in this review. For instance, how are these bioactive peptides coordinate with each other to support the release of sperm at spermiation *and* restructuring of the blood-testis barrier (BTB) to facilitate the transport of preleptotene spermatocytes across the BTB? This is of interest physiologically since both events take place at stage VIII of the epithelial cycle in rat and mouse testes [29,30]. We focus our discussion using the testis of adult rodents as a study model since much of the information is derived from studies in rats, as well as genetic models in mice based on deletion of corresponding laminin genes (Table 1). It is noted that studies on the role of laminins in human spermatogenesis remain to be investigated. Yet studies have shown that mutations and/or genetic variations of laminin genes lead to various pathological conditions (Table 2). These findings thus illustrate the physiological significance of laminin chains in humans.

In this review, we do not elaborate on the role of laminins (and/or their receptors such as integrins) on unipotent spermatogonial stem cell biology [31–36] since this topic has been reviewed by others [37,38] including in this Special Issue to avoid redundancy. Instead, we focus our discussion on the biologically active laminin fragments produced endogenously at the two specific sites across the seminiferous epithelium, namely the basement membrane and the apical ES, during the epithelial cycle that serve as crucial regulatory components in a local regulatory network to support and coordinate cellular events of spermatogenesis (Fig. 1). These include BTB function, and Sertoli cell and germ cell (most notably haploid spermatids) adhesion.

2. Laminins in the testis

2.1. Background

Laminin is a heterotrimeric glycoprotein comprised of one α, one ß, and one γ chain to create a functional laminin ligand of ~400–900 kDa (Fig. 2). Five α (with α3 consists of two isoforms of α3A and α3B, thus generating six α chains altogether), three ß and three γ subunits based on genomic analysis are known to date in cells/tissues from humans and rodents (Figs. 2 and 3) [12,39]. Theoretically, at least 54 possible combinations of heterotrimeric $\alpha_x ß_y \gamma_z$ laminins can exist. However, only 17 laminins through different combinations of $\alpha_x ß_y \gamma_z$ have been identified to date in mammalian tissues including ECM and organs, such as the testis (Fig. 2) [40], which are also essential structural scaffolds in numerous tissues of the mammalian body [12,19] (Fig. 2). All the α, ß and γ laminin chains share similar structural features but each chain also possesses unique sequences and functional domains (Fig. 3).

2.2. Structural and functional features

Our discussion here is focused mostly on laminin-α2 and laminin-γ3 chains since the biologically active peptides, namely LG3/4/5- (Figs. 3 and 4) and F5- (Figs. 4 and 5) peptides, are derived from these two laminin chains in the testis, respectively. More important, these two bioactive peptides have different biological effects in the testis, but their concerted actions are crucial to support spermatogenesis which will be discussed based on findings summarized in Fig. 6.

First, laminin-α2 chain is a notable structural component in the

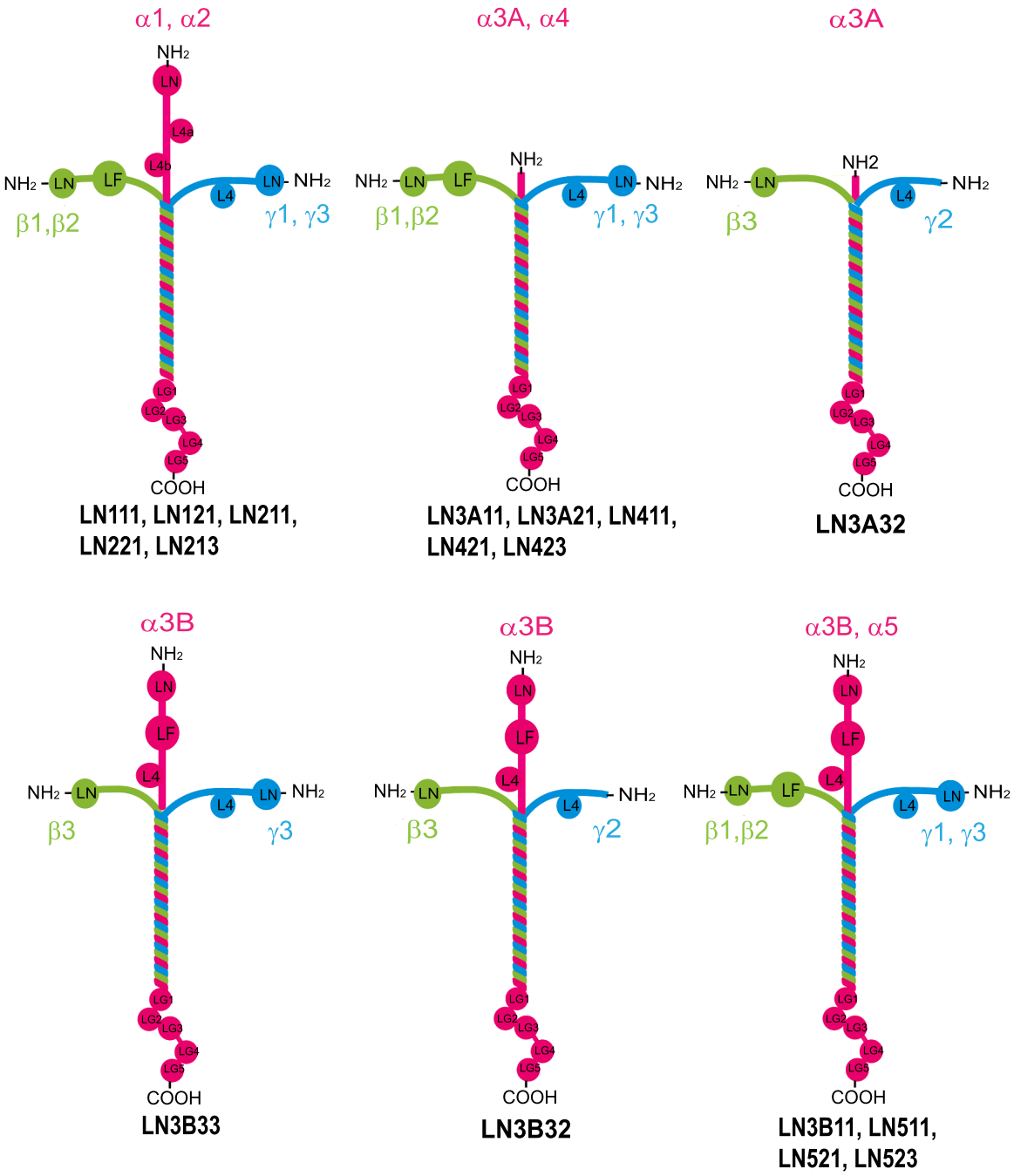


Fig. 2. The functional trimeric laminin ligands in epithelial cells including the seminiferous epithelium of adult rat testes that modulate epithelial function and spermatogenesis. At present, there are six laminin-α chains of α1 through α5 since there are two laminin-α chains of α3A and α3B, three laminin-ß3 chains of ß1, ß2 and ß3, and three laminin-γ chains of γ1, γ2, and γ3 chains. Thus, they can theoretically generate 54 different combinations of αßγ trimeric laminin ligands, and at present, 17 laminin functional ligands are known to exist in different epithelia and/or endothelia, including the laminin-333 (or LN3B33) at the apical ES of the adult rat testes. Detailed of the different functional domains of these laminin chains are shown in Fig. 3.

basement membrane of rodent testes since its deletion in mice led to male infertility [41] (Table 1). The N-terminal region of laminin-α2 chain contains the short arm, which is comprised of laminin globular domains [laminin N-terminal domain (LN) near its N-terminus, to be followed by the laminin 4a domain (L4a) and laminin 4b domain (L4b)], and rod domains of epidermal growth factor (EGF; laminin EGF like domain a) LEa, LEb and LEc (Figs. 3 and 4). The short arm is involved in interacting with other ECM constituent proteins in the basement membrane (BM) to maintain BM stability through scaffolding [12,42] (Figs. 3 and 4). The long arm in the laminin-α2 chain is comprised of the relatively long laminin coiled-coil domain (LCC), to be followed by the five laminin globular (LG) domains of LG1 to LG5 at the C-terminal region (Figs. 3 and 4). The coiled coil domain from each of the laminin α, ß and γ chains in the heterotrimeric functional laminin thus create a structural motif in which the three α-helices are coiled (or twisted) together similar to the three strands of a rope [43] (Fig. 4). Each coiled coil domain contains a heptad repeat of *hxxhcxc* (h and c refer to hydrophobic and charged amino acid residues, respectively). Studies have shown that coiled-coil domains are used to modulate the architecture of organelles (e.g., Golgi, centrioles), tethering of transport vesicles (e.g., endosomes, cellular cargoes), cellular domain conformational changes such as in motor proteins to regulate cargo binding, and to maintain spacing of

Fig. 3. Schematic drawing that illustrates the functional domains of the six laminin α3 chains, three laminin ß chains and three laminin γ3 chains in humans. There are three basic structural domains in the N-terminal region of the laminin chains: LN (laminin N-terminal), LE (laminin-type epidermal growth (EGF) factor-like), and L4/LF (laminin IV) domains. The short arm of laminin chain, such as laminin-α2, is comprised of rod domains of EGF (LEa, LEb, LEc) and globular domains (LN, L4a, L4b). In brief, following the LN domain, the LEa is comprised of a first array of four LE domains of LEa1 to LEa4, then one globular domain L4a (consisting of a long stretch of amino acid residues inserted within one LE domain), followed by the LEb which is comprised of 8 LE domains of LEb1 to LEb8. This is turn followed by another globular domain L4b (consisting of a long stretch of amino acid residues inserted within one LE domain), and finally a stretch of three LE domains of LEc1 to LEc3. Long arm of laminin α2 is comprised of LCC (laminin coiled-coil) domain and 5C-terminal LG domains of LG1, LG2, LG3, LG4 and LG5. Proteolytic cleavage is close to the N-terminus of LG3 to generate the LG3/4/5-peptide (see Fig. 4 for details). It is noted that the laminin-α3A chain consists of only three LE domains without the globular domains of LN, L4a and L4b. The predicted molecular mass (Mr) based on the primary amino acid residues of the polypeptide of each of these laminin chains in humans is also shown.

unique function (e.g., catalytic activities) at fixed distances [44]. Following the coiled coil motif is the LG domains, which are involved in cellular interactions with integrin receptor to induce integrin signaling (Fig. 4). It is noted that this laminin (ligand) and integrin (receptor) interaction is unrelated to the scaffolding function in the basement membrane [45]. However, in this region, it also contains biologically active domain(s) if cleaved from the parent chain via limited proteolysis, such as through the action of MMP9 on laminin-α2 chain, which generates the LG3/4/5-peptide in the testis to promote spermatogenesis [46–48]. More important, this LG3/4/5-peptide was shown to be generated locally in the basement membrane to promote BTB integrity, however, some LG3/4/5-peptide was also transported to the apical ES to maintain its integrity support spermatid adhesion via a microtubule-dependent transport mechanism [47,48].

Second, laminin-γ3 chain, similar to laminin-α2 chain, also has a LN domain, to be followed by the LEa, L4 and LEb domains at its short arm in the N-terminal region (Fig. 3). This short arm is then followed by the long arm comprised of the LCC domain at its C-terminal region which does not contain the LG domains found in laminin-α chains (Figs. 3 and 5). Unlike LG3/4/5-peptide which is derived from the LG domain after the LCC domain located near its C-terminal region (Fig. 4), F5-peptide is derived from domain IV [49,50] near its N-terminal region, which is located inside the L4 domain in laminin-γ3 chain, through proteolytic cleavage by MMP2 [21] (Fig. 5). Most notably, MMP2 was robustly expressed at stage VIII of the epithelial cycle at the apical ES [21]. This thus releases F5-peptide consisting of 50 amino acid residues from domain IV of laminin-γ3 chain [49] at the Sertoli-spermatid adhesion site of apical ES (Fig. 5) [22]. In this context, it is of interest to note that laminin-γ3 is one of the most notable non-basement membrane laminin chains in the murine [18] and rat [40] testes. Laminin-γ3 also plays a crucial role in eye development in mice including retinal lamination, photoreceptor organization and ganglion cell differentiation [51]. In fact, deletion of laminin-γ3 chain in mice led to retinal dysplasia due to an increase in capillary branching in outer retina [18] (Table 1).

2.3. Remarks

In this context, it is of interest to note that laminin chains can also be cleaved via proteolysis through the action of MMPs to generate biologically active fragments in other epithelia. Each of these fragments can have different regulatory roles in modulating cellular function, including cell differentiation, migration, adhesion, vascular function, junction dynamics and others [52–54]. These bioactive fragments are also distinctively different functionally from the parental laminin chains which are scaffolds to confer structural supports.

3. F5-peptide: an endogenously produced regulatory peptide derived from laminin-γ3 chain in the testis

3.1. F5-peptide and spermatogenesis

In the testis, one of the best studied laminin ligands is laminin-333, comprised of α3, ß3 and γ3 chains, which are restrictively but robustly expressed by elongated spermatids at the apical ES in stages VII-VIII [40,

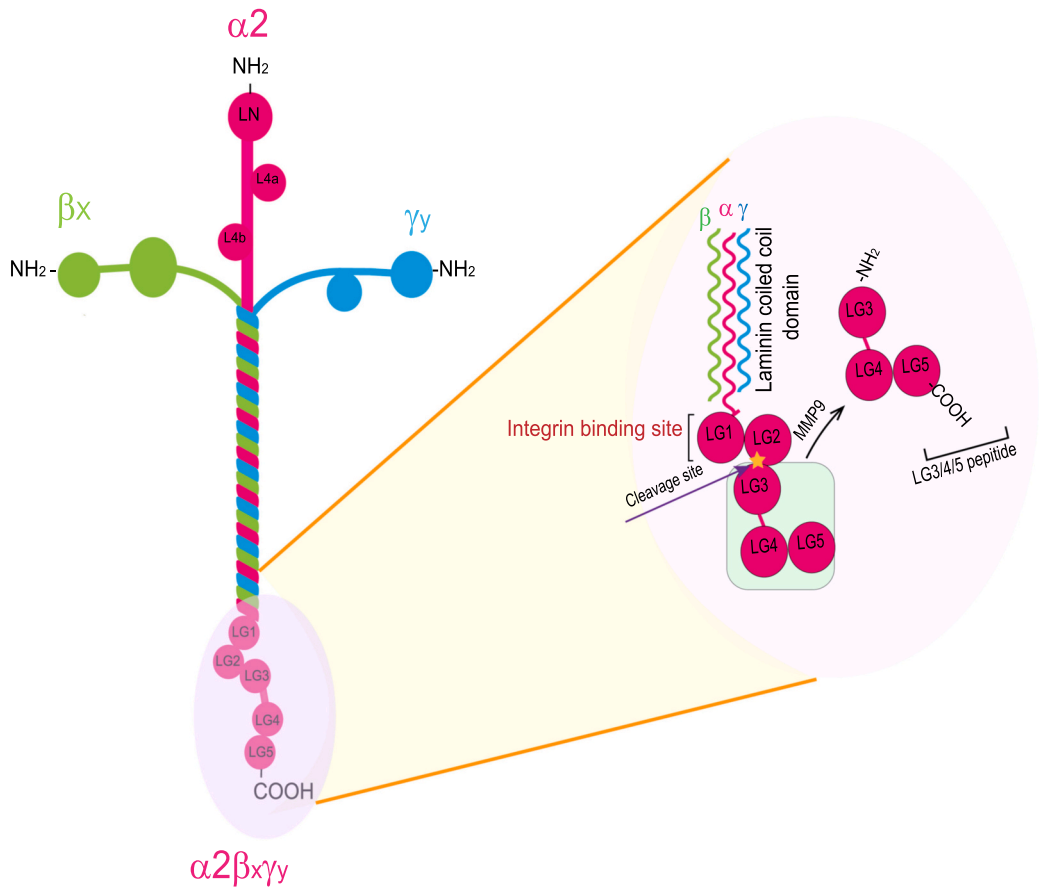


Fig. 4. The functional laminin-α2ßxγy ligand in the basement membrane that generates the LG3/4/5-peptide in the testis. At present, the identity of the ß and γ chains in the basement membrane that generates the LG3/4/5-peptide remain unknown. Studies have shown that the C-terminal LG1 is the predicted integrin binding site for the laminin ligand to activate signaling function downstream of the integrin receptor.

55] (Figs. 1 and 5). This laminin ligand in turn forms an cell adhesion complex with the Sertoli cell expressed α6ß1-integrin [21,56,57] at the apical ES. Most notably, this laminin-333/α6ß1-integrin cell adhesion complex confers one of the strongest cell adhesive in mammalian cells, considerably stronger desmosomes [58]. This is unusual since desmosome plays a prominent role in maintaining skin barrier function in the mammalian body and is considered to be the strongest adhesive anchoring junction [59,60]. Unlike other laminin chains, the laminin-333 is not found at the Sertoli cell-BM interface, but limited to the apical ES at the Sertoli cell-spermatid interface (Fig. 1). In brief, laminin-γ3 chain is a key structural component of the apical ES in the testis. However, studies have shown that laminin-γ3 chain at the apical ES can be cleaved at its domain IV near the N-terminal region via the action of MMP2 in late stage VIII of the epithelial cycle [21], to generate a proteolytic product designated F5-peptide of about 50 amino acid residues [49] (Fig. 5). Studies have been conducted using synthetic F5-peptide or the corresponding cDNA of F5-peptide cloned into mammalian expression vector pCI-neo for its overexpression in vitro with primary cultured Sertoli cells versus testes in adult rats in vivo [49,61]. F5-peptide was found to serve as a potent bioactive peptide to induce apical ES degeneration, thereby facilitating the release of sperm at spermiation in stage VIII tubules [49,61]. On the other hand, F5-peptide also exerts its effects outside the apical ES by inducing remodeling of the basal ES and blood-testis barrier (BTB) at the Sertoli cell-cell interface near the basement membrane [49,50,61] to facilitate the transport of preleptotene spermatocytes across the immunological barrier in stage VIII tubules, acting as an autocrine factor (Figs. 1 and 5). As such, the combined actions of F5-peptide at the apical ES and basal ES/BTB thus coordinate the events of spermiation and preleptotene spermatocyte transport across the immunological barrier that take place simultaneously, but at the opposite ends, across the seminiferous epithelium (Fig. 1). It also represents one of the key regulatory peptides produced locally in the testis to support spermatogenesis. Interestingly, studies have shown that this action of F5-peptide that supports spermatogenesis is mediated through its effects on the organization of actin and microtubule (MT) cytoskeletons via changes in the spatial expression of multiple actin regulatory proteins (e.g., branched actin polymerization proteins Arp2/3 complex and N-WASP vs. actin barbed end capping and bundling protein Esp8) and microtubule regulatory proteins [61]. More important, since LG3/4/5-peptide derived from laminin-α2 chains at the basement membrane has a contrasting biological effect (see Section 4 below) vs. F5-peptide on spermatogenesis, their concerted actions have prompted us to provide a hypothetical model regarding how these two bioactive peptides in the local regulatory network support spermatogenesis as outlined in Fig. 6.

3.2. Laminin fragment (ligand) and integrin (receptor) mediated signaling function

Laminins or their biologically active fragments are ligands, which usually work in concert with integrin receptors through interactions between the ECM and epithelial cells mediated via their C-terminal region that interacts with corresponding integrin receptor, which in turn induces integrin-based signaling to modulate multiple cellular functions [62–67] (Fig. 4). However, emerging evidence has demonstrated several non-integrin receptors for laminins, which include dystroglycan (e.g., α-dystroglycan) and 37/67 laminin receptor (37/67LR) [68,69]. Interestingly, studies by co-immunoprecipitation and dual-labeled

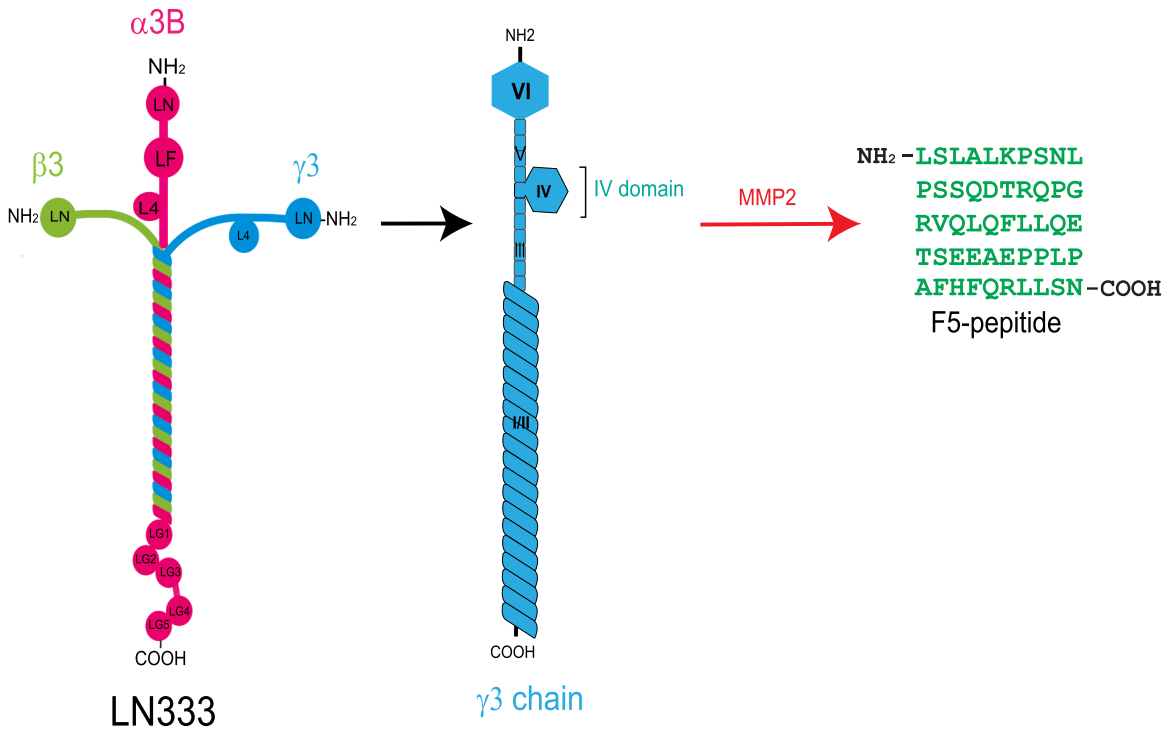


Fig. 5. The functional laminin-333 ligand at the apical ES (ectoplasmic specialization) that generates the F5-peptide derived from laminin-γ3 chain. The F5-peptide is derived from the short arm of laminin-γ3 chain in domain IV, which is comprised of 40 amino acid residues through the proteolytic cleavage of MMP2 at the apical ES, likely at stage VIII of the epithelial cycle (see text for details).

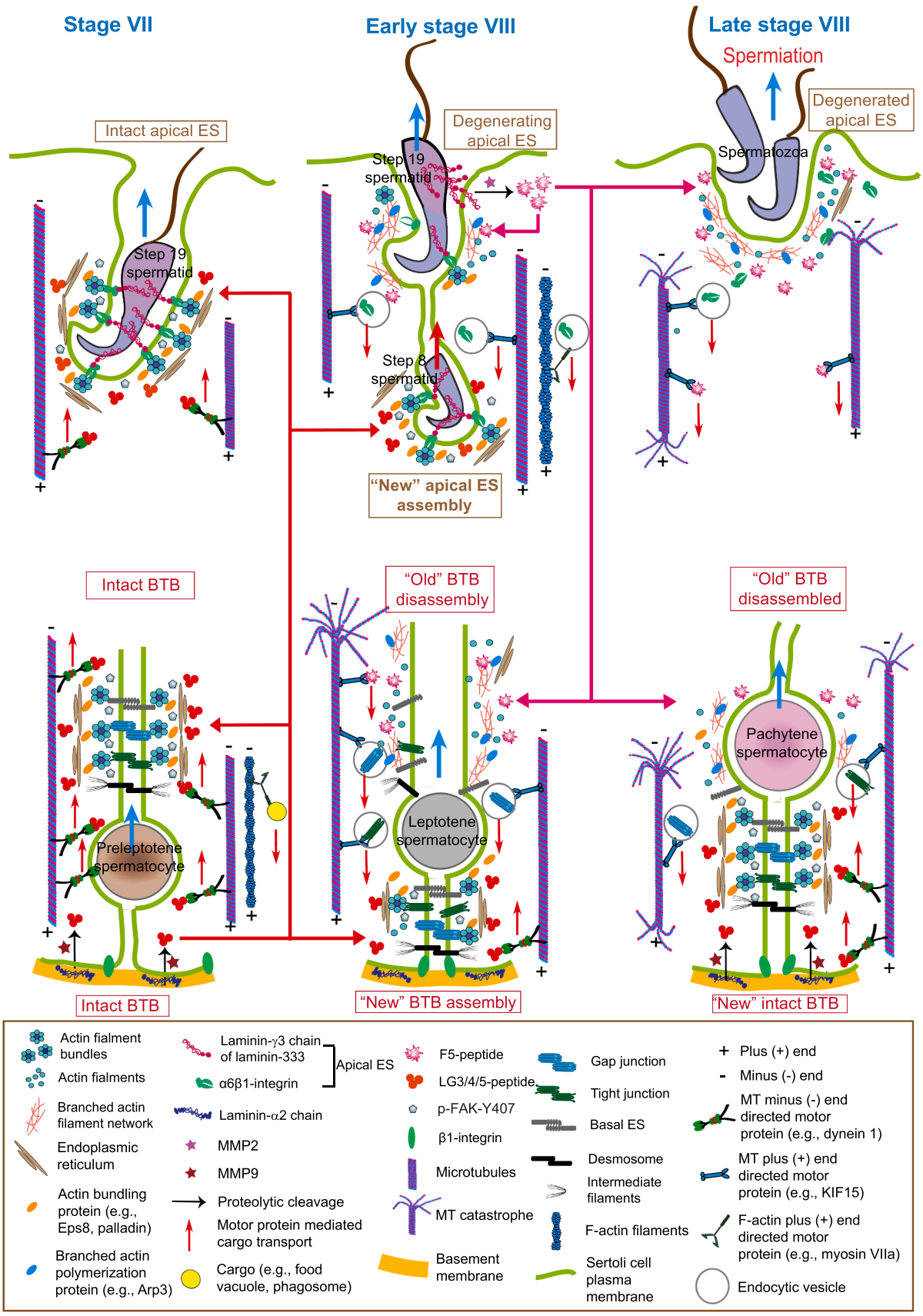
immunofluorescence microscopic analyses have shown that laminin-γ3 forms a protein complex with ß1-integrin [21]. In fact, laminin-333 expressed by elongate spermatids in the rat testis forms an adhesion protein complex with α6ß1-integrin which is specifically expressed by Sertoli cells [70]. Also, α6ß1-integrin is one of the best studied non-basement membrane associated integrin receptor at the apical ES [21,56,57,71]. Additionally, studies have shown that α6ß1-integrin is crucial to maintain spermatid adhesion onto the Sertoli cells at the apical ES in the seminiferous epithelium until at late stage VIII when spermiation takes place in the rat testis, coinciding with the notable down-regulation of α6ß1-integrin expression at the site [72,73]. Studies in other epithelia have also shown that α3ß1, α6ß1, and α6ß4 integrins are putative receptors for a number of laminin ligands such as laminin-511 [74]. In fact, studies have reported that α3ß1, α6ß1, α6ß4, and α7ß1 integrins are receptors for laminin ligands in intestinal epithelial cells under and pathologic conditions [75]. It is likely that α6ß1-integrin expressed by Sertoli cells at the apical ES is the putative receptor for F5-peptide, but since α6ß1-integrin is absent at the BTB site [50], the identity of the receptor for F5-peptide at the BTB remains to be identified. In this context, it is of interest to note that the α6ß1-integrin receptor is strongly expressed by spermatogonial stem cells (SSCs) in a number of mammalian species including rodents, which has been used by investigators to serve as a valuable marker to identify, isolate and culture SSCs [38,76,77]. However, the ligand(s) that interact with SSC α6ß1-integrin to modulate SSCs, PSCs (pluripotent stem cells) or iPSC (induced pluripotent stem cells), and the downstream signaling cascade following integrin/ligand activation remains to be delineated [78,79].

3.3. Remarks and future investigation

Studies through the use of scRNA-Seq will provide much needed information regarding the identity of the integrin receptor for the F5-peptide, its downstream signaling cascade and involving signaling proteins, besides p-FAK-Y407, following activation of the integrin receptor upon its coupling with the F5-peptide. Studies on integrin-based signaling have shown that activation of FAK, RhoA and mTOR/Akt signaling proteins/complexes are some of the most important prevalent cascades [80–82]. Indeed, studies have shown that overexpression of F5-peptide in the testis in vivo that led to defects in spermatogenesis by impeding germ cell adhesion in the seminiferous epithelium was associated with a down-regulation of p-FAK-Y407 [49,61]. This was also associated with notable changes in its distribution at the apical ES and basal ES/BTB sites whereby p-FAK-Y407 no longer robustly expressed at these two sites but mis-distributed [49]. This finding is remarkable since p-FAK-Y047, the activated form of FAK, is a known BTB regulatory protein by conferring BTB integrity through its robust expression at the apical ES and basal ES/BTB to support spermatid and Sertoli cell adhesion at the corresponding site [83].

3.4. A hypothetical model by which F5-peptide regulates spermatogenesis

In brief, F5-peptide is a potent regulator of spermatid and Sertoli cell adhesion by promoting the timely degeneration of apical ES to facilitate the release of sperm at spermiation but also by promoting BTB restructuring to facilitate preleptotene transport at the immunological barrier through a down-regulation of FAK signaling downstream or by perturbing its spatial expression in particular p-FAK-Y407 (Fig. 6). These findings also suggest that an overexpression of p-FAK-Y407 can promote spermatogenesis through an increase in FAK-mediated enhancement in cell adhesion function. Indeed, overexpression of a human or rat p-FAK-Y407 mutant, namely p-FAK-Y407E by making this mutant constitutively active, in primary human [84] or rat [85] Sertoli cell cultures, they are capable of blocking or rescuing Sertoli cell injury induced by PFOS (perfluorooctanesulfonate, an environmental toxicant known to induce Sertoli cell and testis injury [86]).



(caption on next page)

Fig. 6. A hypothetic model depicting the role of F5- and LG3/4/5-peptides in regulating BTB dynamics and cell adhesion across the seminiferous epithelium to support spermatogenesis during the transition of stage VII to VIII of the epithelial cycle. This hypothetic model as shown in the schematic drawings in the top and bottom panel illustrates the role of the F5- and LG3/4/5-peptide derived from the apical ES and basement membrane in modulating the corresponding apical ES (top panel) and basal ES/BTB (bottom panel) remodeling. The left panel represents stage VII, transitioning to early stage VIII (middle panel) and then late stage VIII (right panel) of the epithelial cycle. Intact apical ES is maintained by LG3/4/5-peptide released from laminin-α2 chain in the basement membrane which is transported to the corresponding site via a microtubule (MT)-dependent mechanism (stage VII, left panel). LG3/4/5-peptide also promotes the assembly of new apical ES when step 8 spermatids arise at stage VIII (middle top panel), and the assembly of new BTB and its (middle lower panel) maintenance in early (right lower panel) stage VIII tubule. On the other hand, F5-peptide released from laminin-γ3 chain at the apical ES at stage VIII promotes the disassembly and degeneration of apical ES, but it also promotes remodeling of the old BTB above the preleptotene spermatocyte under transport at the immunological barrier such that the BTB integrity can be maintained during this process. These changes are supported by actin bundling proteins (e.g., Eps8, palladin), branched actin polymerization (or nucleation) proteins (e.g., Arp2/3 complex, N-WASP), and the corresponding MT minus (-) end directed motor proteins (e.g., dynein 1), MT plus (+) end directed motor proteins (e.g., KIF15) and F-actin barbed end or plus (+) end directed motor proteins (e.g., myosin VIIa). Keys to different cellular structures and proteins in the schematic drawing are shown in the bottom boxed panel. See text for details.

4. LG3/4/5-peptide: an endogenously produced regulatory peptide derived from laminin-α2 chain in the testis

4.1. Unique function and structural features of laminin α chains in laminin ligands that interact with integrin receptor

Studies of different laminin ligands have shown that laminin α chains, including laminin-α2, utilize their C-terminal end, i.e., the long arms of laminin α chains, to interact with proteins in the plasma membrane (Figs. 2 and 4). This usually involves integrin-based receptor to induce (or relay) biochemical and/or mechanical communications between intracellular and extracellular signaling, including outside-in and/or inside-out signaling, and the subsequent signal cascades following integrin activation (Fig. 4) [12]. LG1 at the end of the laminin coiled-coil (LCC) domain (from the N-terminal region) is likely the putative integrin binding site (Fig. 4). However, integrin binding to the laminin C-terminal region requires the participation of the LG1, LG2 and LG3 trio and also the coiled-cold domains of the laminin α, ß and γ chains altogether, since separation of the LG1-LG3 trio [87] or disruption of the coiled-coil domains structure [88] perturbs cell adhesion promoting function of laminins. Based on these earlier reports, it is expected that fragments of the laminin-α2 chains at the C-terminus such as the LG3/4/5-peptide also serves as the ligand to induce integrin signaling, but its signaling function can be considerably different from the intact laminin-α2 chains (Fig. 4). Studies have also shown that other cell surface receptors such as GPCRs (G protein-coupled receptors; e.g., GPR126, also known as ADGRG6) interact with laminins (e.g., laminin-211) [89,90]. On the other hand, the N-terminal short arms of the laminin chains (Fig. 4) interact with other basement membrane proteins to elicit assembly, disassembly and/or stabilization of basement membrane in response to stages of epithelial cycle of spermatogenesis. Furthermore, studies have shown that different functional domains along the laminins can interact with small molecules such as cytokines (e.g., TGF-ßs, TNFα) which are also known regulators of BTB dynamics [91,92]. Thus, laminins also serve as a storage site for regulatory cytokines and growth factors to modulate different cellular functions, including cell differentiation, cell movement and others [17,93]. This cytokine binding function of laminins is important since studies have shown that cytokines are important regulators of spermatogenesis and other testis function [91,94–96], and this pool of cytokines maintained by laminins across the seminiferous epithelium would play a crucial role to support spermatogenesis and testis development.

4.2. LG3/4/5-peptide and spermatogenesis

4.2.1. Background

As noted in Fig. 4, laminin-α2 can undergo MMP9-mediated proteolysis in the basement membrane of adult rat testes to generate the biologically active LG3/4/5-peptide from the C-terminal region as recently reported [46,47]. A specific knockdown of laminin-α2 chain by RNAi through the use of a short hairpin RNA (shRNA) of 21 nucleotides against laminin-α2 chain specifically (versus a negative control) was cloned into pGene-Clip hMGFP mammalian expression vector for its overexpression in Sertoli cells cultured in vitro with an established BTB [47]. Overexpression of this laminin-α2 shRNA was found to perturb the Sertoli cell tight junction (TJ)-permeability function when the laminin-α2 expression was silenced by ~60% [47]. This observation thus supports the notion that laminin-α2 promotes BTB integrity under physiological conditions in vivo besides serving as a scaffolding protein in the basement membrane. This observation also suggests that laminin-α2 chain in the basement membrane, similar to laminin-γ3 chain in the apical ES, may generate a bioactive peptide whose action is in sharp contrast to the F5-peptide by promoting BTB remodeling (Figs. 1 and 6). This possibility was subsequently confirmed when different fragments of the laminin-α2 chains were cloned into the mammalian expression vector pCI-neo by mapping the biological activity that promotes Sertoli cell BTB integrity to the 80 kDa fragment of the LG3/4/5-peptide [48]. It is now known that the LG3/4/5-peptide, most notably the LG5-peptide, is a potent bioactive peptide to promote BTB integrity, unlike F5-peptide which perturbs Sertoli cell BTB integrity and affects spermatogenesis in vivo, the LG3/4/5-peptide (and the LG5-peptide) promotes spermatogenesis and it is capable of blocking cadmium-mediated BTB injury in the testis in vivo [48]. In brief, LG3/4/5-peptide is a spermatogenesis promoting biomolecule which promotes both BTB integrity and spermatogenesis as noted in the recent in vivo study [48]. Furthermore, the notion that this laminin-α2 chain generated LG3/4/5-peptide can promote spermatogenesis is also supported by an earlier report using genetics approach wherein seminiferous tubules of laminin-α2 chain-deficient dy^{3k}/dy^{3k} mice were infertile, and seminiferous tubules from these adult mice contained very few spermatids, illustrating defects in spermatogenesis [41] (Table 1).

4.2.2. LG3/4/5-peptide and testis function

Additionally, studies using cross-sections of adult rat testes for immunohistochemistry (IHC) and immunofluorescence analysis (IF) with a specific anti-laminin-α2 chain antibody have obtained some interesting observations. Laminin-α2 chain was found to robustly express in the seminiferous epithelium, but restrictively at the site near the basement membrane at the tunica propria in all stages of the epithelial cycle in adult rat testes [46,47]. Interestingly, a mild but consistent reduction in laminin-α2 expression was noted at the BM site in stages late VII-IX [47], coinciding with the transport of preleptotene spermatocytes across the immunological barrier [30,97,98]. However, in early VIII-IX stages, concomitant with the release of sperm at spermiation which is accompanied by apical ES degeneration [30,97,98], laminin-α2 was also prominently detected at the adluminal (apical) compartment of the seminiferous epithelium, near the apical ES, adjacent to the tubule lumen, but not precisely at the apical ES site, besides its robust expression at the basement membrane [46,47]. The immunofluorescence staining of laminin-α2 chain at the site near the apical ES was virtually non-detectable if the testes were pretreated with Taxol (paclitaxel), an MT-stabilizing reagent that rendered a virtual loss of MT function by prohibiting MT dynamic changes [99,100]. Thus, these findings suggest that the immunoreactive laminin-α2 chain detected

near the apical ES in stage VIII-IX tubules is likely a fragment of laminin-α2 chain derived from the basement membrane which is being transported to the adluminal compartment, away from the BM, through an MT-dependent mechanism [46,47]. It is tempting to speculate that a fragment of laminin-α2 chain may be released from the basement membrane to maintain apical ES function until spermiation when its expression is subsided in stage IX. This concept has now been confirmed in a recent report when different fragments of the LG3/4/5 domain were prepared vs. the LG3/4/5-peptide, and their biological activities to promote Sertoli cell BTB function were compared [48]. It was demonstrated that the biological activity of the laminin-α2 derived LG3/4/5 domains resides mostly at the LG5 fragment (i.e., LG5-peptide) (Fig. 4) [48]. In brief, besides capable of promoting BTB function by maintaining its integrity based on studies in vitro [46,47] and in vivo [48], the LG3/4/5-peptide (and LG5-peptide) is capable of promoting spermatogenesis by blocking the cadmium-induced testis injury [48].

4.2.3. *LG3/4/5-peptide mediated signaling function*

It has been shown that LG3/4/5-peptide exerts its effects through mTORC1/p-rpS6 signaling complex downstream by *inactivating* this signaling complex [46]. This observation is important since earlier studies have shown that an *activation* of the mTORC1/rpS6 signal complex is used by the testis to induce BTB remodeling [101,102]. In fact, overexpression of a quadruple phosphomimetic mutant of p-rpS6, namely p-rpS6-S235E/S236E/S240E/S244E (i.e., by converting Ser 235, 236, 240 and 244 to Glu via site-directed mutagenesis) by making this mutant constitutively active and cloned into pCI-neo mammalian expression vector, in Sertoli cell epithelium with an intact BTB in vitro [103] or the testis in vivo [104] effectively perturbs the barrier function. As such, one can manipulate the BTB function, including drug permeation at the BTB, by modifying the signaling proteins downstream of the LG3/4/5-peptide, namely mTORC1 or rpS6. In fact, several recent reports have shown that overexpression of p-rpS6-mutant alone (or together with F5-peptide, which can effectively induce BTB remodeling as discussed in Section 3) can effectively enhance drug permeation across the BTB in studies in vivo using the non-hormonal male contraceptive adjudin as a candidate drug [105–107]. These findings are important because they illustrate the possible therapeutic use of this LG3/4/5-peptide (or F5-peptide) and their downstream signaling proteins to manage human infertility in particular unexplained infertility without a known etiology. At present, it remains to be determined if the LG3/4/5-peptide exerts its effects to promote spermatogenesis by inducing spermatogonial differentiation to enter meiosis. This possibility should be carefully evaluated in future studies.

5. Contrasting effects of F5- and LG3/4/5-peptides that support spermatogenesis – a hypothetic model

Based on the available data, the contrasting effects of F5- and LG3/4/5-peptides in the testis including its effects on BTB integrity and spermatogenesis provide a unique mechanism to modulate cellular functions across the seminiferous epithelium to support spermatogenesis as noted in Fig. 6. It is likely that F5-peptide induces transient remodeling/restructuring of the BTB and degeneration of spermatid adhesion at the apical ES to support preleptotene spermatocyte transport and the release of sperm at spermiation through changes in p-FAK-Y407 (Fig. 6). On the other hand, LG3/4/5-peptide is utmost important to maintain BTB and apical ES integrity in some stages of the epithelial cycle, in particular the assembly of a “new” BTB behind the preleptotene spermatocytes under transport at the BTB before the “old” BTB is disassembled so that BTB integrity can be maintained (Fig. 6). Additionally, LG3/4/5-peptide is necessary to induce assembly of “new” apical ES when step 8 spermatids appear in stage VIII tubules when the apical ES at the Sertoli-step 19 spermatid interface is undergo degeneration (Fig. 6). Furthermore, LG3/4/5-peptide exerts its effects downstream through the mTORC1/rpS6 signaling complex in contrast to p-FAK-Y407 signaling protein utilized by F5-peptide (Fig. 6). It is conceivable that other regulatory and signaling proteins will be identified in the years to come, and the hypothetical model shown in Fig. 6 will be modified and updated, but it serves as a helpful model for investigators to design functional experiments in future studies.

6. Concluding remarks and future perspectives

As noted herein, much work is needed in future investigations since besides laminin-α2, -α3, -ß3, and -γ3 chains have been studied in the testis as noted in Figs. 4 and 5. Functions of other laminin-α chains (i.e., α1, α4, α5), ß chains (i.e., ß1, ß2), and γ chains (i.e., γ1, γ2) in spermatogenesis or their role in regulating testis function remain to be investigated. Furthermore, unlike laminin-333 in the apical ES, the identity of the other laminin chains that create a functional laminin ligand with laminin-α2 chain in the basement membrane remains to be identified. Additionally, it remains to be examined if any of the laminin heterotrimers as noted in Fig. 2 are present in the testis, in particular in the basement membrane of the seminiferous tubules. Also, if they are found in the testis, do these heterotrimeric laminin ligand exert their effects similar to their counterparts in other tissues? Do these laminin chains produce unique biologically active fragments to support spermatogenesis? Furthermore, the integrin-receptors (plus the downstream signaling cascade) that work in concert with their trimeric laminin ligands, in particular the laminin fragments, such as the F5- and LG3/4/5-peptide, remain to be explored. However, with the advances in bioinformatics technology, in particular scRNA-Seq-based transcriptome profiling, many of these questions will be answered within the next decade. Once additional information is obtained by addressing some of the questions raised here, which coupled with ongoing research in our and other laboratories, it is likely that some of the bioactive peptides (e.g., LG3/4/5-peptide) and their downstream signaling proteins (e.g., p-FAK-Y407E mutant, p-rpS6-mutant) can be used to rescue toxicant-induced male reproductive dysfunction. This possibility is supported by recent studies in which overexpressison of a human p-FAK-Y407E mutant indeed was found to rescue PFOS-mediated Sertoli cell injury in primary human Sertoli cells [84]. Overexpression of a rat p-FAK-Y407E [85] or through the use of an activator of the mTORC1/rp-S6/pAkt1/2 signaling complex [108] also rescued PFOS-mediated Sertoli cell injury in primary rat Sertoli cells cultured in vitro with a functional BTB that mimics the BTB in vivo [85]. Furthermore, the use of LG3/4/5-peptide was shown to block and to rescue cadmium-induced Sertoli cell and testis injury in vitro and in vivo [48].

Conflicts of interest

Authors have nothing to declare.

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