

# An autocrine axis in the testis that coordinates spermiation and blood–testis barrier restructuring during spermatogenesis

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The mechanism(s) that regulate and coordinate the events of spermiation and blood–testis barrier (BTB) restructuring in the seminiferous epithelium that occur concurrently at stage VIII of the seminiferous epithelial cycle of spermatogenesis are unknown. In this report, fragments derived from the laminin complex composed of laminin  $\alpha 3$ ,  $\beta 3$ , and  $\gamma 3$  chains (laminin-333) at the apical ectoplasmic specialization (apical ES) were shown to modulate BTB dynamics directly and/or indirectly via hemidesmosome. Experiments were performed using cultured Sertoli cells with functional tight junction (TJ) barrier and the ultrastructural features of the BTB but not apical ES. Recombinant protein fragments of laminin  $\beta 3$  and  $\gamma 3$  chains were shown to reduce the protein levels of occludin and  $\beta 1$ -integrin dose dependently at the Sertoli–Sertoli and Sertoli–basement membrane interface, respectively, thereby destabilizing the BTB permeability function. These results were corroborated by transient overexpression of laminin fragments in Sertoli cells. To further assess the role of  $\beta 1$ -integrin in hemidesmosome, knockdown of  $\beta 1$ -integrin in Sertoli cells by RNAi was found to associate with occludin redistribution at the Sertoli–Sertoli cell interface, wherein occludin moved away from the cell surface and became associated with endosomes, thereby destabilizing the BTB. In short, an apical ES–BTB–hemidesmosome autocrine regulatory axis was identified in testes, coordinating the events of spermiation and BTB restructuring that occur at the opposite ends of the seminiferous epithelium during spermatogenesis.

ectoplasmic specialization | hemidesmosome | seminiferous epithelium | Sertoli cells | tight junction

During spermatogenesis, preleptotene/leptotene spermatocytes at the basal compartment traverse the blood–testis barrier (BTB) at stages  $\approx$ VIII–IX of the seminiferous epithelial cycle in adult rat testes, entering the adluminal compartment for further development (1). This event takes place concurrently with spermiation, wherein fully developed spermatids (i.e., spermatozoa) detach from the epithelium at the luminal edge, entering the tubule lumen for their eventual maturation in the epididymis. These morphologic changes, which occur at the opposite ends of the Sertoli cell epithelium, were first described in the 1950s (2). In this report, we provide compelling evidence regarding the mechanism that regulates and coordinates these events. The idea was based on a recent study in which a blockade of the laminin function at the apical ectoplasmic specialization (apical ES) by specific antibodies led to spermatid exfoliation and BTB restructuring (3), even though it was unclear how the anti-laminin  $\gamma 3$  IgG traversed the BTB to reach the apical ES. Nonetheless, this study shows that a disruption of the apical ES may lead to a transient BTB disruption, illustrating a plausible physiological link between these two ultrastructures.

The apical ES is a testis-specific adherens junction (AJ) type that anchors developing spermatids to the Sertoli cell in the epithelium during spermatogenesis (4–6). It has properties of both AJ and focal contacts (4, 7). For instance, many proteins that are restricted to the cell–matrix interface at the focal contacts, such as integrins, laminins, and collagens, are found at the apical ES (4). Indeed, laminin  $\gamma 3$  chain was first identified as a nonbasement laminin at

the apical ES in mouse testes (8). Subsequent studies in adult rat testes have shown that laminin  $\gamma 3$ ,  $\alpha 3$ , and  $\beta 3$  chains residing in the elongating/elongated spermatids form a protein complex known as laminin-333 (3), which is the bona fide partner of the  $\alpha 6\beta 1$ -integrin restricted to Sertoli cells (9, 10), constituting the laminin-333/ $\alpha 6\beta 1$ -integrin adhesion complex at the apical ES. Furthermore, this laminin/integrin complex is associated with proteases: matrix metalloprotease-2 (MMP-2), membrane-type 1 (MT1)-MMP, and tissue inhibitor of metalloproteases-2 (TIMP-2) (11). These findings suggested that the activation of MMP-2 by MT1-MMP and TIMP-2 at the apical ES during spermiation could cleave laminin-333 to generate the biologically active laminin fragments. Indeed, there are reports illustrating that fragments of laminin chains that arise under physiological and pathophysiological conditions are biologically active peptides, regulating cell migration, protein production, inflammatory responses, and others (12, 13). Thus, it is possible that fragments of laminin chains released from the apical ES during spermiation could regulate the BTB in the testis (7).

Although  $\beta 1$ -integrin residing in the Sertoli cell is a receptor of laminin-333 at the apical ES (3, 9–11), its precise localization at the basal compartment of the seminiferous epithelium remains unclear. For instance,  $\beta 1$ -integrin was reported to be a component of the basement membrane in the seminiferous tubules (14), perhaps at the hemidesmosomes. Others have reported its localization with the actin-based anchoring junction structure (i.e., basal ES) at the BTB (10, 15). It is also not known whether there is cross-talk between  $\beta 1$ -integrin in the apical ES, the BTB, and hemidesmosome. We report herein an apical ES–BTB–hemidesmosome axis in the testis that coordinates spermiation and BTB restructuring during spermatogenesis.

## Results

**Disruptive Effects of Laminin Fragments on the BTB Integrity.** This study was performed by using primary cultures of Sertoli cells (3) with negligible Leydig and germ cell contamination (16) that were shown to form functional BTB (such as a tight junction-permeability barrier) with the ultrastructural features under electron microscopy that mimicked the *in vivo* barrier (17, 18).

Recombinant proteins corresponding to portions of domain I of laminin  $\beta 3$  and  $\gamma 3$ , as well as domain IV of laminin  $\gamma 3$  chains [supporting information (SI) Fig S1 and Table S1] were expressed in *Escherichia coli* and purified to apparent homogeneity (Fig. 1A). Sertoli cells were cultured alone for 3 days, forming an intact

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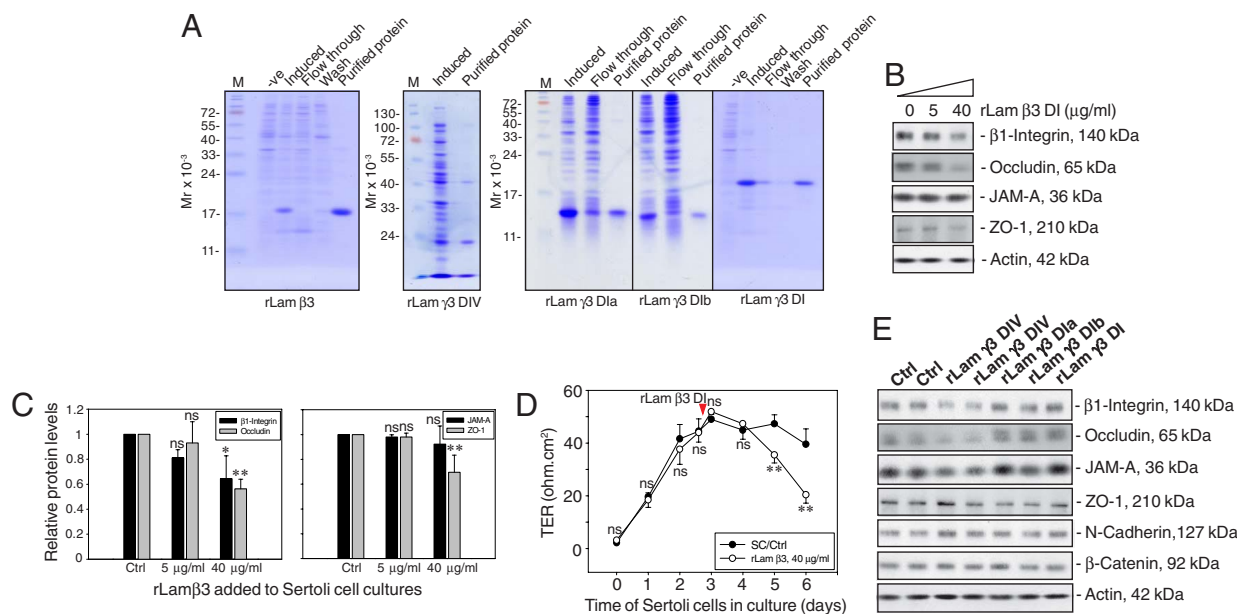
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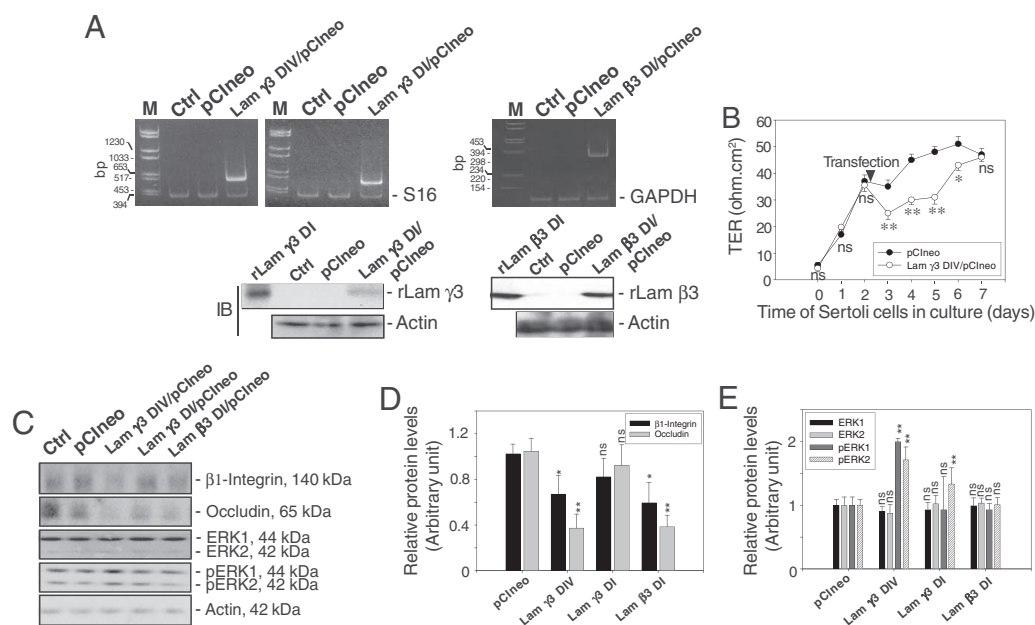
**Fig. 1.** A study to assess the effects of different laminin fragments on the steady-state levels of proteins at the BTB and the BTB integrity in primary Sertoli cell cultures. (A) Purification of the recombinant proteins expressed in *E. coli*. Uninduced (-ve) and induced protein lysates from *E. coli*, fractions collected from the Ni column (both flow through and wash), and purified proteins were resolved by SDS-PAGE with gels stained by Coomassie blue. (B) The 5 and 40  $\mu\text{g/ml}$  recombinant proteins corresponding to part of domain I of laminin  $\beta 3$  chain (rLam  $\beta 3$ ) were added to cultures on day 3 after isolation. Recombinant proteins were included in the daily replacement F12/DMEM for an additional 3 days. Cells were terminated on day 6 and lysates were used for immunoblotting, illustrating a dose-dependent inhibition on the production of  $\beta 1$ -integrin, occludin, and ZO-1 but not JAM-A. Actin served as a loading control. (C) Immunoblot data were densitometrically scanned and compared. Each bar is the mean  $\pm$  SD of  $n = 3$ , normalized against actin, wherein the control was arbitrarily set at 1, against which one-way ANOVA was performed. (D) Effects of the rLam  $\beta 3$  domain I on the TJ barrier. A total of 40  $\mu\text{g/ml}$  rLam  $\beta 3$  domain I on the TJ barrier (red arrowhead), which was also included in the daily replacement F12/DMEM ( $n = 3$ ). (E) Results of representative immunoblots illustrating the effects of other laminin fragments at 40  $\mu\text{g/ml}$  on Sertoli cell target proteins using the experimental conditions of (B). Ctrl, control; rLam, recombinant laminin; DIV, domain IV; rLam  $\gamma 3$  DIa or DIb, recombinant laminin  $\gamma 3$  chain containing Domain Ia or Domain Ib (Table S1); DI, domain I; ns, not significantly different; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

epithelium; thereafter, cells were treated with purified laminin  $\beta 3$  domain I for 3 days with the daily replenishing media containing the same amount of recombinant protein at 0, 5, and 40  $\mu\text{g/ml}$  (Fig. 1B), and the steady-state protein levels of  $\beta 1$ -integrin, occludin, and ZO-1, but not JAM-A, were significantly and dose-dependently reduced (Fig. 1B and C). Since some of these proteins (e.g., occludin and ZO-1) are integral components of the BTB (4, 7), the tight junction (TJ) permeability barrier function was assessed by quantifying the transepithelial electrical resistance (TER) across the Sertoli cell epithelium (17) by treating these cultures with laminin  $\beta 3$  DI recombinant protein, which was added to the apical and basal chambers of the bicameral units on day 2.5 (Fig. 1D, red arrowhead). It was noted that the presence of laminin  $\beta 3$  DI recombinant protein compromised the TJ barrier by inducing a significant decline in TER on days 5 and 6 vs. controls (Fig. 1D). To confirm its specific disruptive effect on BTB function, recombinant proteins of other laminin fragments were also tested under the same conditions as for domain I of laminin  $\beta 3$ . It was noted that only domain IV, but not domain I, of laminin  $\gamma 3$  induced a reduction in the protein levels of  $\beta 1$ -integrin, occludin, and JAM-A, but not ZO-1, N-cadherin, and  $\beta$ -catenin, which are putative BTB proteins in rat testes (Fig. 1E). Collectively, these data illustrate that the disruptive effects of domain I of laminin  $\beta 3$  and domain IV of laminin  $\gamma 3$  chains on the Sertoli cell BTB were not the results of nonspecific protein effects, and only specific laminin fragments are biologically active peptides to modulate BTB function.

**Effects of Overexpression of Laminin Fragments on Sertoli Cell BTB Function.** When DNA constructs containing the same part of the domain I of laminin  $\beta 3$  and  $\gamma 3$ , and the domain IV of laminin  $\gamma 3$ , as recombinant proteins (Fig. S1) were transiently transfected in

Sertoli cells, both mRNAs and proteins of the corresponding laminin fragments were detected by 48 hr thereafter (Fig. 2A). Sertoli cells transfected with vector alone failed to express any laminin chains (Fig. 2A), since laminin-333 is restricted to elongating/elongated spermatids in adult rat testes (3). The effects of overexpression of domain IV of the laminin  $\gamma 3$  chain on the Sertoli cell-TJ barrier were also monitored vs. cells transfected with vector alone. Within  $\approx 24$  hr after transfection, a significant decline in TER was detected, which persisted for the next 4 days, illustrating a loss of BTB function (Fig. 2B). This disruptive effect was transient, because the TER was restored by day 7 (Fig. 2B). Significant reduction in the steady-state protein levels of  $\beta 1$ -integrin and occludin were detected in Sertoli cells with overexpression of domain IV of  $\gamma 3$  and domain I of  $\beta 3$  chains (Fig. 2C and D). The results shown in Fig. 2 are consistent with those of Fig. 1. Besides, an activation of ERK ( $p$ -ERK) was detected following overexpression of domain IV of laminin  $\gamma 3$  (Fig. 2C and E).

**Localization of  $\beta 1$ -Integrin in the Seminiferous Epithelium of Adult Rat Testes.**  $\beta 1$ -Integrin and  $\alpha 6$ -integrin form a functional adhesion complex of  $\alpha 6\beta 1$ -integrin at the apical ES residing in Sertoli cells (9, 10, 15), and  $\alpha 6\beta 1$ -integrin is the receptor of laminin-333 residing in elongating/elongated spermatids (3, 11). However, the precise localization of  $\beta 1$ -integrin at the basal compartment is not well defined. Since specific recombinant laminin chain fragments were shown to perturb the BTB function, we sought to define the precise localization of  $\beta 1$ -integrin in the basal compartment. Results of the study were shown in Fig. 3A-X and Fig. S2. Consistent with the earlier studies,  $\beta 1$ -integrin was localized predominantly at the apical ES in stage VIII tubules (Fig. 3B, J, and N); however, at the basal compartment, relatively little  $\beta 1$ -integrin was colocalized with



**Fig. 2.** A study by transient expression of laminin fragments in Sertoli cells to assess their effects on the BTB integrity and the steady-state levels of different target proteins. (A) RT-PCR and immunoblot analysis showing the expression of domain IV of laminin  $\gamma 3$  (Left), domain I of laminin  $\gamma 3$  (Center), and domain I of laminin  $\beta 3$  (Right) in Sertoli cells after transfection with laminin  $\gamma 3$  DIV/pCneo, laminin  $\gamma 3$  DI/pCneo, and laminin  $\beta 3$  DI/pCneo expression vectors for 2 and 3 days, respectively, vs. cells transfected with pCneo alone (without laminin cDNA constructs) or normal cells (Ctrl). S16, GAPDH, and actin served as loading controls. (B) Changes in Sertoli cell–TJ barrier by quantifying TER across the cell epithelium in bicameral units ( $n = 3$ ) where cells were transfected with laminin  $\gamma 3$  DIV/pCneo vs. pCneo alone on day 2.5 (arrowhead). (C) The protein levels of  $\beta 1$ -integrin, occludin, and ERK/pERK in cells following overexpression with different con-

structs for 3 days vs. controls. (D–E) Bar graphs summarize results of C. Each bar is a mean  $\pm$  SD ( $n = 3$ ) and normalized against actin, wherein cells transfected with pCneo vector alone were arbitrarily set at 1, against which one-way ANOVA was performed. ns, not significantly different; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

the TJ markers: occludin (Fig. 3 C, G, A–D, and E–H) and ZO-1 (Fig. 3 K and I–L). This thus suggests that  $\beta 1$ -integrin is not restricted to the basal ES at the BTB. Instead,  $\beta 1$ -integrin was mostly colocalized with a basement membrane marker, laminin  $\alpha 2$  (Fig. 3 O, S, M–P, and Q–T) (19), and partially colocalized with intermediate filament component vimentin (Fig. 3 W and U–X). Collectively, these results illustrate that besides its presence at the apical ES,  $\beta 1$ -integrin is a component of hemidesmosomes at the Sertoli cell–matrix interface in the basement membrane.

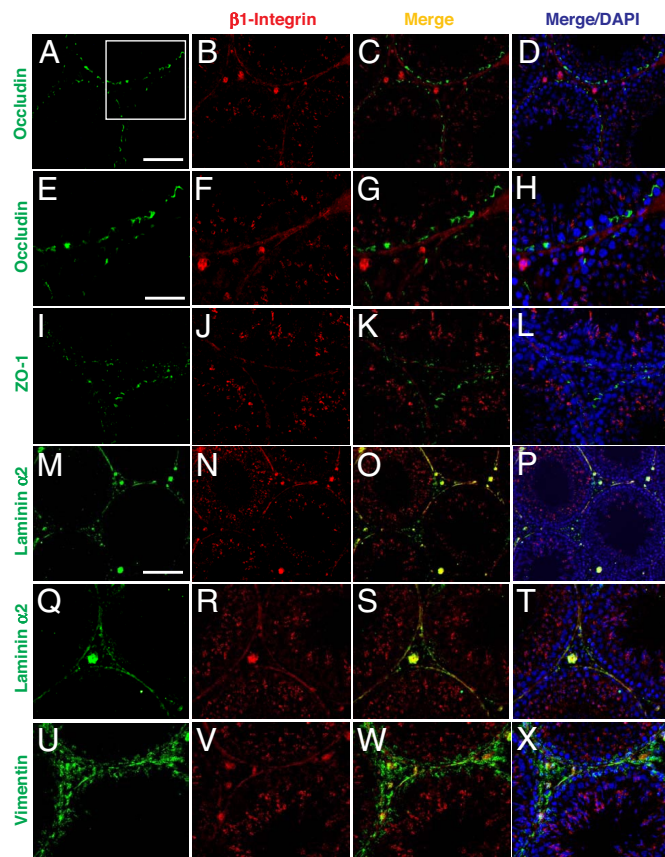
**Roles of  $\beta 1$ -Integrin on BTB Dynamics.** Since fragments of specific laminin chains (Fig. S1) were shown to modulate BTB function and reduce the steady-state level of  $\beta 1$ -integrin (Figs. 1 and 2), and  $\beta 1$ -integrin was confined mostly to the hemidesmosomes rather than the basal ES at the BTB (Fig. 3), we sought to investigate the functional involvement of  $\beta 1$ -integrin in BTB dynamics by using RNA interference (RNAi) techniques with Sertoli cell cultures having functional BTB and cell–matrix junctions, such as hemidesmosomes (Fig. 4A) (18, 20). Sertoli cell cultures seeded on day 0 were subjected to a hypotonic treatment on day 2 (21) to remove any residual germ cells, eliminating all apical ES, such that the  $\beta 1$ -integrin studied herein was predominantly restricted to the Sertoli cell–matrix interface. RNAi was performed on day 4 in Sertoli cell epithelium, and cells were terminated on days 7 and 9 (data not shown) by using  $\beta 1$ -integrin–specific small interfering RNA (siRNA) (a 21-bp double-stranded RNA) vs. scrambled control siRNA. For Sertoli–germ cell cocultures, total germ cells isolated from adult rat testes on day 4 were plated to the Sertoli cell epithelium to allow the assembly of functional AJ (e.g., apical ES) as verified by light and electron microscopy (6, 18), RNAi was performed on day 5, and cultures were terminated on day 8. The steady-state protein level of  $\beta 1$ -integrin in these Sertoli cell cultures following RNAi vs. controls (i.e., Sertoli cells cultured alone and cells treated with nontargeting control siRNA duplex) was monitored by immunoblotting, as shown in Fig. 4 A and C. Cells transfected with  $\beta 1$ -integrin siRNA duplex had a  $\approx 70\%$  reduction in  $\beta 1$ -integrin protein vs. controls (Fig. 4 A and C) on day 7, which persisted up to day 9 (data not shown). However, there was no significant change in the steady-state levels of TJ (e.g., occludin) and basal ES (e.g., N-cadherin and  $\beta$ -catenin) proteins, nor its

putative binding partner  $\alpha 6$ -integrin (Fig. 4 D and E and G and H) on day 7, which also persisted until day 9 (data not shown), except for a  $\approx 40\%$  decline in the level of ZO-1, a TJ adaptor, on day 7 (Fig. 4F) and day 9 (data not shown). When  $\beta 1$ -integrin was knocked down in Sertoli–germ cell cocultures, a significant decline,  $\approx 70\%$ , in  $\beta 1$ -integrin was also detected (Fig. 4 B and C), but other markers remained relatively stable, similar to controls (Fig. 4 B–H).

**A Knockdown of  $\beta 1$ -Integrin at the Cell–Matrix Interface by RNAi Leads to a Disruption of Occludin-Based TJ Fibrils at the BTB.** Even though there was no significant alteration in the steady-state protein levels of occludin and N-cadherin at the BTB when  $\approx 70\%$  of  $\beta 1$ -integrin was silenced at the cell–matrix interface, a significant decline in ZO-1 was noted (Fig. 4 A and F). We next examined whether there were any changes in the redistribution of proteins at the Sertoli–Sertoli cell interface (Fig. 5Aa–Ae). When Sertoli cells were transfected with Cy-3-labeled  $\beta 1$ -integrin siRNA duplex (red fluorescence in Fig. 5Ab and Ac), but not control siRNA duplex (red fluorescence in Fig. 5Aa), there was a significant disruption of the immunoreactive “beltlike” structures of occludin (green fluorescence) at the cell–cell interface corresponding to the TJ fibrils, wherein occludin appeared to move away from the cell–cell interface and became cytosol bound (Fig. 5Ab and Ac vs. Aa). This effect was not detectable when these cells were stained for N-cadherin (Fig. 5Ae vs. Ad). Occludin vesicles were concentrated around the nucleus (white arrowheads in Fig. 5Ba), apparently as a result of protein endocytosis, and had remarkable tendency to colocalize with an early endosome marker, EEA-1 (Fig. 5Bc and Bd vs. Ba and Bb). This morphologic observation was further confirmed by the biochemical study shown in Fig. 5 C and D to assess the effects of  $\beta 1$ -integrin knockdown on occludin endocytosis. Consistent with the data shown in Fig. 5 A and B, a significant reduction,  $\approx 40\%$ , in total biotinylated Sertoli cell surface occludin was detected (Fig. 5C, top) following a  $\beta 1$ -integrin knockdown vs. controls (Fig. 5 C and D), with no change in the steady-state occludin level (Fig. 5C).

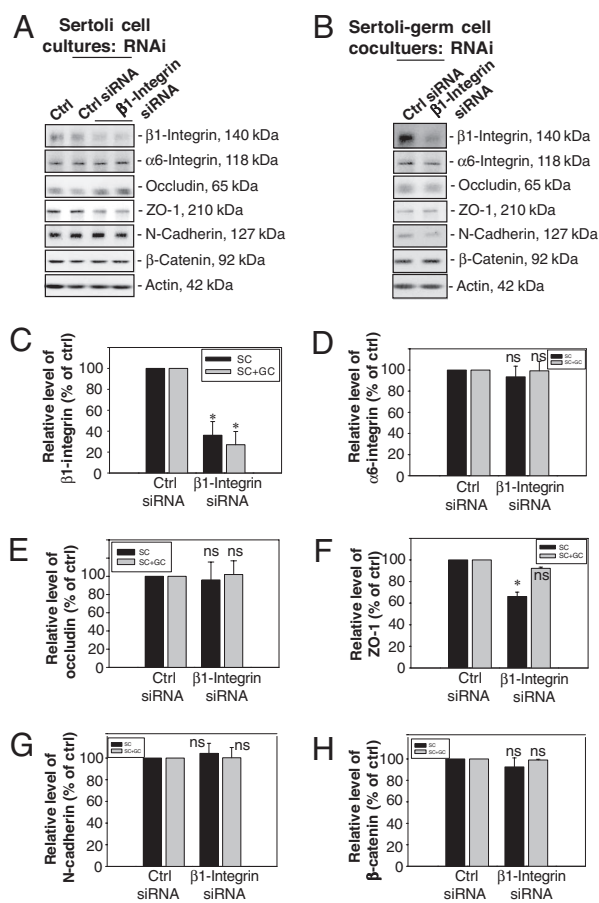
## Discussion

The apical ES and the BTB are two ultrastructures in the seminiferous epithelium that are located at the opposite ends of the adjacent Sertoli cells (Fig. 6). Junctional complexes at these two



**Fig. 3.** A study by immunofluorescent microscopy to examine the localization of  $\beta$ 1-integrin in rat testes illustrating its presence at the apical ES and hemidesmosome. (A–D) Occludin (green, FITC) was localized at the BTB in the seminiferous epithelium of normal testes.  $\beta$ 1-Integrin (red, Cy3) was predominantly localized at the apical ES, and it was also localized in the basal compartment of the epithelium. However, the signal of  $\beta$ 1-integrin in the basal compartment was predominantly found underneath occludin in the merged image (C). (D) DAPI staining of nuclei. Boxed imaged in A was magnified in E–H, illustrating most  $\beta$ 1-integrin was localized below the BTB (occludin) (see G and H vs. E and F). (I–L) ZO-1 (green, FITC) was also localized at the BTB above  $\beta$ 1-integrin (red, Cy3). (M–P) Laminin  $\alpha$ 2 (green, FITC, a known basement membrane protein in rodent testes (19) was localized along the basement membrane of the seminiferous tubules as well as Leydig cells in the interstitium. It was shown to colocalize with  $\beta$ 1-integrin in the basal compartment, as shown in the merged image (O, see yellowish color). (Q–T) Higher magnification showing the colocalization of  $\beta$ 1-integrin with laminin  $\alpha$ 2 at the hemidesmosomes. (U–X) Vimentin (green, FITC; an intermediate filament structural protein), was localized at both basement membrane as well as at the BTB.  $\beta$ 1-integrin (red, Cy3) was found to colocalize with vimentin at the basement membrane (see yellow color in W). (Scale bar in A: 100  $\mu$ m, which applies to B–D, I–L, and Q–T; in M: 120  $\mu$ m, which applies to N–P; in E: 50  $\mu$ m, which applies to F–H and U–X.)

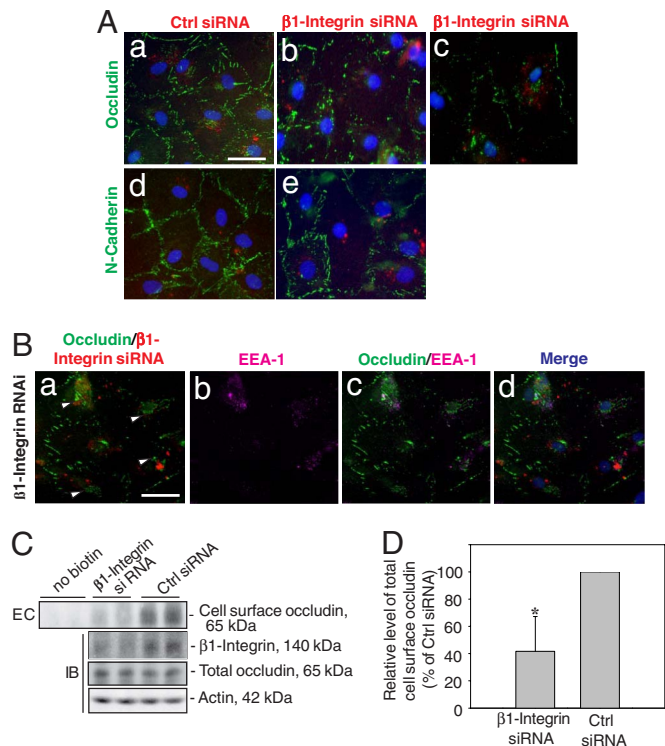
sites undergo restructuring concurrently at stages VIII–IX of the epithelial cycle to facilitate (i) spermiation and (ii) the transit of preleptotene/leptotene spermatocytes across the BTB. In adult rat testes, one of the major cell adhesion complexes at the apical ES is the laminin-333/ $\alpha$ 6 $\beta$ 1 integrin complex (4, 5, 7). In the present study, fragments of laminin-333 were shown to regulate BTB function near the basal lamina. First, recombinant proteins corresponding to specific domains of the laminin chains were shown to perturb the Sertoli cell–TJ barrier *in vitro*. These proteins also down-regulated the steady-state levels of occludin at the BTB as well as  $\beta$ 1-integrin at the Sertoli cell–basement membrane interface, perhaps at the hemidesmosomes. It is important to note that primary Sertoli cell cultures are an established *in vitro* system to monitor TJ barrier function that mimics the BTB *in vivo* morpho-



**Fig. 4.** A study to assess the effects of  $\beta$ 1-integrin knockdown by RNAi on the steady-state levels of junction-associated proteins in primary Sertoli cell and Sertoli–germ cell cocultures. (A–B) Immunoblots showing changes in the steady-state levels of several junction proteins after  $\beta$ 1-integrin RNAi in Sertoli cell cultures (A) or Sertoli–germ cell cocultures (B), with actin serving as a loading control. From both sets of cultures, lysates were used for immunoblot analysis as shown in A and B, and data from three experiments were scanned and shown in C–H. Each bar is the mean  $\pm$  SD ( $n = 3$ ) and normalized against actin, wherein control cultures were arbitrarily set at 100%, against which one-way ANOVA was performed. ns, not significantly different; \*,  $P < 0.01$ .

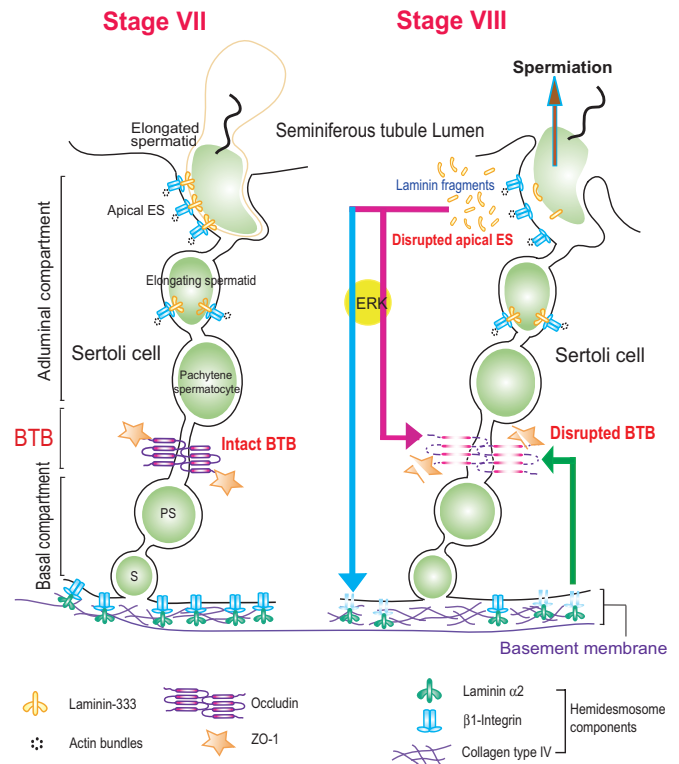
logically and physiologically based on earlier reports (18, 22, 23), illustrating the significance of these findings. Second, these results are in agreement with data from overexpression experiments. Furthermore, laminin fragments apparently mediated the effects via the ERK signaling pathway. Third, to delineate the functional significance of the laminin fragment–mediated reduction in the  $\beta$ 1-integrin level, primary Sertoli cell cultures were transfected with  $\beta$ 1-integrin-specific siRNA to confirm the functional linkage along the apical ES–BTB–hemidesmosome axis. Indeed, when  $\beta$ 1-integrin was knocked down by RNAi, a redistribution of occludin at the Sertoli–Sertoli cell interface was detected, moving away from the TJ fibrils, undergoing enhanced endocytosis, and associating with early endosomes via its interaction with EEA-1.

The mechanism by which fragments of the laminin chains are formed in the seminiferous epithelium *in vivo* remains unknown. However, recent studies have illustrated that the laminin  $\gamma$ 3 chain is a substrate of matrix metalloprotease 2 (MMP-2) (11). Also, membrane-type 1 (MT1)-MMP, and tissue inhibitor of metalloproteases-2 (TIMP-2), which are known to activate MMP-2 (24), are localized at the apical ES and are associated with the  $\beta$ 1-integrin–laminin  $\gamma$ 3 complex (11). It is plausible that at spermiation the activation of MMP-2 by the MT1-MMP/TIMP-2 complex may



**Fig. 5.** A study to assess changes in the distribution of occludin following RNAi of  $\beta 1$ -integrin in Sertoli cell cultures. Sertoli cell epithelium was transfected with siRNA duplex on day 4 and cultured for two additional days before being used for fluorescent microscopy. (Aa and Ad) Sertoli cells were transfected with Cy3-labeled nontargeting control siRNA (red fluorescence in cells) and stained for occludin (green, FITC) and *N*-cadherin (green, FITC), which was restricted mostly at the cell–cell interface. (Ab, Ac, and Ae) Cells were transfected with Cy3-labeled  $\beta 1$ -integrin siRNA duplex, illustrating a disappearance of occludin, but not *N*-cadherin, from the cell surface (Aa–Ac vs. Ad–Ae), and occludin appeared to become internalized (see Ab and Ac from two different experiments vs. Aa). (Scale bar: Aa, 10  $\mu$ m, which applies to Ab–Ae.) (Ba) A loss of occludin (green) from the cell–cell interface was noted where cells were transfected with Cy3-labeled  $\beta 1$ -integrin siRNA (red; see Ba vs. Aa). Most internalized occludin proteins (green) that appeared in cell cytosol (white arrowheads; Ba) associated with an early endosome marker (EEA-1, purple, Cy5; Bb), and the merged images are shown in Bc; Bd shows the merged images of Ba–Bc and with DAPI staining for Sertoli cell nuclei. (Scale bar: Ba, 10  $\mu$ m, which applies to Bb–Bd.) (C) Endocytosis (EC) assay (see *SI Methods*) to assess the effects of  $\beta 1$ -integrin knockdown on occludin internalization vs. controls. Significantly less biotinylated occludin was detected at the cell surface after  $\beta 1$ -integrin RNAi vs. control (Top), whereas the total cellular occludin did not change considerably when assessed by immunoblotting (IB) (Bottom) wherein the endogenous  $\beta 1$ -integrin level was reduced following RNAi (Middle). Actin served as the loading control. (D) A bar graph that summarizes results of C. Each bar is the mean  $\pm$  SD ( $n = 3$ ) wherein vehicle control was arbitrarily set at 100%. \*,  $P < 0.01$  by Student *t* test.

lead to proteolytic cleavage of specific laminin chains at the apical ES. This would generate the biologically active fragments, which in turn regulate the steady-state levels of the integral membrane proteins at the BTB and/or mediated via its effects on the hemidesmosomes as supported by the results reported herein (Fig. 6). The hypothetical scheme shown in Fig. 6 presents a model for the mechanism that coordinates the events of spermiogenesis and BTB restructuring that occur concurrently at stage VIII of the epithelial cycle. This hypothesis is further supported by two lines of evidence. First, proteolytic fragments of laminin chains are known biologically active peptides and are capable of regulating cell migration, protein production, activation of signal transducers, organelle formation, and others (for reviews, see 4,7). For instance, recombinant LG4 module of the laminin  $\alpha 3$  chain in laminin-5 (laminin-332) and



**Fig. 6.** A schematic drawing illustrating an autocrine regulatory loop in adult rat testes. At spermiogenesis, biologically active laminin fragments released from the laminin/integrin complex at the apical ES, (e.g., via MMP-2), significantly reduce the level of occludin at the BTB (red arrow). These fragments can also reduce the level of  $\beta 1$ -integrin at the hemidesmosomes (blue arrow), destabilizing the BTB via a feedback mechanism (green arrow). Collectively, these combined effects facilitates preleptotene spermatocyte transit at the BTB. This autocrine regulatory mechanism thus coordinates spermiogenesis and BTB restructuring that occur at the opposite ends of the Sertoli cell epithelium.

a 19-amino acid residue synthetic peptide within this LG4 domain were shown to stimulate the expression of MMP-1 in keratinocytes and fibroblasts (13). Second, the migration of monocytes across the blood–brain barrier was recently shown to be associated with an activation of proteases, such as MMPs. This activation led to proteolytic breakdown of occludin at the blood–brain barrier to facilitate monocyte migration (25), analogous to the possible cleavage of laminin-333 by MMP-2 at the apical ES during spermiogenesis, and the transit of spermatocytes across the BTB facilitated by laminin fragments. Also, occludin possesses a putative extracellular MMP cleavage site (26), and the endothelial cells that constitute the blood–brain barrier express high levels of MMP-2 and MMP-9 (27). Again, this is comparable with the spatial relationship of the laminin 333– $\alpha 6\beta 1$ -integrin complex and MMP-2, MT1-MMP, and TIMP-2 (4), and the presence of proteases/protease inhibitors at the BTB (7). Furthermore, the model depicted in Fig. 6 offers an explanation for the earlier observation that local administration of anti-laminin  $\alpha 3$  or  $\gamma 3$  IgG to the testis perturbed apical ES and BTB function (3), even though they might not have reached the apical ES by traversing the BTB. For instance, laminin fragments generated at the apical ES and translocated to the site of hemidesmosomes and BTB could have been “trapped” and “immobilized” by the antibodies (3). This thus disrupts the local regulatory loop and the BTB.

Although  $\beta 1$ -integrin was localized predominantly at the apical ES, some  $\beta 1$ -integrin was found at the BTB as previously reported (10). However, most of the  $\beta 1$ -integrin staining in the epithelium outside the apical ES was at the hemidesmosomes. Since the primary Sertoli cell cultures used for our studies had negligible

germ cell contamination (16), the laminin fragment-induced  $\beta$ 1-integrin reduction in these cultures can be attributed mostly to the loss of  $\beta$ 1-integrin at the hemidesmosomes instead of at the apical or basal ES. In short, these findings illustrate the presence of a functional apical ES-BTB-hemidesmosome axis in the seminiferous epithelium, which is used to coordinate the events of spermiation and BTB restructuring during spermatogenesis (see Fig. 6).

## Materials and Methods

**Animals.** The use of animals was approved by The Rockefeller University Laboratory Animal Use and Care Committee with protocol numbers 03017 and 06018.

**Primary Sertoli Cell Cultures and Treatment of Cells with Recombinant Proteins.** Sertoli cells were isolated from 20-day-old Sprague–Dawley rat testes (3). Functional BTB that mimicked the *in vivo* barrier was formed in these cultures within  $\approx$ 3 days when assessed by quantifying the TER across the cell epithelium (17) and by electron microscopy (18). These cultures were used to assess the effects of purified recombinant proteins corresponding to different fragments of the laminin chains (*SI Methods*).

**Transient Overexpression of Laminin Fragments in Sertoli Cells.** Laminin fragments corresponding to part of domain I of laminin  $\beta$ 3 and  $\gamma$ 3 chains and domain IV of laminin  $\gamma$ 3 chain (Fig. 1) were cloned into pCl-neo mammalian expression vector (Promega) at the restriction enzyme sites between *NheI* and *EcoRI* by using specific primers (Table S1). The authenticity of these clones was confirmed by direct nucleotide sequencing (Genewiz). On day 2 after Sertoli cell isolation,  $\approx$ 1 or 0.5  $\mu$ g of DNA of each construct was transfected to Sertoli cells plated on Matrigel-coated bicameral units or 12-well plates at a cell density of  $1.2 \times 10^6$  or  $0.45 \times 10^6$ , respectively, by using Effectene Transfection reagent (Qiagen). Transfection mixture was removed 24 hr later and replaced with fresh F12/DMEM. RNA and protein lysates were extracted 2 and 3 days thereafter, respectively. The TJ barrier integrity after transient expression of laminin  $\gamma$ 3 domain IV vs. pClneo vector alone was also assessed by TER measurement as described (17).

**Sertoli Cell-Specific Knockdown of  $\beta$ 1-Integrin by RNAi.** On day 2 after isolation, cultures were subjected to a hypotonic treatment to remove residual germ cells. This step also eliminated any remaining  $\beta$ 1-integrin at the apical ES between Sertoli cells and elongating/elongated spermatids, of which  $\beta$ 1-integrin is a putative component (4, 5, 7). Thus,  $\beta$ 1-integrin detected in these Sertoli cell-only cultures was derived mostly at the cell–matrix interface, because relatively little  $\beta$ 1-integrin was detected at the Sertoli–Sertoli cell interface at the basal ES (Fig. 3 and Fig. S2), which is also part of the BTB (4). On day 4, Sertoli cells were transfected with 100 nM ON-TARGETplus 21-mer double-stranded siRNAs: sense

5'-GGUAGGUCCAACGGCUUAAU-3' and antisense: 5'-PUAAGCCGUUGGACCUAUCGUU-3' (cat. no. J-089600-10), targeting rat  $\beta$ 1-integrin (NM-017022; Dharmacon, Inc.) by TransIT-TKO (cat. no. MIR 2150; Mirus) according to the manufacturer's instructions. Transfection mixture was replaced 24 hr after transfection with F12/DMEM supplemented with growth factors (3), and cells were then terminated 3 and 5 days thereafter (i.e., on days 7 and 9). In all RNAi experiments, negative controls were included in which cells were transfected with equal amounts of ON-TARGETplus siCONTROL nontargeting siRNA 5'-UGUUUACAUGUCGACUAA-3' (cat. no. D-001810-01; Dharmacon). Selected experiments were conducted by using Sertoli–germ cell cocultures (18), wherein total germ cells isolated from 90-day-old rat testes were added to the Sertoli cell epithelium on day 4 by using a Sertoli–germ cell ratio of 1:2. Cells were then transfected with siRNA (100 nM) on day 5 after anchoring junctions (e.g., apical ES) were formed when examined by electron microscopy (18). Cocultures were terminated on day 8.

**Dual-Labeled Immunofluorescent Analysis.** The distribution of integral membrane proteins in cells after  $\beta$ 1-integrin knockdown was examined by fluorescent microscopy. In brief, Sertoli cells were isolated and cultured on Lab-TekChamber Slide System (Nalge Nunc International) coated with Matrigel at a cell density of  $\approx 0.1 \times 10^6$  cells per 1.8 cm<sup>2</sup>. To ascertain that cells were indeed transfected with the siRNA when distribution of integral membrane proteins at the cell–cell interface were examined, siRNA was labeled with Cy3 by the Label IT siRNA tracker intracellular localization kit (cat. no. MIR 7212; Mirus) 1 day before transfection. On day 4, cells were transfected with 100 nM fluorescently labeled siRNA by TransIT-TKO transfection reagent. Transfection mixture was removed 24 hr thereafter, cells were then cultured for an additional 24 hr and fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100. After blocking with 10% normal goat serum (vol/vol), cells were incubated with 1:100 rabbit anti-occludin (cat. no. 71-1500, lot 51202542; Invitrogen, Zymed) and 1:100 mouse anti-early endosomal antigen 1 (EEA-1; cat. no. 610456, lot 76250; BD Biosciences). Following overnight incubation, secondary antibodies conjugated with FITC or Cy-5 (Zymed) diluted in PBS at 1:50 were incubated with cells on slides for  $\approx$ 30 min. Cells were mounted in Vectashield Hardset, and fluorescent micrographs were obtained (3).

**General Methods.** Endocytosis assay, RT-PCR, immunoblot analysis, fluorescent microscopy, Sertoli cell TJ-barrier function monitored by TER measurement, and statistical analyses were performed as described (3,17) (*Table S1, SI Methods*).

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