

# NC1 domain of collagen $\alpha 3(IV)$ derived from the basement membrane regulates Sertoli cell blood-testis barrier dynamics

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The blood-testis barrier (BTB) is an important ultrastructure for spermatogenesis. Delay in BTB formation in neonatal rats or its irreversible damage in adult rats leads to meiotic arrest and failure of spermatogonial differentiation beyond type A. While hormones, such as testosterone and FSH, are crucial to BTB function, little is known if there is a local regulatory mechanism in the seminiferous epithelium that modulates BTB function. Herein, we report that collagen  $\alpha 3(IV)$  chain, a component of the basement membrane in the rat testis, could generate a noncollagenous (NC1) domain peptide [Col $\alpha 3(IV)$  NC1] via limited proteolysis by matrix metalloproteinase-9 (MMP-9), and that the expression of MMP-9 was upregulated by TNF $\alpha$ . While recombinant Col $\alpha 3(IV)$  NC1 protein produced in *E. coli* failed to perturb Sertoli cell tight junction (TJ)-permeability barrier function, possibly due to the lack of glycosylation, Col $\alpha 3(IV)$  NC1 recombinant protein produced in mammalian cells and purified to apparent homogeneity by affinity chromatography was found to reversibly perturb the Sertoli cell TJ-barrier function. Interestingly, Col $\alpha 3(IV)$  NC1 recombinant protein did not perturb the steady-state levels of several TJ- (e.g., occludin, CAR, JAM-A, ZO-1) and basal ectoplasmic specialization- (e.g., N-cadherin,  $\alpha$ -catenin,  $\beta$ -catenin) proteins at the BTB but induced changes in protein localization and/or distribution at the Sertoli cell-cell interface in which these proteins moved from the cell surface into the cell cytosol, thereby destabilizing the TJ function. These findings illustrate the presence of a local regulatory axis known as the BTB-basement membrane axis that regulates BTB restructuring during spermatogenesis.

## Introduction

In the mammalian testis, the blood-testis barrier (BTB) located near to the basement membrane in the seminiferous epithelium is crucial for spermatogenesis.<sup>1-4</sup> First, it divides the epithelium into the basal and adluminal compartments to establish a unique microenvironment in the adluminal compartment for spermiogenesis by limiting paracellular and transcellular transport of substances across the barrier by performing its gate-keeping function. This gatekeeping function of the BTB is manifested by the unique composition of proteins, ions, electrolytes, sugars and biomolecules (e.g., hormones) in the fluid of the seminiferous tubule lumen and the rete testis lumen vs. the blood plasma/serum.<sup>2,5-7</sup> Second, the BTB also confers cell polarity by restricting the movement of membrane proteins and lipids between apical and basolateral membranes (i.e., fence function).<sup>8</sup> Lastly, irreversible disruption of the BTB by treatment of adult rats with toxicants (e.g., glycerol, cadmium)<sup>9-14</sup> is known to cause sterility. These findings collectively illustrate the significance of the BTB in spermatogenesis.

Most blood-tissue barriers (e.g., blood-brain barrier and blood-retinal barrier) are conferred by the endothelial tight junction

(TJ) of microvessels, which is located at the apical region of the endothelial cells, furthest away from the basal lamina.<sup>15,16</sup> The BTB, however, is constituted by co-existing TJ, basal ES [ectoplasmic specialization, a testis-specific filamentous (F)-actin-rich adherens junction (AJ)<sup>17</sup>], desmosome and gap junction, located adjacent to the basement membrane, which is a modified form of extracellular matrix (ECM).<sup>18</sup> This unique morphological setting thus suggests that the basement membrane may modulate the BTB function during the epithelial cycle.<sup>19</sup> In fact, studies have shown that a disruption of the basement membrane function via the use of antibodies impedes spermatogenesis and the Sertoli cell TJ-barrier,<sup>20-22</sup> implicating there is a functional link between the BTB and the basement membrane.

In the mammalian testis, such as the testis in rodents and bulls, the basement membrane is composed of two major building blocks namely type IV collagen and laminins.<sup>19</sup> Type IV collagen is a triple helical structure composed of three  $\alpha$  chains of  $\alpha 1(IV)$  to  $\alpha 6(IV)$  chains with  $\alpha 3(IV)$  as the most predominant chain in the testis.<sup>23,24</sup> Each collagen chain is characterized by an N-terminal noncollagenous 7S domain of ~15-amino acid residues, a middle collagenous domain of ~1,400 residues of G-X-Y repeats, and a C-terminal noncollagenous (NC1) domain of

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**Table 1.** Antibodies used for different experiments in this report

Antibody	Vendor	Catalog no.	Applications/working dilution
Rabbit anti-Col IV	Abcam	ab6586	IHC* (1:100)
Rabbit anti-MMP-9	Abcam	ab7299	IB (1:1000)
Rabbit anti-Col $\alpha$ 3(IV) NC1	Wong & Cheng (current report)	Not applicable	IB (4 $\mu$ g IgG/ml)
Rabbit anti-His	Roche Applied Science	04905318001	IB (1:1000)
Rabbit anti-Flag M2	Stratagene	200470-21	IB (1:1000)
Rabbit anti-Occludin	Life Technologies	71-1500	IB (1:200)
Rabbit anti-CAR	Santa Cruz Biotechnology	sc-15405	IB (1:200), IF (1:50)
Rabbit anti-JAM-A	Life Technologies	36-1700	IB (1:150)
Rabbit anti-ZO-1	Life Technologies	61-7300	IB (1:150)
Mouse anti-ZO-1	Invitrogen	33-9100	IF (1:50)
Rabbit anti-N-cadherin	Santa Cruz Biotechnology	sc-7939	IB (1:200), IF (1:50)
Rabbit anti- $\alpha$ -catenin	Santa Cruz Biotechnology	sc-7894	IB (1:200)
Rabbit anti- $\beta$ -catenin	Santa Cruz Biotechnology	sc-7199	IB (1:200)
Mouse anti- $\beta$ -catenin	Chemicon/EMD Millipore	MAB2081	IF (1:50)
Goat anti-actin	Santa Cruz Biotechnology	sc-1616	IB (1:200)
Goat anti-rabbit IgG-Alexa Fluor 555 conjugated	Invitrogen	A21429	IF (1:250)
Goat anti-mouse IgG-Alexa Fluor 488 conjugated	Invitrogen	A11029	IF (1:250)

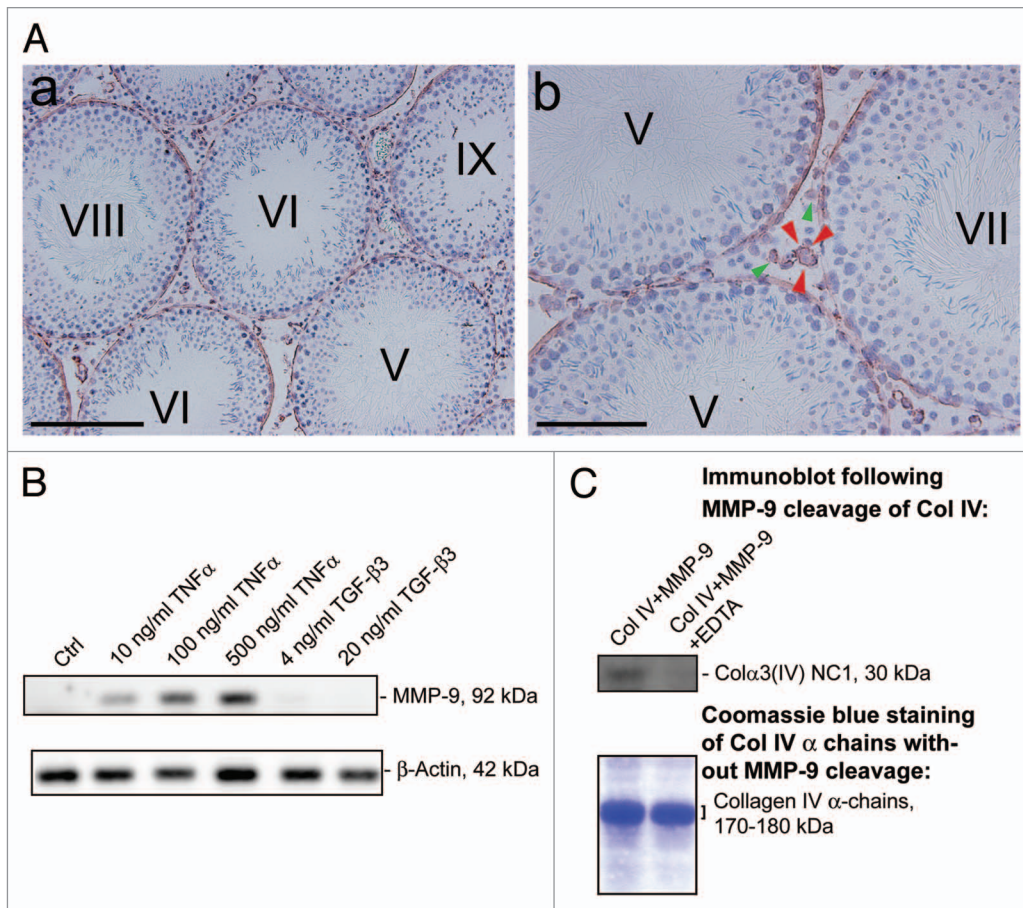
\*, IHC, immunohistochemistry; IB, immunoblotting; IF, immunofluorescence microscopy. It is noted that antibodies used for various experiments in this report cross-reacted with the corresponding proteins in the rat as indicated by the manufacturers.

~230 amino acid residues.<sup>19</sup> Collagens are scaffolding proteins known to provide support to epithelial and endothelial cells. However, previous studies have shown that NC1 fragments of collagen chains generated by limited proteolysis by matrix metalloproteinases (MMPs), such as MMP-9, are physiologically active peptides.<sup>25</sup> For instance, NC1 domain of collagen IV, also known as tumstatin, was shown to regulate cell adhesion, proliferation, angiogenesis and apoptosis in different cell types via its interactions with cell surface receptors, such as integrins.<sup>25-28</sup> Furthermore, it was reported that using Sertoli cells cultured *in vitro*, TNF $\alpha$ , a cytokine produced by Sertoli and germ cells in the testis,<sup>3,29</sup> was capable of inducing the production of activated MMP-9 by Sertoli cells,<sup>22</sup> which might be used to induce limited proteolysis of collagen  $\alpha$ 3(IV) chains to generate the NC1 domain peptide—the biologically active peptide that possibly regulates Sertoli cell TJ-barrier function.<sup>22</sup> Herein, we report findings that NC1 peptide derived from collagen  $\alpha$ 3(IV) chain was capable of perturbing Sertoli cell TJ function via its effects on protein localization at the Sertoli cell-cell interface, demonstrating unequivocally a functional relationship between the BTB and the basement membrane.

## Results

**Cleavage of Col IV to generate NC1 domain by MMP-9.** Using a specific anti-Col IV antibody (Table 1) for immunohistochemistry, Col IV was detected at the base of the seminiferous epithelium in seminiferous tubules of adult rat testes, consistent with its localization at the basement membrane (Fig. 1A). This finding is consistent with earlier studies showing that Col IV is one of the components of the basement membrane in mammalian

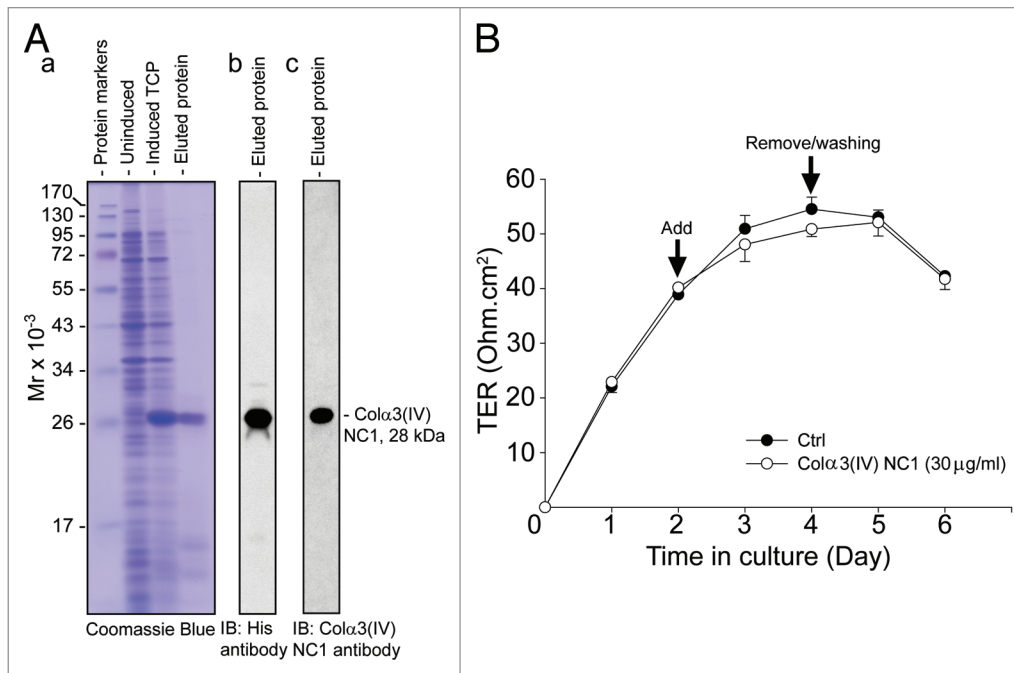
testes including rodents,<sup>30-32</sup> and  $\alpha$ 3,  $\alpha$ 4 and  $\alpha$ 5 chains of Col IV are recruited to the basement membrane of the tubules during postnatal development,<sup>23,30</sup> and  $\alpha$ 3(IV) and  $\alpha$ 4(IV) chains of Col IV are known to constitute almost 80% of collagen chains in the basement membrane of tubules in bovine testes.<sup>24</sup> In addition, no stage-specific expression of Col IV during the epithelial cycle was detected, consistent with its role as a structural basement membrane protein (Fig. 1A). Col IV was also detected in the interstitium in adult rat testes, associated with Leydig cells and endothelial cells of the microvessels (Fig. 1A, green and red arrowheads respectively), which is in agreement with previous studies. It is known that collagens including Col IV are expressed by Leydig cells in the mouse testis<sup>33</sup> and endothelial cells as well as basement membrane of microvessels.<sup>34-37</sup> Next, we investigated the upstream protease(s) that could possibly cleave the NC1 domain from Col IV in the basement membrane. MMP-9 is one of the best studied proteases known to cleave collagens.<sup>38,39</sup> Also, MMP-9 was found near the basement membrane in the seminiferous epithelium and is a secretory product of Sertoli cells.<sup>22</sup> To examine this possibility, Sertoli cells were cultured at low cell density ( $8 \times 10^4$  or  $0.2 \times 10^6$  cells/cm<sup>2</sup>) on dishes without Matrigel. At this lower cell density, the amount of extracellular matrix substances (e.g., Col IV and nidogen) secreted by Sertoli cells and deposited on the dishes was sufficient to support their attachment and growth as earlier reported.<sup>40</sup> Two days after isolation, 10–500 ng/ml human recombinant TNF $\alpha$  was added to F12/DMEM serum-free medium without bacitracin (which is a protease inhibitor and a polypeptide antibiotic). Sertoli cells were incubated with TNF $\alpha$  for 2 d before protein lysates, conditioned medium and extracellular matrix were collected. In line with our earlier results, TNF $\alpha$  was found to significantly induce the steady-state level of MMP-9



**Figure 1.** TNF $\alpha$  upregulates MMP-9 to promote cleavage of collagen (Col) IV to generate Col $\alpha$ 3(IV) NC1 domain. **(A)** Localization of Col IV in the seminiferous epithelium of adult rat testis was studied using frozen cross-sections fixed in ice-cold methanol and visualized with a specific anti-Col IV antibody (Table 1). Low magnification (A:a) micrograph illustrates that Col IV was expressed in the seminiferous epithelium of adult rat testes at all stages of the epithelial cycle. Higher magnified image shown in (A:b) illustrates that Col IV was restricted to the basal region of the seminiferous epithelium, consistent with its localization at the basement membrane. Immunoreactive Col IV was also found in Leydig cells (see green arrowheads in A:b), as well as endothelial cells and/or basal lamina of the microvessels (see red arrowheads in A:b) in the interstitium of adult rat testes. Scale bar, 100  $\mu$ m and 25  $\mu$ m in A:a and A:b, respectively. **(B)** Sertoli cells were cultured at  $8 \times 10^4$  cells/cm $^2$  on 6-well dishes without Matrigel. Different concentrations of TNF $\alpha$  and TGF- $\alpha$ 3 were added 2 d after Sertoli cell isolation. Sertoli cell-conditioned medium was harvested 2 d thereafter, and 25  $\mu$ g protein for each sample was used for SDS-PAGE and immunoblotting. TNF $\alpha$ , but not TGF- $\beta$ 3, was found to cause a dose-dependent increase in MMP-9 secretion by Sertoli cells as shown by immunoblotting (upper panel). The lower panel is the blot immunostained with an anti- $\beta$ -actin antibody using corresponding Sertoli cell lysates (25  $\mu$ g protein) to assess equal protein loading. **(C)** Recombinant MMP-9 (0.5  $\mu$ g protein) was used to examine if it induced cleavage of recombinant mouse Col IV (BD Bioscience) (10  $\mu$ g protein for each lane) contained mostly Col  $\alpha$ 3(IV) and Col  $\alpha$ 4(IV) of Mr 170–180 kDa in a ratio of 1:20 (MMP9:Col IV) with or without EDTA (an inhibitor of MMP-9) to generate Col $\alpha$ 3(IV) NC domain, which was detected by using a specific anti-Col $\alpha$ 3(IV) NC domain antibody (Table 1) by immunoblotting [see upper panel in (C)]. The lower panel in (C) is the Coomassie blue-stained SDS-polyacrylamide gel of 10  $\mu$ g purified recombinant Col IV protein per lane used for the experiment shown in the upper panel to demonstrate equal amount of Col IV was used for this experiment. Data shown herein are representative findings of an experiment. Each experiment, however, was repeated three times, excluding pilot experiments to optimize the experimental conditions, using testes of  $n = 3$  rats (A), Sertoli cells isolated from different groups of 20-d-old male pups (B) or different batches of Col IV and MMP-9, respectively. Similar data were obtained in different experiments.

protein in the conditioned medium when cells were cultured at  $8 \times 10^4$  cells/cm $^2$  since MMP-9 is a secretory product of Sertoli cells (Fig. 1B).<sup>22</sup> Similar effect was observed at higher cell density of  $0.2 \times 10^6$  cells/cm $^2$  (data not shown). A dose-dependent induction in MMP-9 by TNF $\alpha$  was detected and this effect was specific for TNF $\alpha$  because the inclusion of TGF- $\beta$ 3, another cytokine also known to disrupt the BTB, did not induce MMP-9 production by Sertoli cells (Fig. 1B). Using recombinant MMP-9 protein, it was shown that MMP-9 effectively cleaved Col IV to release the NC1 domain, which was readily detected by our antibody against

Col $\alpha$ 3(IV) NC1 (Fig. 1C, Table 1). This digestion was specific to MMP-9 because the presence of EDTA, an inhibitor of MMP-9, blocked the cleavage of Col IV to generate NC1 domain (Fig. 1C). We had tried to detect NC1 domain peptide in Sertoli cell lysates, conditioned medium and extracellular matrix by immunoblotting after treatment of TNF $\alpha$  using our anti-Col $\alpha$ 3(IV) NC1 antibody (Table 1). However, it is likely that due to the titer of the antibody, the level of endogenous NC1 that was cleaved by TNF $\alpha$ -induced MMP-9 was too low to be detected in these samples (data not shown).



**Figure 2.** Expression of recombinant Colα3(IV)NC1 protein in bacteria, its purification, and its effects on the Sertoli cell TJ-permeability barrier function. **(A)** BL21 (DE3) competent *E. coli* cells transformed with pET46 Ek/LIC-Colα3(IV) NC1 vector was induced with 0.1 mM IPTG to express the recombinant protein. Strong expression of Colα3(IV) NC1 domain (28 kDa) was noted when compared the un-induced total cell protein (TCP) to induced TCP as shown in a representative Coomassie blue stained gel (A:a). Recombinant protein was purified by Ni<sup>2+</sup>-Sephacrose chromatography in which the histidine (His, H) tag at the N-terminus of the recombinant protein specifically bound to the Ni-column. The eluted protein was purified to apparent homogeneity (A:b), and it was recognized by both anti-His (A:b) and anti-Colα3(IV) NC1 antibody (A:c) (see Table 1). **(B)** Primary Sertoli cells were plated at  $1.2 \times 10^6$  cells/cm<sup>2</sup> on Matrigel-coated bicameral units at time 0 to allow the assembly of a functional TJ-permeability barrier. Purified bacterial Colα3(IV) NC1 recombinant protein was refolded and dialyzed against PBS as described in Materials and Methods, and it was included in the F12/DMEM medium on day 2 at a concentration of 30 μg/ml (~1 μM). Mild but not statistically significant perturbation of the Sertoli cell BTB was observed when compared with PBS control (Ctrl) (Fig. 2B). Bacterial recombinant Colα3(IV) NC1 protein was removed 2 d after incubation. Each data point had n = 3 bicameral units. This experiment was repeated three times using different batches of Sertoli cells as well as recombinant protein, and yielded similar results.

**Production of mammalian recombinant Colα3(IV) NC1 domain protein.** Since TNFα was found to induce MMP-9, which led to the cleavage of Col IV to generate NC1 domain (Fig. 1), these findings are consistent with earlier reports that Col IV is a putative substrate of MMP-9.<sup>38,39</sup> Moreover, NC1 domain derived from this cleavage as shown in other epithelia was found to be biologically active, capable of modulating cell adhesion, cell proliferation and apoptosis;<sup>25</sup> we thus sought to examine if NC1 domain has any biological effects on the BTB. Previous studies have shown that Colα3(IV) is one of the major chains expressed in the basement membrane of seminiferous tubules.<sup>30,32</sup> We thus attempted to use bacterial cells to produce recombinant Colα3(IV) NC1 domain, which was purified to apparent homogeneity as shown in Figure 2A. The solubility and the yield of recombinant Colα3(IV) NC1 produced in bacteria were found to be poor, which may have resulted from the lack of glycosylation of the NC1 domain produced in *E. coli*. Nonetheless, we tested the ability of this recombinant protein in disrupting the Sertoli cell BTB by quantifying TER across the Sertoli cell epithelium with cells cultured onto Matrigel-coated bicameral units (Fig. 2B). Perhaps due to the lack of glycosylation of the recombinant NC1 domain protein, rendering it less effective to modulate the BTB function, we did not observe a

statistically significant disruption on the TJ-permeability barrier in multiple experiments, one of which was shown herein (Fig. 2B). These findings also illustrate that recombinant Colα3(IV) NC1 domain proteins produced in *E. coli* based on three separate experiments with different batches of purified NC1 protein were not biologically active. We next switched to produce recombinant Colα3(IV) NC1 domain protein in mammalian 293T cells. BM40 signal peptide (SP) and Flag tag were genetically engineered at the 5'-end of Colα3(IV) NC1 domain using primers shown in Table 2 and described in Materials and Methods. The presence of the SP allowed the secretion of the recombinant protein into the conditioned medium, whereas the Flag tag facilitated its purification by using anti-Flag M2 agarose beads. Using this approach based on the use of secreted recombinant protein harvested in the conditioned medium vs. the use of cell lysates also provided the advantage of dealing with soluble protein, instead of insoluble protein which resulted from the *E. coli* system. After affinity chromatography using the anti-Flag M2 resin, recombinant Colα3(IV) NC1 protein was purified to apparent homogeneity as visualized by Coomassie blue stained gels (Fig. 3A). The purified protein was recognized by the anti-Flag antibody (Fig. 3B) and anti-Colα3(IV) NC1 antibody (Fig. 3C) (Table 1).

**Table 2.** Primers used to construct pTracer-CMV2-Col $\alpha$ 3(IV) NC1 expression vector

Sense primer 1*	5'-AAGGATGATGACGACAAG	ACAAGAATGAGAGGCTTCATCT-3'	
	Flag	NC1	
Sense primer 2	5'-CTGGCAGCCCTCTGGCA	GATTATAAGGATGATGACGACAAG	A-3'
	BM40 SP	Flag	NC1
Sense primer 3	5'-TTTCTCCTTTGCCTGGCCGGGAGGGCCCTGGCAGCCCT-3'		
	BM40 SP		
Sense primer 4	5'-CAGATATC	ATGGCTAGGGCCTGGATCTTCTTCTCCTTTGCCTGGC-3'	
	BM40 SP		
Anti-sense primer	5'-CATCTAGATTAGTGTCTTTTCTTCATGCACAC-3'		

\*, four overlapping PCRs were performed by using sense primer 1 and anti-sense primer in the first round and so on. Primer sequences were underlined to indicate the added BM40 signal peptide (SP) and Flag sequences at the 5'-end of Col $\alpha$ 3(IV) NC1 coding sequence. The Flag encoding sequence is DYKDDDK while that for BM40 SP is MARAWIFFLLCLAGRALAAPLA. An extra Ala (A) amino acid was added after the start codon to form a Kozak sequence for high level of mRNA expression in Lenti-X 293T cells. Four more amino acids (APLA) from the rat BM40 coding sequence immediately after the SP were included to aid cleavage of the SP from the recombinant protein.

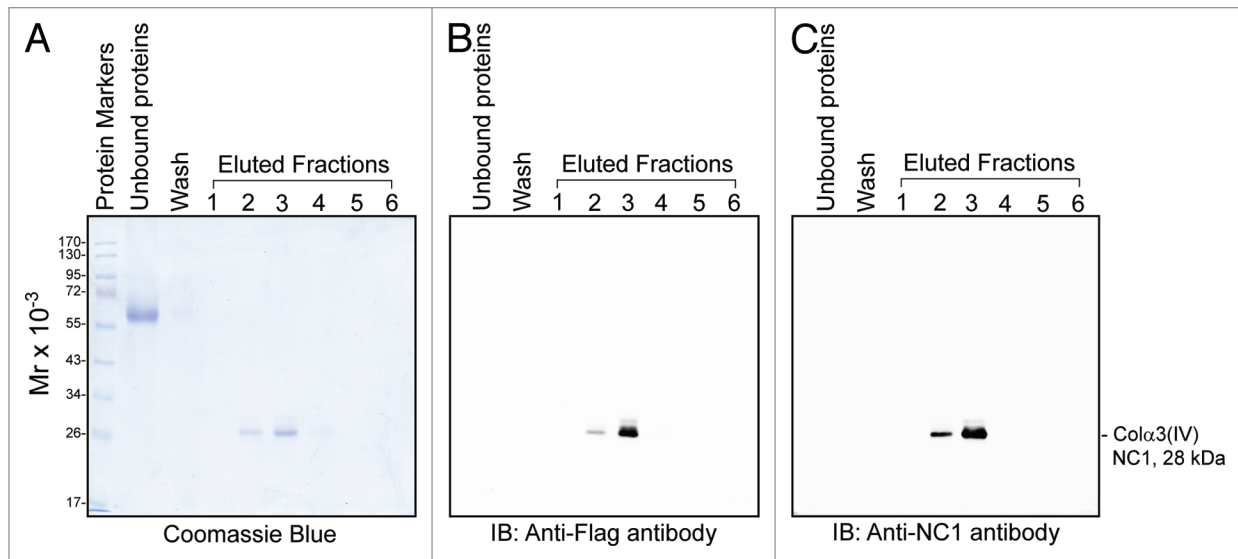
**Col $\alpha$ 3(IV) NC1 domain reversibly disrupts the Sertoli cell TJ-permeability barrier function.** Sertoli cells were cultured alone for 2 d to allow the establishment of a functional TJ-permeability barrier, manifested by a relatively stable TER across the cell epithelium as shown in **Figure 4A**. The inclusion of purified recombinant Col $\alpha$ 3(IV) NC1 protein at 30  $\mu$ g/ml (1  $\mu$ M) in F12/DMEM in the apical and basal compartment of the bicameral units was found to perturb the Sertoli TJ-permeability barrier (**Fig. 4A**). This disruptive effect was reversible since the removal of the recombinant protein by washing the Sertoli cell epithelium with fresh F12/DMEM without the recombinant protein allowed the “resealing” of the disrupted TJ-barrier (**Fig. 4A**). Interestingly, while the recombinant Col $\alpha$ 3(IV) NC1 domain protein reversibly perturbed the TJ-barrier, it failed to downregulate the steady-state levels of both TJ and basal ES proteins at the Sertoli cell BTB, when cell cultures were terminated 2 d after addition of the recombinant protein in the medium for immunoblotting (**Fig. 4B and C**). We next assessed if there were any changes in the localization and/or distribution of junction proteins at the Sertoli cell-cell interface after treatment of Col $\alpha$ 3(IV) NC1 (**Fig. 4D**). Treatment of Sertoli cells cultured on Matrigel-coated coverslips with 30  $\mu$ g/ml Col $\alpha$ 3(IV) NC1 for 2 d was found to induce mis-localization of junction proteins. For instance, TJ proteins (e.g., CAR and ZO-1) and basal ES proteins (e.g., N-cadherin and  $\beta$ -catenin) were no longer localized predominantly at the Sertoli cell-cell interface, instead these proteins moved away from the cell-cell interface and appeared to be in the cell cytosol (**Fig. 4D**). It is noted that some CAR staining was detected in the nucleus (**Fig. 4D**).

## Discussion

Restructuring of the BTB that occurs near the basement membrane takes place simultaneously with spermiation that takes place at the adluminal edge of the seminiferous epithelium at stage VIII of the epithelial cycle. It is presently unknown how these two cellular events are being coordinated. An earlier study has shown that laminin fragments produced at the apical ES during spermiation via the action of MMP-2 were capable of perturbing the Sertoli cell BTB function near the basement membrane.<sup>41</sup>

A more recent report has identified the biologically active fragment of the laminin chain.<sup>42</sup> Also, a knockdown of  $\beta$ 1-integrin, an integrated component of the hemidesmosome in the testis by RNAi, was also found to perturb the Sertoli cell TJ barrier function.<sup>41</sup> These findings thus support the notion that there is a local autocrine-based regulatory axis in the seminiferous epithelium, known as the “apical ES-BTB-hemidesmosome” axis, that regulates and/or coordinates cellular events across the epithelium during the epithelial cycle.<sup>41,42</sup> The presence of this local functional axis was also supported by studies using a Sertoli cell injury model, based on the use of phthalates, which showed that a disruption of the apical ES involving cleavage of laminin- $\gamma$ 3 chains during spermatid loss also led to a BTB disruption.<sup>43-45</sup>

Herein, we report findings that support the presence of an autocrine-based axis between the BTB and the basement membrane. It is likely that biologically active Col $\alpha$ 3(IV) NC1 domain peptides are generated at the basement membrane, plausibly via the action of TNF $\alpha$ -induced MMP-9 production that causes limited proteolysis of collagen  $\alpha$ 3(IV) chain in the basement membrane. More important, the perturbation of the Sertoli cell TJ-permeability barrier function by biologically active Col $\alpha$ 3(IV) NC1 domain peptide is not mediated by downregulating the expression of integral membrane proteins nor their adaptors at the site since such changes would require minutes, if not hours, for changes in phenotypes. Instead, this biologically active collagen-based peptide is readily available to alter cellular localization and/or distribution at the Sertoli cell-cell interface rapidly. This, in turn, induces BTB restructuring at the site in response to the stages of the epithelial cycle during spermatogenesis and these changes are not mediated via de novo protein synthesis and/or gene expression as illustrated in this report. While the Col $\alpha$ 3(IV) NC1 domain recombinant protein that was used in our studies to confirm its effects on the Sertoli cell TJ-permeability function and also protein distribution at the Sertoli cell-cell interface was purified to apparent homogeneity, it is still possible that other peptides and/or biomolecules can be produced in the basement membrane to affect the Sertoli cell TJ barrier function. Work is now in progress to examine this possibility.

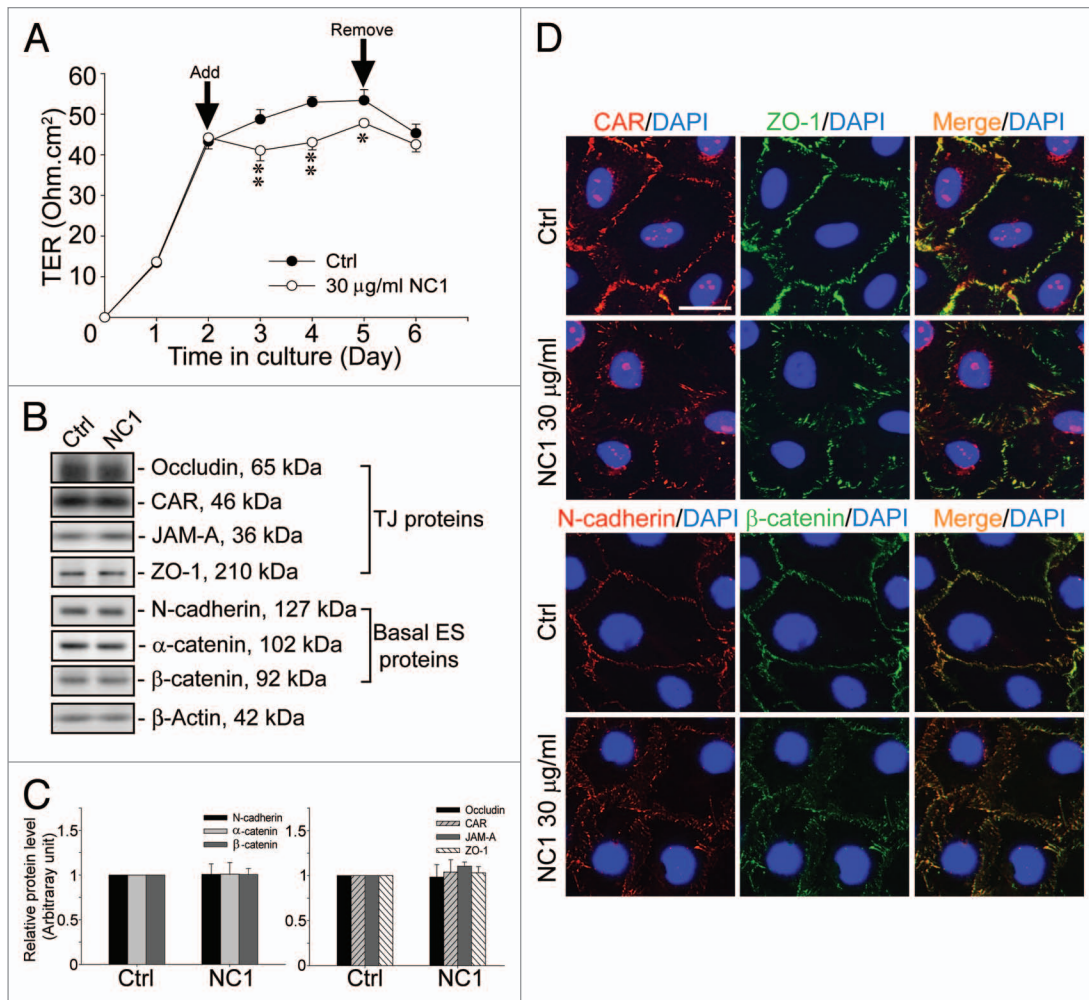


**Figure 3.** Production of recombinant Col $\alpha$ 3(IV)NC1 domain protein in mammalian cells. Recombinant Col $\alpha$ 3(IV)NC1 domain protein was expressed from pTracer-CMV2-Col $\alpha$ 3(IV)NC1 stably transfected in Lenti-X 293T mammalian cells. Serum-free conditioned medium was harvested and Col $\alpha$ 3(IV)NC1 domain was purified using anti-Flag M2 resin. Recombinant Col $\alpha$ 3(IV)NC1 domain protein was detected in fractions 2 and 3 eluted from the affinity chromatography and it was purified to apparent homogeneity in a single step when its purity was analyzed by Coomassie blue stained SDS-polyacrylamide gel (A), and by immunoblotting using an anti-Flag (B) and an anti-Col $\alpha$ 3(IV)NC1 antibody (C). These findings are representative data from three different experiments, using different batches of recombinant protein excluding pilot experiments to establish the optimal conditions, over a 2-y period which yielded similar results.

It was noted that transient perturbation of the Sertoli cell TJ-permeability barrier induced by the Col $\alpha$ 3(IV) NC1 domain peptide as reported herein was not as drastic as those induced by toxicants (e.g., cadmium),<sup>46,47</sup> even though these changes were statistically significant. This is likely due to the possibility that Col $\alpha$ 3(IV) NC1 domain peptide, which is released from the basement membrane via the action of MMP-9 during spermatogenesis, such as at stage VIII of the epithelial cycle, is not being used to “disrupt” the BTB analogous to the disruption induced by cadmium<sup>46,47</sup> or bisphenol A (BPA).<sup>48</sup> Instead, it is being used to disrupt the “old” BTB that are found above the preleptotene spermatocytes, which are located adjacent to the basement membrane as these germ cells are being transported across the BTB over a ~29 h period, the duration of stage VIII during the epithelial cycle,<sup>49,50</sup> while “new” BTB is being assembled behind these spermatocytes. This possibility is supported by the findings that the steady-state protein levels of TJ (e.g., CAR, ZO-1)- and basal ES (e.g., N-cadherin,  $\beta$ -catenin)-proteins were not downregulated following treatment of the Sertoli cell epithelium with Col $\alpha$ 3(IV) NC1 domain peptide as reported herein. Instead, this mild but statistically significant perturbation of the TJ-permeability barrier is mediated by changes in the localization of TJ- and basal ES-proteins at the Sertoli cell-cell interface, possibly via an increase in protein endocytosis at the “old” BTB, so that they can be transcytosed from the apical to the basal region of the preleptotene spermatocytes and these proteins can be recycled to assemble “new” BTB. This also represents a novel and physiologically efficient system to regulate BTB function during the transit of preleptotene spermatocytes across the BTB while the immunological barrier is still maintained.

It is noted that the TJ protein complex CAR-ZO-1 and the basal ES protein complex N-cadherin- $\beta$ -catenin were co-localized almost to the same site at the Sertoli cell-cell interface as reported herein, which are also the structural protein complex of the BTB. This is not entirely unexpected since it is known that TJ coexists with basal ES, localized to the same site at the BTB in the testis when examined by electron microscopy.<sup>3,4,51-54</sup> In this context, it is of interest to note that CAR, a TJ-integral membrane protein known to serve as a receptor for coxsackievirus and adenovirus<sup>55,56</sup> to facilitate their crossing at the tissue barriers<sup>57,58</sup> was also found in Sertoli cell nuclei besides its localization at the Sertoli cell-cell interface, corresponding to its localization at the TJ. A recent study has shown that CAR can indeed be internalized via protein endocytosis and associated with EEA1 (early endosome antigen 1, an early endosome marker), and it is involved in endocytic vesicle-mediated protein trafficking events such as protein endocytosis.<sup>59</sup> Furthermore, an earlier study in human bladder cell lines has shown that CAR is also involved in cell cycle regulation in the nucleus to elicit its growth-inhibitory activity.<sup>60</sup> Thus, it is not entirely unusual that CAR can be detected in the nucleus; however, it remains to be determined if the Col $\alpha$ 3(IV) NC1 domain peptide is involved in regulating endocytic vesicle-mediated trafficking, and its functional relationship with CAR and other proteins at the BTB. Additional studies, such as the use of immunogold electron microscopy and/or biochemical assays of endocytosis and recycling, will need to be performed in future studies to assess the involvement of Col $\alpha$ 3(IV) NC1 domain peptide in protein trafficking events.

As hemidesmosome is an integrated component at the Sertoli cell-basement membrane interface, we now rename this axis as the “apical ES-BTB-basement membrane” axis. This functional axis



**Figure 4.** Disruption of the Sertoli cell TJ-permeability barrier function by recombinant Col $\alpha$ 3(IV)NC1 domain protein mediated via changes in protein distribution and/or localization at the Sertoli cell-cell interface. **(A)** Sertoli cells were plated at  $1.2 \times 10^6$  cells/cm<sup>2</sup> on Matrigel-coated bicameral units with an established TJ-permeability barrier by day 2, and 30  $\mu$ g/ml recombinant Col $\alpha$ 3(IV) NC1 (1  $\mu$ M) in PBS was included in the F12/DMEM. The presence of the NC1 domain recombinant protein was found to significantly perturb the TJ-barrier function vs. PBS control (Ctrl) until the recombinant protein was removed on day 5, and the partially disrupted TJ-barrier was “resealed.” \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ . **(B)** Sertoli cells were plated at  $0.5 \times 10^6$  cells/cm<sup>2</sup> for 2 d before 30  $\mu$ g/ml (1  $\mu$ M) of the recombinant NC1 domain was included in the F12/DMEM. Two d later, there was no significant change in steady-state levels of TJ and basal ES proteins when compared with PBS control (Ctrl). **(C)** This histogram summarizes results shown in **(B)** where protein levels from Ctrl were arbitrarily set at 1. Each bar is a mean  $\pm$  SD of  $n = 3$  independent experiments. **(D)** Sertoli cells were cultured at  $0.045 \times 10^6$  cells/cm<sup>2</sup> on Matrigel-coated coverslips, and treated with 30  $\mu$ g/ml NC1 or PBS Ctrl for 2 d. Cells were fixed and stained for CAR (red) and ZO-1 (green) or N-cadherin (red) and  $\beta$ -catenin (green) by dual-labeled immunofluorescence analysis using the corresponding primary antibodies and Alexa Fluor 555- or 488-conjugated secondary antibodies (see **Table 1**). Sertoli cell nuclei were visualized by DAPI (blue). CAR, ZO-1, N-cadherin and  $\beta$ -catenin were all seen at the Sertoli cell-cell interface in Ctrl cells while treatment of cells with 30  $\mu$ g/ml NC1 led to changes in distribution of these BTB-associated cell adhesion proteins, moving from the cell-cell interface into the cell cytosol. Scale bar, 5  $\mu$ m, which applies to all micrographs. Findings of this dual-labeled immunofluorescence analysis experiment reported herein were results of a representative experiment, which was repeated three times using different Sertoli cell cultures and yielded similar results.

can now be vigorously investigated since components across this axis, including the crucial regulatory proteins, such as Col $\alpha$ 3(IV) NC1 domain peptide reported herein, can now be examined in functional experiments. Thus, future studies should include a careful examination on the production of Col $\alpha$ 3(IV) NC1 domain peptide during the epithelial cycle. Besides, the role of cytokines (e.g., TNF $\alpha$ ), either alone or together with MMP-9 (and other MMPs) on its production should be evaluated. Furthermore, Col $\alpha$ 3(IV) likely acts as a ligand, its receptor in Sertoli cells, such as integrins (and/or other molecules) should also be identified.

## Materials and Methods

**Animals and antibodies.** Sprague-Dawley rats, 18-d-old pups or adult rats (~270–300 g b.w.) were obtained from Charles River Laboratories. The use of animals reported here was approved by the Rockefeller University Institutional Animal Care and Use Committee (Protocol Numbers 09-016 and 12-506). Antibodies used for different experiments reported herein were obtained commercially, unless otherwise specified, are listed in **Table 1**.

**Production of antibody against Col $\alpha$ 3(IV) NC1 domain.** Recombinant Col $\alpha$ 3(IV) NC1 domain was produced using the pET30 Ek/LIC vector kit in BL21 (DE3) bacteria (EMD Millipore). Col $\alpha$ 3(IV) NC1 domain was amplified by sense 5'-GACGACGACA AGATCACAAG AATGAGAGGC TTCATCTTCA-3' and antisense 5'-GAGGAGAAGC CCGGTGTGTC TTTTCTTCAT GCACACCTGA-3' primers using 90-d-old rat testis cDNA as template. This corresponded to the 4,321–5,010 nt of rat full-length Col $\alpha$ 3(IV) (NM\_001135759), which is the NC1 domain. The stop codon in the original full-length sequence was omitted due to the presence of in frame stop codon in the vector. Purity of the recombinant protein expression in *E. coli* isolated by affinity chromatography was confirmed by SDS-PAGE with the gel stained by Coomassie blue. ~200  $\mu$ g purified protein emulsified with Freund's complete adjuvant was used to immunize a female white New Zealand rabbit, to be followed by two booster injection of purified protein (200  $\mu$ g each) emulsified with Freund's incomplete adjuvant. Blood (~20–50 ml) was collected ~4-wk thereafter, and then every 10-d over a ~10-wk period. Serum was obtained by centrifugation (2,000 g, 10 min, 4 °C, twice) after blood was allowed to clot overnight at 4 °C. IgG was isolated by sequential ammonium sulfate precipitation and DEAE affinity chromatography.<sup>61</sup>

**Purification of Col $\alpha$ 3(IV) NC1 domain recombinant protein produced in bacteria.** Col $\alpha$ 3(IV) NC1 was produced in bacteria using the pET46 Ek/LIC expression vector (EMD Millipore). Col $\alpha$ 3(IV) NC1 coding sequence was amplified by sense primer: 5'-GACGACGACA AGATCACAAG AATGAGAGGC TTCATCTTCA-3' and antisense primer: 5'-GAGGAGAAGC CCGGTTTAGT GTCTTTTCTT CATGCACA-3' using pET30 Ek/LIC-Col $\alpha$ 3(IV) NC1 as template. The authenticity of pET46 Ek/LIC-Col $\alpha$ 3(IV) NC1 was confirmed by direct nucleotide sequencing (at Genewiz) and transformed into BL21 (DE3) (EMD Millipore) for recombinant protein production. Five ml overnight bacterial culture was used to inoculate 500 ml LB supplemented with 100  $\mu$ g/ml carbenicillin (Life Technologies). Bacteria were allowed to grow until the OD<sub>600</sub> reached ~0.65 and 0.1 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) was used to induce production of recombinant protein for 3.5 h at 37°C. Bacteria were harvested at 6,000  $\times$  g 10 min. All bacterially expressed Col $\alpha$ 3(IV) NC1 was found in the inclusion bodies (insoluble fraction). Bacteria were lysed and inclusion bodies were extracted by BugBuster Plus Lysonase kit (EMD Millipore) according to the manufacturer protocol. 6 M guanidine HCl, 0.1 M NaH<sub>2</sub>PO<sub>4</sub> and 10 mM TRIS-HCl, pH 8.5 was used to dissolve inclusion bodies. Ni<sup>2+</sup>-Sepharose high performance resin (GE Healthcare) was used to purify the histidine (His)-tagged Col $\alpha$ 3(IV) NC1 domain in a packed column (Bio-rad). To wash the resin, increasing amount of imidazole (10 mM and 25 mM) in 8 M urea, 0.1 M NaH<sub>2</sub>PO<sub>4</sub> and 10 mM TRIS-HCl, pH 8 was used. Col $\alpha$ 3(IV) NC1 domain was eluted with 250 mM imidazole, 8 M urea, 0.1 M NaH<sub>2</sub>PO<sub>4</sub> and 10 mM TRIS-HCl, pH 8 and refolded according to Gu et al.<sup>62</sup> Briefly, eluted protein was dialyzed against decreasing concentration of urea: 1) 4 M urea, 0.1 M NaCl and 20 mM TRIS-HCl, pH 6 overnight at 4°C; 2) 2 M urea, 0.1 M NaCl, 2 mM reduced glutathione, 0.2 mM

oxidized glutathione, 1 mM EDTA and 20 mM TRIS-HCl, pH 6.5 for 8 h 4°C and 3) PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub> and 1.47 mM KH<sub>2</sub>PO<sub>4</sub>) pH 7.4 overnight at 4°C.

**Collagen (Col) IV cleavage by MMP-9.** Murine MMP-9 (EMD Millipore) was first activated by treatment with 0.5 mg/ml TPCK-trypsin (Thermo Scientific) in an activation buffer (50 mM TRIS-HCl, 150 mM NaCl, 5 mM CaCl<sub>2</sub> and 0.05% Brij-35, pH 7.5 at 22°C) for 30 min at 37°C. Thereafter, trypsin activity was inhibited by adding aprotinin (Sigma-Aldrich) (1 mg/ml) to activation buffer. 0.5  $\mu$ g activated MMP-9 was then used to digest 10 g recombinant mouse Col IV [BD Biosciences, derived from Engelbreth-Holm-Swarm (EHS) lathritic mouse tumor, a major component of the basement membrane which contained mostly Col  $\alpha$ 3(IV) and  $\alpha$ 4(IV) chains of Mr 170–180 kDa in a buffer of 1 M Tris (pH 8 at 22°C) with or without 200 mM EDTA, for 3 h at 37°C. The digested proteins were resolved by SDS-PAGE and probed for Col $\alpha$ 3(IV) NC1 by immunoblotting.

**Production of recombinant Col $\alpha$ 3(IV) NC1 domain in mammalian cells.** pTracer-CMV2 (Life Technologies) expression vector carrying rat Col $\alpha$ 3(IV)NC1 domain was constructed by PCR using AccuPrime Pfx Taq (Life Technologies) and pET30 EK/LIC-Col $\alpha$ 3(IV) NC1 as a template. Four sequential overlapping PCRs were performed to add a rat BM40 (NM\_012656.1) signal peptide (SP) and Flag sequence at the 5'-end of Col $\alpha$ 3(IV) NC1 coding sequence to allow extracellular secretion and aid purification. Sequences of the primers used for preparing the cDNA construct were listed in Table 2. The resulting DNA fragment was digested with EcoRV and XbaI for 7 h at 37°C and ligated into pTracer-CMV2 overnight at 15°C. Correct clone was confirmed by DNA sequencing (Genewiz). pTracer-CMV2-Col $\alpha$ 3(IV) NC1 vector was linearized by ScaI for 16 h at 37°C. A human embryonic kidney cell line Lenti-X 293T cells (Clontech) was used to produce the recombinant Col $\alpha$ 3(IV) NC1 domain protein. Cells were maintained in high glucose DMEM supplemented with 10% FBS (Life Technologies), 3.7 g/L NaHCO<sub>3</sub>, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 g/ml streptomycin, 2 mM GlutaMAX and 0.1 mM MEM non-essential amino acids (Life Technologies), pH 7.4 at 37°C in humidified 10% CO<sub>2</sub>/95% air (v/v) atmosphere. Cells seeded at 4  $\times$  10<sup>4</sup> cells/cm<sup>2</sup> in 12-well dishes (with each well contained ~4-ml medium) were transfected with 1 g linearized DNA using Fugene HD reagent (Roche) according to manufacturer's protocol. One day after transfection, cells were split 1:4 into 100-mm dish and 600  $\mu$ g/ml zeocin (Life Technologies) were included in culture medium. Fresh medium was changed every 3 d until foci were formed. Individual colonies were trypsinized and cultured in 12-well dishes with 50  $\mu$ g/ml zeocin. Positive clones were selected by RT-PCR using sense primer 1 and antisense primer (Table 2) as well as fluorescence microscopy to detect the GFP tag in the vector. A clone with the strongest Col $\alpha$ 3(IV) NC1 domain expression was expanded into three 150-mm dishes, grown until confluency and replaced with serum-free high glucose DMEM. During the production of recombinant Col $\alpha$ 3(IV) NC1 domain, zeocin was omitted. After 40 h of incubation, supernatant was collected and centrifuged at 2,000 g for 10 min to remove cell debris. Recombinant Col $\alpha$ 3(IV)NC1 domain was purified using anti-Flag M2 affinity gel (Sigma-Aldrich) by incubating cleared



supernatant with the resin at 4°C for 3 h in the presence of 0.2 M NaCl in a laboratory rocking platform (Clay Adams Nutator) to reduce non-specific binding. Resin was then packed into an EconoColumn (Bio-Rad), washed with TBS (50 mM TRIS-HCl, 150 mM NaCl, pH 7.4) and recombinant Col $\alpha$ 3(IV) NC1 domain protein eluted with 0.1 M glycine HCl, pH 3.5. Eluted protein was neutralized with 1 M Tris, pH 8 and equilibrated in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub> and 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4), and the purified was confirmed by SDS-PAGE using Coomassie blue-stained gel with an apparent Mr of 30 kDa. Four batches of recombinant proteins were produced over a 2 y period with consistent results.

**Primary Sertoli cell cultures.** Primary Sertoli cells were isolated from 20-d-old rat testes and cultured in serum-free F12/DMEM medium supplemented with insulin, transferrin, epidermal growth factor (EGF) and bacitracin as described.<sup>40</sup> These Sertoli cells were differentiated and ceased to divide<sup>63</sup> unless serum (such as 5% fetal calf serum) was present in the media.<sup>64</sup> These cells were functionally indifferent from Sertoli cells isolated from adult rat testes based on earlier studies.<sup>65,66</sup> But the purity of Sertoli cells isolated from adult testes was only ~85% reported in our earlier studies<sup>65,66</sup> consistent with an earlier report.<sup>67</sup> However, Sertoli cells isolated from 20-d-old rat testes had a purity of > 98% with negligible contamination of germ and Leydig cells when cell contaminants were assessed by RT-PCR and/or immunoblotting using the corresponding specific primers and/or specific antibodies against unique markers of these cells.<sup>40,68</sup> Thus, Sertoli cells from 20-d-old rat testes were used in our experiments. To investigate the induction of MMP-9 by TNF $\alpha$  (R&D Systems, Minneapolis, MN) or TGF- $\alpha$ 3 (Calbiochem-EMD Millipore), Sertoli cells were seeded at  $8 \times 10^4$  or  $0.2 \times 10^6$  cells/cm<sup>2</sup> on 6-well-dishes (without Matrigel) for 2 d in F12/DMEM with other supplements but without bacitracin. Sertoli cell-conditioned medium and cell lysates were collected 2 d after treatment. To extract the extracellular matrix deposited by Sertoli cells, cells were seeded on 100-mm dishes without Matrigel and processed as earlier described<sup>69</sup> with minor modifications. In brief, Sertoli cells were washed with PBS containing 1 mM phenylmethylsulfonyl fluoride and aminocaproic acid (1 mg/ml) for three times, to be followed by two successive incubations with the wash buffer and 5 mM EGTA for 15 min at 37°C to remove all Sertoli cells. Remaining cell debris was removed by three washes with PBS alone, to be followed by three washes of double distilled water. Four hundred  $\mu$ l of SDS sample buffer [0.125 M Tris, pH 6.8 at 22°C containing 1% SDS (w/v), 1.6% 2-mercaptoethanol (v/v) and 10% glycerol (v/v)] was added and dishes were scraped thoroughly by using a rubber policeman. To assess the effect of recombinant Col $\alpha$ 3(IV) NC1 domain on the Sertoli cell BTB function, primary Sertoli cells were seeded at  $0.5 \times 10^6$  cells/cm<sup>2</sup> on Matrigel-coated 12-well dishes (for protein lysates preparation) or  $1.2 \times 10^6$  cells/cm<sup>2</sup> on Matrigel-coated bicameral unit [for transepithelial electrical resistant (TER) measurement] to assess the TJ-permeability barrier function. For cell staining, Sertoli cells at  $4.5 \times 10^4$  cells/cm<sup>2</sup> were seeded on Matrigel-coated coverslips, so that cell nuclei were evenly interspersed, and changes in the distribution and/or localization of BTB-proteins at the Sertoli cell-cell interface following treatment with Col $\alpha$ 3(IV) NC1 domain recombinant

protein could be easily assessed by dual-labeled immunofluorescence analysis. Recombinant Col $\alpha$ 3(IV) NC1 domain protein in PBS (30  $\mu$ g/ml, at 1 M) was added 2 d after cell isolation. PBS was used as a vehicle control. Recombinant protein was removed 2 d (for protein lysates and cell staining) or 3 d (for TER measurement to assess TJ function) after treatment.

**Assessment of Sertoli cell TJ-permeability barrier function.** The Sertoli cell TJ-permeability barrier function was monitored by quantifying the TER across the Sertoli cell epithelium as described.<sup>40,70</sup> Sertoli cells were plated on Matrigel-coated bicameral units on day 0. Within ~2 d, Sertoli cells established a functional TJ-permeability barrier, with visible ultrastructures of TJ, basal ES, gap junction and desmosome under electron microscopy,<sup>71,72</sup> which mimicked Sertoli BTB in vivo, and this system has been used by other investigators to study BTB regulation.<sup>46,73-78</sup> Recombinant Col $\alpha$ 3(IV) NC1 domain protein was added to apical and basal compartment of the bicameral units on day 2 at 1  $\mu$ M (30  $\mu$ g/ml). This concentration was selected based on pilot experiments. F12/DMEM was replaced daily and Col $\alpha$ 3(IV) NC1 domain protein was also included in the replacement medium until day 5, when F12/DMEM without recombinant protein was used. Each time point has triplicate bicameral units in both the treatment and the control group. Each experiment was repeated at least three times using different batches of Sertoli cells.

**Immunohistochemistry, immunofluorescence microscopy and immunoblotting.** Frozen cross-sections of testes were obtained in a cryostat at -22°C, collected on poly-L-lysine-coated microscopic slides, fixed in methanol for 10 min at -20°C. Immunohistochemistry was performed using Histostain-SP kit (AEC, rabbit, Life Technologies) with 1:100 anti-Col IV (Abcam) (Table 1) as described.<sup>70</sup> Immunofluorescence staining was performed as described.<sup>79</sup> For cell staining, primary Sertoli cells were either fixed in methanol at -20°C for 10 min (for basal ES-proteins N-cadherin and  $\beta$ -catenin) or 4% paraformaldehyde/PBS (w/v) (for TJ-proteins CAR and ZO-1) at room temperature for 10 min, and then permeabilized with 0.2% Triton X-100 (v/v) for 5 min. Dual-labeled immunofluorescence analysis was performed using rabbit anti-CAR or rabbit anti-N-cadherin vs. mouse anti-ZO-1 or mouse anti- $\beta$ -catenin to detect the corresponding TJ- and basal ES-proteins at the Sertoli cell-cell interface (see Table 1). Negative controls were performed using normal rabbit IgG, diluted in blocking solution to substitute the primary antibody. Cells were then incubated with Alexa Fluor-conjugated secondary antibodies of the corresponding species (Alexa Fluor 555 for red fluorescence; Alexa Fluor 488 for green fluorescence) (Table 1) to visualize the corresponding target proteins. Fluorescence microscopy was performed using an Olympus BX61 microscope equipped with DP71 12.5 megapixel digital camera, and images were acquired using MicrosuitFive software package. All images were obtained in TIFF image format and analyzed (such as image overlay) using Adobe Photoshop. Immunoblotting was performed by using 25  $\mu$ g protein and 5  $\mu$ g protein of Sertoli cell lysates and Sertoli cell-conditioned media, respectively, as described.<sup>79</sup> All the antibodies used herein are listed in Table 1. Results of immunohistochemistry, dual-labeled immunofluorescence analysis and immunoblotting reported herein were findings of a representative experiment.

Each experiment was repeated at least four times, excluding pilot experiments to establish experimental conditions, using at least  $n = 3$  rats or different batches of Sertoli cells over a 3 y period.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Data acquisition and Statistical Analysis

Data presented here were results from at least three independent experiments using different batches of Sertoli cells and recombinant proteins. Statistical Analysis was performed by ONE-way ANOVA followed by Dunnett's test using the GB-STAT software package (Version 7.0, Dynamic Microsystems).

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#### Author Contributions

Conceived the ideas of this project: CYC. Designed experiments: EWPW, CYC. Performed the experiments: EWPW, CYC. Analyzed the data: EWPW, CYC. Prepared the reagents, materials and analysis tools: EWPW, CYC. Wrote the paper and final editing of the manuscript: EWPW, CYC.

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