A study to assess the assembly of a functional blood-testis barrier in developing rat testes

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The blood-testis barrier (BTB) is an important ultrastructure in the seminiferous tubule of the mammalian testis that segregates the events of spermatogenesis, in particular post-meiotic germ cell development, from the harmful substances in the environment including toxicants and drugs, as well as from the unwanted hormones and biomolecules in the systemic circulation. It is known that the BTB is assembled by ~15–21 days postpartum (dpp) in rats coinciding with the onset of late cell cycle progression, namely the formation of zygotene and pachytene spermatocytes by day 15–18 dpp. This is to prepare for: (1) the differentiation/transformation of pachytene spermatocytes to diplotene and dictyate spermatocytes and (2) meiosis I and II, which take place by 23–26 and 26 dpp, respectively. Recent findings have shown spermatogonia/spermatogonial stem cells (SSC) in the tubules failed to re-initiate spermatogenesis by differentiating spermatogonia beyond type A spermatogonia in the absence of a functional BTB, leading to meiotic arrest. These studies thus illustrate that a functional BTB is crucial to the initiation and/or re-initiation of spermatogenesis. Herein, we sought to examine the precise time window when a functional and intact BTB is established in the developing rat testis during the final stage of cell cycle progression and meiosis. Using the techniques of: (1) dual-labeled immunofluorescence analysis to assess the distribution of integrated proteins at the tight junction (TJ), basal ectoplasmic specialization [basal ES, a testis-specific atypical adherens junction (AJ) type] and gap junction (GJ) at the BTB, (2) functional assay to assess the BTB integrity in vivo, (3) immunoblot analysis to monitor changes in steady-state levels of adhesion proteins at the BTB, and (4) co-immunoprecipitation to assess changes in protein-protein interactions at the BTB, it was shown that a BTB was being assembled by day 15–20 dpp, but a functional BTB was not fully established until day 25 dpp in Sprague-Dawley rats, tightly associated with the onset of meiosis I and II. These findings thus illustrate the significance of the BTB on cell cycle progression and the preparation for meiosis, such as germ cell differentiation beyond type A spermatogonia.

Introduction

The blood-testis barrier (BTB) is an important ultrastructure in the seminiferous tubules of the mammalian testis. It segregates the seminiferous epithelium into the basal and the adluminal (apical) compartments so that post-meiotic germ cell development takes place in a unique microenvironment. This thus prevents the production of anti-spermatid antibodies, many of which express transiently during spermiogenesis.^{1–3} Besides conferring the testis its immune-privilege status,^{2,4} BTB also serves as a “gatekeeper” to restrict paracellular flow and/or transport of biomolecules, water, electrolytes, ions, and hormones across the barrier, as well as maintaining cell polarity in the epithelium.^{2,5} BTB was reported to be established in rats by age 15–18^{6,7} or 21⁸ day postpartum (ppd), and this time also coincides with the cell cycle progression from zygotene to pachytene spermatocytes by 15–18 dpp, and then diplotene and dictyate spermatocytes by 23–26 dpp, to be followed immediately by secondary spermatocytes and round spermatids to complete both meiosis I and II by 26 dpp.⁹ These findings thus implicate the likely

involvement of a functional BTB and cell cycle progression and meiosis.

We recently reported that the fertility of adult rats treated with adjuvin, 1-(2,4-dichlorobenzyl)-1H-indazole-3-carbohydrazide (formerly called AF-2364, a potential male contraceptive), failed to recover using an acute high-dose at 250 mg/kg b.w. vs. the low-dose group received adjuvin at 50 mg/kg b.w. where fertility was found to rebound.¹⁰ Earlier studies have shown that adjuvin disrupts germ cell adhesion in the seminiferous epithelium, most notably elongating/elongated spermatids, to be followed by round spermatids and spermatocytes.^{11–13} It was noted that in rats from the low-dose treatment group, spermatogonia/spermatogonial stem cells (SSC) were not depleted so that spermatogenesis could re-initiate to repopulate the voided tubules with germ cells of all classes gradually after adjuvin was metabolically cleared from the host animals and fertility could rebound.^{11–15} Interestingly, although the fertility failed to recover in rats from the high-dose group, the population of spermatogonia/SSC in the tubules was found to be similar to that of the low-dose treated group and untreated control rats.¹⁰ Detailed analysis of these animals

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revealed that in the low-dose group, the BTB was only transiently disrupted, yet the BTB was irreversibly damaged in the high-dose group, and spermatogonia/SSC were found to fail to differentiate beyond type A spermatogonia in rats from the high-dose group.¹⁰ These findings thus illustrate for the first time that a functional BTB is crucial to spermatogonia/SSC differentiation beyond type A spermatogonia, and to prepare for meiosis. This postulate is supported by an earlier study in which neonatal rats treated with diethylstilbestrol, a synthetic nonsteroidal estrogen, that delayed the BTB assembly by ~4-wk was found to delay the initiation of cell cycle progression and meiosis by 4-wk since type A spermatogonia failed to differentiate to type B spermatogonia and preleptotene spermatocytes.⁸

Since developmental data have shown that (1) late cell cycle progression to diplotene and dictyate spermatocytes does not occur until 23–26 dpp; (2) meiosis I and II do not complete until 26 dpp in rats,⁹ and (3) in light of the recent findings using the adjudin model regarding the onset of diakinesis and meiosis I/II that failed to occur in the absence of a functional and intact BTB,¹⁰ we thought it pertinent to re-assess the precise time window when a functional BTB is being established during postnatal development in rats. This study should help to reassess the physiological significance of BTB in cell cycle progression and the onset of meiosis during spermatogenesis. This is the subject of the present report.

Results

Changes in the steady-state levels of constituent proteins at the BTB during post-natal development. In order to determine the time when a functional BTB is being established in the testis during development, the steady-state levels of several BTB proteins including (1) TJ proteins: occludin, claudin-11 and ZO-1; (2) basal ES proteins: N-cadherin and β -catenin; (3) GJ proteins: connexin-43; and (4) non-receptor protein tyrosine kinases that are known to regulate BTB: FAK and c-Src were assessed by immunoblotting (Fig. 1) using the corresponding specific antibodies (see Table 1). These proteins were selected for the present study because they are the known components of TJ, basal ES, and GJ, which, in turn, constitute the functional Sertoli cell BTB via their coexistence at the site in the rat testis, and more importantly, these antibodies are well characterized as reported earlier.^{2,15–18} It was noted that all the TJ and GJ proteins examined as reported herein showed a transient surge in their steady-state level during development and was peaked by ~17–25 dpp. The surge of these proteins was apparently being used as building blocks to assemble the BTB, consistent with earlier reports that BTB was assembled at ~15–18 dpp based on electron microscopy.^{6–8} Interestingly, the steady-state levels of two BTB regulatory protein kinases known to be involved in BTB dynamics^{19,20} also induced at 17–20 dpp, but the steady-state levels of c-Src and FAK remained elevated well into the adulthood versus immature rats at 12–15 dpp (Fig. 1A and B). On the other hand, the protein levels of basal ES proteins N-cadherin and β -catenin were high by day 12–15 dpp, but gradually declined during development (Fig. 1). In short, it is obvious from these findings that the

BTB appears to initiate its assembly beginning on ~15–20 dpp (as illustrated by claudin-11) and persisted through ~25 dpp as illustrated by ZO-1, N-cadherin, occludin, connexin-43, FAK and c-Src (Fig. 1A and B). The levels of occludin, ZO-1, N-cadherin, β -catenin, connexin-43, FAK and c-Src remained steady after 25 dpp because they need to maintain the BTB integrity during the seminiferous epithelial cycle, such as during BTB restructuring at stage VIII of the epithelial cycle to facilitate the transit of preleptotene spermatocytes at the site to enter the adluminal compartment for further development.

Changes in the patterns of localization and co-localization of BTB proteins in the seminiferous epithelium of rat testes during postnatal development. We next examined changes in the patterns of localization and co-localization of several BTB-associated proteins in the seminiferous epithelium during postnatal development. This was to assess their distribution in developing rat testes vs. adult rat testes when a functional BTB is known to be present. For instance, besides inducing the constituent proteins during development as illustrated by immunoblot analysis shown in Figure 1, the expressed constituent proteins require proper localization such that a continuous belt-like structure is formed near the basement membrane in the tubules.^{7,21} Thus, dual-labeled immunofluorescence analysis using fluorescent microscopy was used to study the localization of TJ proteins occludin and claudin-11 (Fig. 2A and B), basal ES protein N-cadherin (Fig. 3) and GJ protein connexin-43 (Fig. 4) vs. the adaptor protein ZO-1 (Figs. 2–4) which is known to interact with TJ, basal ES, and GJ proteins in the rat testes.^{22,23} It was noted that the TJ proteins occludin and claudin-11 formed a weak but diffused belt-like structure at the base of the seminiferous epithelium beginning on 15 dpp, and with obvious co-localization with ZO-1 by 17 dpp (Fig. 2A and B). The “occludin-ZO-1 belt-like structure” and the “claudin-11-ZO-1 belt-like structure” became more and more defined during development (Fig. 2A and B) and by 25 dpp, occludin and ZO-1 were more confined to the BTB site, similar to those found in adult testis by 120 dpp (Fig. 2A). However, claudin-11 was restrictively localized to the BTB site only by 38 dpp (Fig. 2B). On the other hand, unlike occludin, a belt-like structure with a relatively diffused pattern was observed by 20 dpp for the basal ES protein N-cadherin (Fig. 3), and a restrictive distribution that confined to the BTB site was not detected until 30 dpp, and only at this age that N-cadherin was found to co-localize with ZO-1, displaying a pattern similar to rats by 38 and 120 dpp (Fig. 3). The GJ protein connexin-43 began to form a diffused belt-like structure near the basement membrane by 15 dpp, and connexin-43 was found to co-localize with ZO-1 by 17 dpp (Fig. 4). But a well defined belt-like structure of connexin-43 that was restricted to the base of the seminiferous epithelium and co-localized with ZO-1 was not apparent until 25 to 30 dpp (Fig. 4). Thus, the findings based on dual-labeled immunofluorescence analysis to assess the distribution of TJ-, basal ES-, and GJ-proteins illustrate that BTB began to be assembled from 15 dpp, but not all the BTB-associated proteins were restricted to the BTB site until at least by 25 dpp. For instance, occludin and ZO-1 were found to be restrictive to the BTB site by ~20 dpp, but this pattern of restrictive localization at the BTB was not found

for claudin-11, N-cadherin and connexin-43 until ~25 to 30 dpp (Figs. 3 and 4 vs. 2).

A study by using an *in vivo* assay to assess the BTB integrity in rat testes during postnatal development. To further define the relative timing regarding the establishment of a functional BTB in developing rat testes, an *in vivo* functional assay was used to assess the presence of an effective BTB that blocked the entry of fluorescence tag from entering the apical compartment following its administration via the jugular vein, and that semi-quantitative data can be obtained from such an assay for statistical comparison and analysis (Fig. 5). It was found that by 12 dpp at the time the BTB remained to be assembled, it was almost as “leaky” as rats from the cadmium-treated group which is known to induce BTB disruption^{21,24,25} (Fig. 5). Even though by 15 dpp when the BTB begins its assembly based on dual-immunofluorescence analysis data shown in Figures 2–4, the BTB remained ineffective to block the movement of fluorescence tag across the barrier into the adluminal compartment (Fig. 5A). Although statistical analysis illustrates that a functional BTB was established by day 15 and 17 dpp (Fig. 5B), but many tubules remained “leaky” to the fluorescence tag (Fig. 5A), consistent with dual-labeled immunofluorescence data shown in Figure 2–4. These findings, nonetheless, are in agreement with earlier reports that a functional BTB was being established by 16–18 dpp, but it was not formed until 21 dpp.^{6–8,26} However, Figure 5 has convincingly demonstrated that a fully effective, intact and functioning BTB was only established until at least day 25 dpp, since only at this age, most of the tubules displayed the ability to block the transit of fluorescence tag across the BTB, blocking their entry into the adluminal compartment (Fig. 5A and B). The findings depicted in Figure 5 are also in agreement with data obtained by dual-labeled immunofluorescence analysis shown in Figures 2–4, depicting the relative patterns of localization of TJ-, basal ES- and GJ-proteins and their co-localization with ZO-1 at the BTB in developing rat testes vs. adult rats at 120 dpp, demonstrating a functional BTB was not established until 25 dpp, coinciding with the final steps of meiosis I and the completion of II which occurs by 26 dpp.⁹

Changes in the interaction between occludin and connexin-43 with FAK. Since FAK was recently shown to be an integrated component of the occludin-ZO-1 adhesion protein complex

Figure 1. Change in the steady-state levels of BTB-associated proteins in rat testes during post-natal development. Rats on 12-, 15-, 17-, 20-, 25-, 27-, 30- and 38-D (day postpartum, dpp) with $n = 3$ rats per age group were terminated to obtain lysates of testes for immunoblot analysis of BTB-associated proteins, including TJ proteins: occludin, claudin-11 and ZO-1; basal ES proteins: N-cadherin and β -catenin; GJ protein: connexin-43; and BTB regulatory proteins: FAK and c-Src. Actin served as a protein loading control. The results shown here are representative data from three independent experiments with $\sim 100 \mu\text{g}$ protein per lane. (B) Histograms summarizing results of immunoblot analysis such as those shown in (A) in which each data point was normalized against actin. The steady-state level of a target protein at 12 dpp was arbitrarily set as 1 against which comparison was performed for statistical analysis using 2-way ANOVA. Each bar = mean $\pm$ SD of $n = 3$ rats. * $p < 0.05$; ** $p < 0.01$.

at the BTB, and it is known to confer proper phosphorylation status to occludin at the BTB,^{20,27} we thus sought to examine if the pattern of protein-protein interactions between FAK and two BTB integral membrane proteins correlated with the establishment of the BTB in the testis during development. Connexin-43 was also selected in this study because recent studies have shown that besides being an integrated component of the GJ at the

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

Antibody	Vendor	Catalog no.	Application	Working dilution for IB, (IF) or [IP]
Rabbit anti-connexin-43	Cell signaling technology (Danvers, MA)	3512	IB, IF, IP	1:1,000 (1:100), [1:62.5]
Rabbit anti-N-cadherin	Santa Cruz Biotechnology (Santa Cruz, CA)	sc-22839	IB	1:1,000
Rabbit anti-FAK	Santa Cruz Biotechnology	sc-558	IB	1:200
Mouse anti-cSrc	Santa Cruz Biotechnology	sc-8056	IB	1:200
Goat anti-actin	Santa Cruz Biotechnology	sc-1616	IB	1:300
Rabbit anti-claudin-11	Invitrogen (Carlsbad, CA)	36-4500	IB, IF	1:125, (1:100)
Rabbit anti-occludin	Invitrogen	71-1500	IB, IF, IP	1:125, (1:100), [1:62.5]
Rabbit anti- β -catenin	Invitrogen	71-2700	IB	1:200
Rabbit anti-ZO-1	Invitrogen	61-7300	IB	1:250
Mouse anti-ZO-1	Invitrogen	33-9188	IF	(1:100)

*Antibodies used in this study cross-reacted with the corresponding rat proteins as indicated by the manufactures. IB, immunoblotting; IF, immunofluorescence microscopy; IP, co-immunoprecipitation.

BTB,²³ connexin-43 also regulates reassembly of the TJ-barrier²⁸ which is essential to maintain the immunological barrier during the transit of preleptotene spermatocytes at the BTB to prepare for meiosis I. Using the technique of co-immunoprecipitation, the association between FAK and occludin as well as between FAK and connexin-43 gradually increased during development and peaked by day 20 at the time of BTB assembly (see Figs. 1–5), perhaps being used to maintain the proper phosphorylation status of the TJ and GJ proteins, so that they can be assembled to the TJ-fibrils and the coexisting GJ, respectively, to be used to establish the BTB (Fig. 6).

Discussion

In this study, we have assessed the time in which a fully functional BTB is assembled in the rat testis. We sought to use a combination of techniques including (1) immunoblotting by assessing the steady-state levels of a number of adhesion and constituent proteins at the BTB, (2) dual-labeled immunofluorescence analysis of BTB-associated proteins including proteins at the TJ, basal ES, and GJ by fluorescent microscopy, (3) an in vivo BTB integrity assay, and (4) co-immunoprecipitation. Collectively, these results have demonstrated that the BTB begins its assembly by 15–20 dpp, however, a fully functioning BTB is not established until 25 dpp. First, this conclusion is supported by the results from immunoblotting that illustrated the transient increase of TJ proteins necessary for BTB assembly including occludin,²⁹ claudin-11^{30,31} and ZO-1³² was at 15–20 dpp. Moreover, GJ protein connexin-43 which is important for maintaining BTB integrity^{23,28} was also transiently increased at 15–20 dpp. Second, proper localization of integral membrane proteins and their adaptors can also affect the BTB integrity. Earlier studies have shown that a functional BTB is associated with a continuous belt-like structure formed by TJ proteins that constitute the TJ-fibrils near the basement membrane.^{7,21} Additionally, several studies have demonstrated that in pathological conditions, such as during tumorigenesis associated with male reproductive dysfunction when the BTB was disrupted and

compromised, mis-localization of claudin-11^{33,34} or ZO-1³² was detected by immunohistochemistry. Moreover, a recent study has shown that although occludin and claudin-11 were induced significantly at the BTB after acute doses of adjuvant in adult rats, BTB was still disrupted because the significantly induced occludin and claudin-11 were mis-localized,¹⁰ illustrating proper localization of proteins at the BTB is crucial to maintain its integrity. Based on dual-labeled immunofluorescence analysis as reported herein, well-defined and co-localized “occludin-ZO-1,” “claudin-11-ZO-1” and “connexin-43-ZO-1” belt-like structures were only apparent by 25 dpp. This thus demonstrates that a fully functioning BTB is established by 25 dpp. Third, based on findings from the in vivo BTB assay, a drastic reduction of fluorescence signals in the adluminal compartment was found to be diminished by 25 dpp vs. pups at 12, 15, 17 and 20 dpp. Thus, this finding, coupled with results of the co-immunoprecipitation experiment further suggests that the BTB is only functionally intact by 25 dpp. However, it is noteworthy that by 20 dpp, some of the tubules displayed a significantly reduced fluorescent signal penetration beyond the BTB vs. other tubules in the same cross-sections, and statistical analysis also supported the notion that the BTB integrity was established by 20 dpp, yet the BTB functionality in the testis as a “whole” was not truly established until 25 dpp when the findings depicted herein were analyzed collectively. Since by the age of 25 dpp, the ability of BTB to restrict the movement of FITC-inulin to the basal compartment is analogous to that of an adult rat testis by 120 dpp, illustrating no fluorescence was detected at the apical compartment in >98% of the tubules scored. This finding, nonetheless, indicates that the final phase of BTB establishment is taking place on 20 dpp, and some tubules are better established vs. others at that age. This observation, however, is in agreement with a previous study indicating that the BTB is not established synchronously along the cords, its establishment is depending on the stage of meiosis of germ cells present in tubules.⁷

Previous studies have indicated that the BTB is established by 16–21 dpp in rats,^{6–8,26} which is slightly earlier than the time estimated in this study. This slight discrepancy can be due to

Figure 2. Changes in the cellular localization of TJ proteins in the seminiferous epithelium of rat testes during post-natal development. (A) Co-localization of occludin (a TJ-integral membrane protein, red fluorescence) and ZO-1 (an adaptor associated with TJ-, basal ES-, and GJ-proteins at the BTB, green fluorescence) in frozen sections of testes obtained from rats on 12-, 15-, 17-, 20-, 25-, 30-, 38- and 120D (D, day postpartum, dpp) were shown. Occludin was found to have a diffused pattern of localization across the seminiferous epithelium in the testes of rats on 12D, and was not co-localizing with ZO-1. By 15D, occludin began to localize at the basal compartment and displayed some co-localization with ZO-1. Then from day 17D onward, these two proteins were found to localize closer to the basement membrane as denoted by the white broken-line and having a more defined localization consistent with their localization at the BTB where they showed their co-localization at the same site. (B) Co-localization of claudin-11 (red) and ZO-1 (green) in frozen sections of testes obtained from rats on 12-, 15-, 17-, 20-, 25-, 30-, 38- and 120D. Changes in the localization pattern of claudin-11 vs. ZO-1 in the seminiferous epithelium were similar to that of occludin-ZO-1. Based on these findings, it is apparent that these proteins were not properly localized to the BTB site near the basement membrane until ~25 to 30D. White broken-line circles indicate the relative location of the basement membrane adjacent to the BTB in the seminiferous tubule. The data shown herein are representative results of a dual-labeled immunofluorescence analysis experiment which was repeated three times using samples from different sets of rat testes and yielded similar results. Bar in 12D:a = 25 μ m, which applies to all micrographs of 12D to 20D; and bar in 25D:a = 50 μ m, which applies to all micrographs of 25D to 120D.

the differences in methods that were used in different studies. For example, an earlier study performed by Russell et al. to determine the BTB integrity during postnatal development was based on initial washing of the testis with hypertonic solution

prior to perfusion using fixative, to be followed by light microscopic examination; and it was noted that the BTB was formed by 18 dpp.⁶ However, another study performed by Bergmann et al. using hypertonic fixative coupled with electron microscopy

Figure 3. Changes in the cellular localization of basal ES proteins N-cadherin and ZO-1 in the seminiferous epithelium of rat testes during post-natal development. Co-localization of N-cadherin (red) and ZO-1 (green) in frozen sections of testes obtained from rats on 12-, 15-, 17-, 20-, 25-, 30-, 38- and 120D (D, dpp) were shown. White broken-line denotes the relative location of the basement membrane, lying right behind the BTB in the seminiferous epithelium of the tubule. N-Cadherin was found to have a diffused pattern of localization across the seminiferous epithelium in the testes on 12 to 17D and was not co-localized with ZO-1. By 20 to 30D, N-cadherin was found to localize more closely to the basal compartment, consistent with its localization at the BTB and displayed some co-localization with ZO-1 (20D: *a-d*). But only by 30D, N-cadherin was found to have a more defined localization at the BTB and better co-localization with ZO-1 at the BTB. The data shown herein are representative results of an experiment which was repeated three times using samples from different sets of rat testes and yielded similar results. Bar in 12D: *a* = 25 μ m, which applies to all micrographs of 12 to 20D; and bar in 25D: *a* = 50 μ m, which applies to all micrographs of 25 to 120D.

to assess the BTB integrity during development indicated that the BTB was established by 19 dpp.⁷ In a more recent study by Toyama et al. based on the use of cytochrome *c* coupled with light and electron microscopy, the BTB was shown to be established by 21 dpp.⁸ Herein, we have shown unequivocally that that depending on the BTB markers that were selected for dual-labeled immunofluorescence microscopy, the timing of BTB establishment could be slightly different. For instance, occludin and ZO-1 was assembled to the BTB by as early as 17 dpp, but for claudin-11, N-cadherin and connexin-43, it was 38, 30 and 38 dpp, respectively. These observations thus illustrate the constituent proteins at the BTB that are being assembled to the site do not occur uniformly during development.

Earlier studies have shown that the BTB is composed of an array of TJ-, basal ES-, and GJ proteins' and in addition to these proteins, desmosomes are also key integrated components at the BTB,^{2,16} making the BTB a unique blood-tissue barrier among other barriers in the mammalian body, including blood-brain barrier, blood-retina barrier, blood-epididymal barrier, blood-biliary barrier and the gut-epithelial barrier. Since in these latter barriers, they are constituted almost exclusively by the TJ between endothelial and/or epithelial cells instead of coexisting TJ, basal ES, GJ and desmosome, thus a loss of any TJ-proteins can significantly impede the functioning of these blood-tissue barriers. Hence, a fully functional BTB in the testis does not require the presence of all the proteins from TJ, basal ES, GJ and desmosome simultaneously, and changes in the expression and localization of different BTB-associated proteins in the seminiferous epithelium during testicular development as reported herein also support this argument since the steady-state of some proteins are declining in adulthood (e.g., claudin-11), whereas others remain relatively constant following the establishment of a functional BTB (e.g., connexin-43, occludin). However, it is likely that a set of proteins is necessary to confer its functionality. Perhaps this is physiologically necessary and important to maintain the unique features of the BTB since unlike other blood-tissue barriers, the BTB undergoes extensive restructuring during spermatogenesis, such as at stage VIII of the epithelial cycle, to facilitate the transit of preleptotene spermatocytes. Thus, the "old" BTB site above the transiting preleptotene spermatocytes can be "gradually" degenerating while the "new" BTB site behind the spermatocytes can also be "gradually" assembling since a "loss" of either TJ, basal ES, GJ or desmosome is not going to lead to a "dissolution" of the immunological barrier. It is obvious that many of the signaling events that coordinate the proper functioning of these junction types that constitute the BTB remain unknown. Nonetheless, the findings report herein does not contradict to earlier reports regarding the assembly of the BTB that occurs by age 16–21 dpp in rats.^{6-8,26} For instance, the use of different marker proteins at the BTB indeed supports these earlier observations, such as the localization of occludin at the BTB. However, a fully functional BTB, based on a semi-quantitative in vivo assay, is only formed by 25 dpp in rats as depicted in Figure 7. This timing of BTB assembly also coincides precisely with the occurrence of meiosis I and II which is known to take place by ~26 dpp in rats,⁹ illustrating the significance of BTB and meiosis. This conclusion is

indeed supported by findings in humans in which infertile men with oligospermia were having a disrupted BTB, and spermatogonia and spermatocytes failed to enter meiosis.³⁵

In summary, we have shown that an intact BTB is relatively established by 20 dpp during postnatal development in rats, however, a fully functioning BTB is not assembled until 25 dpp. Figure 7 summarized the key findings in this report.

Materials and Methods

Animals and antibodies. Sprague-Dawley (outbred) rats were purchased from Charles River Laboratories and housed at the Rockefeller University Comparative Bioscience Center (CBC). The use of Sprague-Dawley rats for studies reported herein was approved by the Rockefeller University Institutional Animal Care and Use Committee (Protocols 06018 and 09016). Antibodies were obtained commercially from different vendors and the working dilutions of these antibodies for various experiments are listed in Table 1.

BTB integrity assay. The BTB integrity in vivo in rats was assessed by an assay established earlier in our laboratory as described.^{10,36,37} This is a semi-quantitative assay to assess the BTB integrity based on the ability of an intact BTB that blocks the diffusion of a small fluorescence tag, such as FITC-inulin, from the basal to the apical compartment of the seminiferous epithelium when the tag is administered to live animals at the jugular vein. In short, rats were under anesthesia with ketamine HCl (60 mg/kg b.w.) in the presence of an analgesic xylazine (10 mg/kg b.w.) (Sigma-Aldrich) administered i.m. A small incision, about 1-cm, was made in the area over the jugular vein with a surgical scissors to expose the blood vessel and depending on the age of rats, about 0.5–1.5 mg FITC-conjugated inulin (M_r 4.6 kDa) (Sigma-Aldrich) in 100–300 μ l PBS, depending on the age and testis weight of the animal, was administered into the jugular vein with a 28-gauge needle, such that the fluorescence tag could reach the testis, attempting to traverse the BTB. Thereafter, the skin was closed with two wound clips (9-mm Autoclip from Clay Adams, Becton Dickinson). Rats were allowed to recover, and about 30–45 min thereafter, rats were euthanized by CO₂ asphyxiation in a carbon dioxide rat chamber. Testes were removed immediately and snap-frozen with liquid nitrogen. Rats treated with CdCl₂ at 5 mg/kg b.w. i.p. for 3-d were used as positive control since the BTB was reported to be irreversibly damaged by this treatment.^{21,24,25} The distribution of FITC (green fluorescence) in the seminiferous epithelium among tubules was monitored by fluorescence microscopy using a Nikon Eclipse 90i Fluorescence Microscope and images were obtained using a built-in Nikon DS-Qi1Mc digital camera at 12.5-Megapixel (Mpx) with Nikon NIS Elements Advanced Research Imaging Software (Version 3.2) (Nikon Instruments Inc.) in an HP xw8600 Workstation. Images were exported to TIFF format and analyzed in PhotoShop using Adobe Creative Suite (Version 3.0), such as image overlay. BTB was considered not fully formed when fluorescence signal was no longer confined to the basal compartment but detected in the adluminal compartment since in normal adult rat testes, all the

Figure 4. Changes in the cellular localization of gap junction proteins connexin-43 and ZO-1 in the seminiferous epithelium of rat testes during postnatal development. Co-localization of connexin-43 (red) and ZO-1 (green) in frozen sections of testes obtained from rats on 12-, 15-, 17-, 20-, 25-, 30-, 38- and 120D (D, dpp) were shown. White broken-line illustrates the relative location of the basement membrane in the seminiferous epithelium of the tubule, adjacent to the BTB site. Connexin-43 was weakly stained in the seminiferous epithelium of testes of rats on 12D, 30-, which remained diffusely localized in the epithelium until 30D. However, connexin-43 was found to co-localize with ZO-1 by only 17D and thereafter. And only by 30D, connexin-43 displayed a proper and well defined localization at the BTB, co-localizing with ZO-1 in the seminiferous epithelium. Bar in 12D:a = 25 μ m and 25D:a = 50 μ m, which applies to micrographs of 12D to 20D and micrographs of 25D to 120D respectively.

Figure 5. A study of using an in vivo functional assay to assess the BTB integrity during postnatal development. (A) Localization of inulin-FITC (green) in frozen sections of testes of rats on 12-, 15-, 17-, 20-, 25-, 30-, 38- and 120-D (day postpartum, dpp) following administration of the fluorescence tag (inulin-FITC) at the jugular vein to assess the BTB integrity. A functional BTB effectively blocks the movement of inulin-FITC from the basal to the apical compartment in the epithelium, see testes from rats at 25-, 30-, 38- and 120-d-old rats. In short, inulin-FITC was administered to rats at the jugular vein at time 0 and the diffusion of inulin-FITC was monitored ~45 min thereafter (see tubules in (A):a). Rats received CdCl₂ (5 mg/kg b.w., via i.p.) at the corresponding age at time 0 and used for BTB integrity assay on day 3, which served as positive control (see tubules in (A):b). White broken-line encircles the basement membrane which illustrates the relative location of the BTB in the seminiferous epithelium. White brackets illustrate the relative distance traveled by inulin-FITC from the BTB (i.e., D_{Signal}). Arrowheads indicated inulin-FITC found in the lumen of the seminiferous tubules, illustrating the BTB remained non-functional in these tubules. Asterisk (*) in 20D:a indicates a tubule with a relatively more defined BTB when compared with other tubules at this time point. Scale bar = 50 μ m in 12D:a which applies to all micrographs in 15–20D, bar = 100 μ m in 25D:a which applies to all micrographs in 30–120D. (B) This histogram provides the semi-quantitative data of the BTB integrity assay by quantifying the distance traveled by inulin-FITC from the BTB near the basement membrane vs. the radius of a seminiferous tubule. Each bar = mean $\pm$ SD of $n = 60$ –80 tubules that were randomly selected and scored from testes of each rat for each time point with a total of 2–3 rats. Black brackets, comparison between rats from 12D vs. all other groups; blue brackets, 15D vs. all other groups; green brackets, 20D vs. all other groups; brown brackets, 25D vs. all other groups; pink brackets, 30D vs. all other groups. The findings shown herein thus demonstrate that BTB was assembled by 20D, but the majority of the tubules were not having a functional BTB until 25D and thereafter. * $p < 0.05$; ** $p < 0.01$; ns, not significantly different.

fluorescence was blocked from entering the adluminal compartment. To obtain semi-quantitative data on the BTB integrity, the ratio between the distance traveled by the fluorescence signal from the basement membrane in a seminiferous tubule (D_{Signal}) and the radius of the tubule (D_{Radius}) was obtained for each tubule and each time point is the mean $\pm$ SD from a total of 180–240 tubules from 3 rats (i.e., 60–80 tubules from each

rat). For instance, in the positive control group in which rats were treated with CdCl₂ in which fluorescence was found in the center of a tubule in virtually all tubules, the ratio of D_{Signal}/D_{Radius} was at 1. If the cross-section of a seminiferous tubule was oval-shaped because it was obliquely sectioned, D_{Radius} was obtained by averaging the shortest and the longest distance from the basement membrane.

Lysate preparation and immunoblotting. Lysates of testes were prepared in IP lysis buffer [50 mM Tris, pH 7.4, at 22°C, containing 0.15 M NaCl, 1% Nonidet P-40 (vol/vol), 1 mM EGTA, 2 mM N-ethylmaleimide, 10% glycerol (vol/vol)] supplemented with protease inhibitor mixture (Sigma-Aldrich) and phosphatase inhibitor mixture I and II (Sigma-Aldrich) at a dilution of 1:100 to block protease and phosphatase activities using a Cole Parmer Ultrasonic Processor as described.^{18,38} Protein concentration was estimated using a BioRad D_c Protein Assay kit in 96-well plates with a BioRad Model 680 Spectrophotometry Reader. Approximately 100 µg protein were used for immunoblotting and ECL images were captured using a FujiFilm LAS-4000 mini chemiluminescence imaging system, with images captured using FujiFilm Multi Gauge software package (Version 3.1), converted to TIFF format and analyzed with Scion image as earlier described.³⁹ All data were normalized against actin to correct for possible differences in protein loading between samples in a given experiment. All samples within an experimental group were processed simultaneously to eliminate inter-experimental variations. Antibodies used for immunoblot analysis were listed in Table 1. Each immunoblotting experiment was repeated at least three times with three different set of testis samples for statistical analysis.

Dual-labeled immunofluorescence analysis. Frozen sections (7 µm) were obtained in a cryostat at -20°C, which were fixed immediately in either Bouin's fixative or 4% paraformaldehyde (wt/vol) for 10 min. Sections were then permeabilized with 0.1% Triton X-100 in PBS (10 mM sodium phosphate, 0.15 M NaCl, pH 7.4 at 22°C) (vol/vol) for 4 min. Sections were blocked using 1% BSA (wt/vol) for 30 min, to be followed by an overnight incubation of primary antibodies diluted in blocking solution at room temperature. Sections were then incubated with Alexa Fluor—conjugated secondary antibodies (Invitrogen; red fluorescence, Alexa Fluor 555; green fluorescence, Alexa Fluor 488) at 1:250 diluted with the corresponding blocking solution at room temperature. Sections were mounted with Vectorshield Antifade mounting media with DAPI (4',6-diamidino-2-phenylindole, for staining cell nucleus) (Invitrogen) for fluorescence microscopy. Fluorescence images were captured using an Olympus DP71 12.5 Mpx digital camera interface to an Olympus BX61 fluorescence microscope using the Olympus MicroSuite Five Imaging software package (Version 1226) to obtain images in TIFF format. Image overlay and analysis were performed using PhotoShop in Adobe Creative Suite Design Premium software package (Version 3.0). All staining experiments were performed 2–3 times with different sets of testes. To reduce inter-experimental variations, testes from all time points within an experimental group were processed simultaneously in a single experimental session by placing 2–3 cross-sections of testes on a single microscopic slide. Negative control included the use of normal mouse or normal rabbit IgG diluted in PBS to substitute the primary antibody.

Co-immunoprecipitation. To assess changes in protein-protein interactions between occludin and FAK as well as between connexin 43 and FAK during testicular development, 500 µg of testis lysates from rats on 12-, 17-, 20-, 25- and 30-dpp were

Figure 6. Changes in the interaction between occludin and connexin-43 with FAK at the BTB in rat testes during post-natal development. (A) Rats on 12-, 17-, 20-, 25- and 30-D (day postpartum, dpp) were terminated to obtain lysates of testes for co-immunoprecipitation using an anti-occludin or anti-connexin-43 IgG (see Table 1), and the immunocomplexes were extracted for immunoblotting using an anti-FAK antibody to examine the interaction of TJ protein occludin and GJ protein connexin-43 with FAK, a known BTB regulatory protein tyrosine kinase. The results shown here are representative data from three independent experiments. (B) Histogram summarizing results from (A) with each data point (mean $\pm$ SD, $n = 3$) normalized against actin which served as a protein loading control to correct differences in protein loading see (A), illustrating changes in protein-protein association. The interaction between occludin and FAK or connexin-43 and FAK in rats at 12D was arbitrarily set at 1 against which statistical comparison was performed. * $p < 0.05$; ** $p < 0.01$.

initially incubated with 1.5 µg normal IgG, to be followed by an incubation with 15 µl Protein A/G Plus (Santa Cruz) for 2 h in each sample, thereafter, the supernatant was obtained following a brief centrifugation (1,000 g, 5 min) which was used for subsequent co-immunoprecipitation. This pre-clearing step is important to eliminate unwanted non-specific interactions between the target protein [and its binding partner(s)] with IgG in the corresponding antibody. Lysates after the pre-clearing step were then incubated with 2 µg of primary antibody, either anti-occludin or anti-connexin-43 IgG, overnight at room temperature (and for negative control, primary antibody was substituted with 2 µg of normal IgG). Thereafter, each sample was incubated with Protein A/G Plus (15 µl) so that the immunocomplexes containing IgG-target protein/binding partner(s) were bound to the resin and were obtained following centrifugation, which were then extracted in SDS-sample buffer (0.125M Tris, pH 6.8 at 22°C, containing 1% SDS, wt/vol, 1.6% 2-mercaptoethanol, vol/vol, and 20% glycerol, vol/vol) at 100°C for 5 min, to be followed by SDS-PAGE and immunoblot analysis using an anti-FAK antibody. Antibodies used for this coimmunoprecipitation experiment were listed in Table 1 and this experiment was

Figure 7. A schematic drawing illustrating the different stages pertinent to the establishment of a functional BTB during postnatal development in the rat testis.

repeated at least three times with three different set of testes to obtain sufficient data for statistical comparison.

Statistical analysis. Statistical analysis of data derived from immunoblotting, co-immunoprecipitation and BTB integrity assay was performed with GB-STAT software package (Version 7.0; Dynamic Microsystems) using two-way ANOVA followed by Newman-Keul's test. For data obtained from the BTB integrity assay, Student's t-test was used to compare corresponding groups annotated by brackets of different colors, such as 12 dpp vs. 12-, 17-, 20-, 25-, 30-, 38- or 120 dpp group.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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