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In search of suitable in vitro models to study germ cell movement across the blood-testis barrier

Dolores D. Mruk* and C. Yan Cheng
Center for Biomedical Research; The Population Council; New York, NY USA

The movement of preleptotene/leptotene spermatocytes across the blood-testis barrier, also known as the Sertoli cell barrier, during stages VIII to XI of the seminiferous epithelial cycle is one of the most important cellular events taking place in the mammalian testis. Without the passage of spermatocytes, spermatogenesis would be halted, resulting in transient or permanent sterility. Unfortunately, we have very little knowledge on how preleptotene/leptotene spermatocytes cross the blood-testis barrier. While we know cytokines, proteases and androgens to mediate Sertoli cell junction restructuring, most data continue to be derived from experiments using Sertoli cells cultured alone in two dimensions. Thus, additional in vitro models which include germ cells must come into use. In this Commentary, we hope to shed new light on how we may better study spermatocyte movement across the BTB.

Keywords: germ cell, Sertoli cell, blood-testis barrier, testis, cell junction

Abbreviations: BTB, blood-testis barrier; ECM, extracellular matrix; ES, ectoplasmic specialization; FAK, focal adhesion kinase; TJ, tight junction

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*Correspondence to: Dolores D. Mruk; Email: mruk@popcbr.rockefeller.edu

The mechanism behind the movement of preleptotene/leptotene spermatocytes across the blood-testis barrier (BTB, also known as the Sertoli cell barrier) during stages VIII to XI of the seminiferous epithelial cycle has only been partly elucidated after nearly two decades of continuous research. In our seminal paper on the topic, which was published in 1997, we showed that seeding freshly isolated germ cells onto a layer of pre-cultured Sertoli cells triggered the production of proteases,[1] which seemed to illustrate that contact sites need to be primed by 'molecular scissors' before stable Sertoli-germ cell junctions can be established. These biochemical findings were significant because they were published at a time when testis cell junctions were understood to only be mere morphological structures with little needed regulation. Today, however, we understand cell junctions in the testis to be sophisticated and dynamic ultrastructures capable of mediating or participating in diverse signaling cascades that regulate many aspects of cell function. For instance, cell adhesion facilitated by the ectoplasmic specialization (ES), a unique type of adhesive junction present only in the testis, was found to involve focal adhesion kinase (FAK) signaling.[2,3] These results are unprecedented but nonetheless interesting because FAK, a non-receptor protein tyrosine kinase, was long thought to be a major regulator of cell migration and integrin-mediated adhesion which together form the basis of the focal contact, a type of junction that attaches a cell to its substratum.[4] Thus, these biochemical studies greatly revolutionized the biology of cell junctions in the testis, and they paved the way for other important studies.

The movement of preleptotene/leptotene spermatocytes across the BTB in the mammalian testis is a critical cellular event because barrier integrity cannot be compromised. If it were to be compromised, even transiently, spermatogenesis would be halted and infertility would ensue. Eloquent morphological studies from the 1980s have shown preleptotene/leptotene spermatocytes to cross the BTB while enclosed within an intermediate compartment that is sealed at north and south poles by cell junctions.[5-8] As preleptotene/leptotene spermatocytes transit upwards to gain entry into the adluminal compartment of the seminiferous epithelium, two simultaneous events occur: 'old'

junctions present above these cells disassemble, while 'new' junctions assemble below these cells, revealing that an enormous amount of restructuring is taking place at the BTB during stages VIII to XI. Equally important are the findings that the BTB is comprised of intermingled junction types [i.e., tight junctions (TJs), basal ESs, desmosomes and gap junctions], and that this unique organization of junctions is needed to reinforce barrier integrity, especially during the movement of germ cells when 'old' and 'new' junctions are not yet stable and robust. It is also worth noting that junction assembly and disassembly during germ cell movement involves strict coordination across the different structures. Case in point, when one junction type was affected by knocking down the function of a key structural ES, desmosome or gap junction protein, the integrity of the Sertoli cell barrier was found to be compromised,[9,10] suggesting that an "all-or-none" regulatory mechanism is at the core of BTB function. So how can one reasonably investigate the movement of preleptotene/leptotene spermatocytes across the Sertoli cell barrier and acquire meaningful results when this cellular event is so complicated in vivo? We focus our remaining discussion on addressing this important question.

Two well-characterized in vitro models are currently available to study junction dynamics in the testis. The first model involves isolating highly-pure Sertoli cells from pre-pubertal rodent testes and culturing these cells at high density on Matrigel^{TM}-coated substrata after which time cells polarize and assemble functional cell junctions that structurally and biochemically mimic the BTB in vivo.[6] The second model is similar to the first one just described, except that freshly isolated germ cells are seeded on top of the Sertoli cell epithelium at an appropriate Sertoli: germ cell ratio and co-cultured. From these models, we have acquired an enormous amount of useful information relating to how these junctions are assembled and regulated. For example, we know that cytokines can trigger integral membrane protein (e.g., occludin, N-cadherin) internalization and/or recycling at the Sertoli cell barrier,[7,11,12] providing an efficient mechanism of junction disassembly and reassembly during germ cell movement. Unfortunately, there are several drawbacks associated with the use of these two in vitro models. First, Sertoli cells are cultured in two-dimensions on Matrigel^{TM}-coated substrata, but we know cell biology to manifest itself in three-dimensions. This is critical because germ cells depend on Sertoli cells and on their immediate surroundings to function properly. Second, while Sertoli cells are known to establish a functional TJ permeability barrier when cultured on Matrigel^{TM}-coated bicameral units, this barrier is not very tight, suggesting that it may not accurately reflect the biology of the BTB in vivo. Thus, additional research is needed to identify the missing factor(s). Finally, Sertoli-germ cell co-cultures do not support germ cell migration in vitro. These findings, when taken collectively, suggest that a new generation of experimental models is needed that more closely relate in vitro findings with in vivo events taking place in the seminiferous epithelium of the mammalian testis.

Three dimensional (3D) cell culture platforms are valuable tools for biologists because they better mimic physiological conditions. Here, cells or tissues are embedded within a scaffold consisting of either a protein- [e.g., extracellular matrix (ECM), collagen, chondroitine sulfate] or a polymer- (e.g., polyethylene glycol, poly-D-lysine) based material and cultured. For example, epithelial cells that are encapsulated within a protein scaffold that sits on top of a nylon mesh of large pore size (i.e., 10 or 20 μm) can grow in-between pores and achieve a 3D conformation. Our own unpublished studies showed Sertoli cells (isolated from testes of 20 d old rats) to fill up pores when they were cultured for extended periods of time on top of a thin layer of Matrigel^{TM} within a transwell system. In another study, Legendre et al., also used a transwell system to recapitulate the architecture of the seminiferous epithelium in vivo when peritubular myoid cells were cultured on the underside of the insert, and a mixture of Sertoli and germ cells was embedded within the ECM and co-cultured inside of the insert.[13] Interestingly, Sertoli cells aggregated to form a TJ-bearing tubular-like structure (somewhat similar to a seminiferous tubule) with germ cells concentrating at the center of the structure, and these observations are in agreement with a report published by Gassei et al.[14] Interactions between Sertoli and peritubular myoid cells are known to be essential for spermatogenesis,[15,16] and the incorporation of myoid cells into 2D or 3D cell culture platforms is likely to strengthen our understanding of Sertoli-germ cell interactions. For example, peritubular myoid cells have been reported to be targets of androgen action, and the effects of androgens on Sertoli and germ cell function are mediated partly by myoid cells.[17-19] Peritubular myoid cells in rodent testes are also known to contribute to BTB function.[20,21] Moreover, Sertoli cell function is known to be regulated by components of the ECM, which is deposited by both Sertoli and peritubular myoid cells.[22-25] It is also worth noting that myoid cells produce peritubular factor that modulates Sertoli cell function (PModS), which is known to affect the secretion of transferrin, inhibin and androgen-binding protein by Sertoli cells.[26] Being that peritubular myoid cells regulate many different aspects of Sertoli cell function throughout spermatogenesis, they are also likely to play an important role in germ cell migration.

Perhaps 3D experiments should be expanded by using Sertoli cells isolated from adult testes because inherent mechanisms that support germ cell movement would already be in place. Furthermore, these cells are also known to be extensively contaminated by germ cells upon isolation, which in this case may be to the investigator's advantage, and it would be interesting to see if germ cells sandwich in between Sertoli cells during aggregation/tubule assembly since this would more accurately reflect the organization of the seminiferous epithelium in the testis. While this model would not be ideal to study de novo junction assembly (nor would it be useful for recording trans-epithelial electrical resistance [TER] measurements) being that adult Sertoli cells are contaminated with germ cells, it may provide new insight on the mechanism behind the disassembly of Sertoli-germ cell junctions. Three dimensional scaffolds are

also known to support germ cell differentiation in the presence of Sertoli cells and gonadotropins.[27] Furthermore, others have used sophisticated bioreactors to culture cells in 3D.[28-34] In one particular system, a slowly-rotating vessel is filled with culture medium within which ECM-coated porous beads are suspended. After cells attach to ECM-coated beads and achieve a 3D conformation, they can be easily removed and transferred into multi-well plates or dishes for continued experimentation. In the context of the testis, isolating staged seminiferous tubules and culturing them within Matrigel™ may provide new insights on the movement of preleptotene/leptotene spermatocytes across the Sertoli cell barrier. For instance, unique signaling pathways may be triggered in seminiferous tubules cultured under 3D conditions as opposed to those cultured under 2D conditions so that the former model should be investigated in future studies. Another approach may involve isolating Sertoli, germ and peritubular myoid cells and then incubating these cells on a rotator or rocking platform to allow reaggregation. However, it is not known if this in vitro system can recreate in vivo cell-cell interactions.

When Sertoli cells are cultured at high density on a Matrigel™-coated transwell system, a functional TJ permeability barrier is known to be formed by day 3 in vitro when its assembly is monitored by TER readings.[35] Morphological studies by electron microscopy confirmed these findings and showed extensive junctions between Sertoli cells,[10,36-38] which closely resembled those found at the BTB in vivo.[7,38-42] However, there is at least one major difference between in vitro and in vivo systems currently being used to study BTB dynamics: the Sertoli cell permeability barrier in vitro is categorized as being leaky, but the BTB is one of the tightest blood-tissue barriers in the mammalian body,[43] illustrating that a critical factor(s) is missing from Sertoli cell cultures. While previous studies have shown follicle stimulating hormone (FSH) and testosterone to tighten the Sertoli cell TJ permeability barrier even further,[35,44] TER readings were still far below those reported for other epithelia. Germ cells, as well as peritubular myoid cells, may constitute the missing factor because they are known to contribute to BTB function directly or indirectly, as well as to engage actively in crosstalk with Sertoli cells.[6,20,45] At this point, it would be interesting to see if germ cells at different stages of development can regulate barrier function positively, negatively or not at all. For instance, germ cells lying closer to the BTB may play a greater role in barrier restructuring, whereas those residing farther away may play a lesser, or possibly a different, role. Thus, 2D and 3D co-cultures of Sertoli cells and preleptotene/leptotene spermatocytes are likely to yield intriguing observations, and both models should be used to acquire as much information as possible on the role of germ cells in BTB dynamics. On a final note, it is possible that Sertoli cells are not extensively polarized in vitro, which is likely to contribute to a leaky permeability barrier. Adding germ cells on top of the Sertoli cell epithelium may enhance cell polarity, upregulate critical junction proteins and strengthen the Sertoli cell permeability barrier. Alternatively, Sertoli cells can be briefly cultured at high density on Matrigel™-coated dishes to trigger the columnar phenotype, after which time cells can be coated with another (thicker?) layer of Matrigel™ which may help Sertoli cells to polarize more extensively without forming aggregates or tubules.

Presently, there is no in vitro model of germ cell movement. Although we have been unsuccessful in establishing an in vitro system that would recapitulate at least some of the events of germ cell movement, this model is still achievable in our opinion. In one of our experiments, Sertoli cells were cultured at high density inside of Matrigel™-coated transwell systems and allowed to form an epithelium. Thereafter, total germ cells were cultured on the underside of transwell systems and co-cultures were maintained for extended periods of time to see if germ cells would migrate upwards. In another experiment, germ cells were seeded on a thick layer of Matrigel™ within a transwell system, followed shortly by the plating of Sertoli cells on top of germ cells. However, in both cases just described, no disassembly of the Sertoli cell barrier and no movement of germ cells were observed when transwell systems were processed for electron microscopy. Instead, Sertoli cell cytoplasmic processes moved downwards and filled up membrane pores in the former experiment, possibly in an attempt to engulf and/or establish junctions with germ cells that were cultured on the underside of culture inserts (unpublished observations). While germ cell migration was not observed, these models may provide unique opportunities to study the events leading up to germ cell movement. For example, this model can be used to study the formation of filopodia or lamellipodia, actin-rich protrusions where actin polymerization, nucleation, elongation and bundling occur.[46] In this context, it is worth noting that spermatogonial stem cells, when transplanted into germ cell-depleted testes via the efferent duct, can colonize the niche, communicate with Sertoli cells, proliferate/differentiate, migrate past the BTB and restore spermatogenesis.[47-49] As such, this in vivo system has the potential to significantly improve our understanding of germ cell movement during spermatogenesis. At this point, it is also hoped that studies from other scientific fields such as cancer biology provide new insights on how these experiments can be expanded in future. The success of these in vitro models will almost certainly depend on the inclusion of hormones, androgens and other factors that are critical for spermatogenesis.

Herein, we have discussed Sertoli and Sertoli-germ cell in vitro models that have been, and continue to be, used to study important cellular events as they relate to spermatogenesis. While these two systems are far from ideal, they have provided a wealth of useful information. For example, we understand the role of proteases and protease inhibitors in junction assembly and disassembly better today than we did in the 1990s, thanks to technological advancements in the field. Still, it is important that new in vitro models are introduced in the future as these will be invaluable in our understanding of spermatogenesis in the mammalian testis.

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