Tubulobulbar complexes (TBCs) are actin-related double-membrane invaginations formed at intercellular junctions in the seminiferous epithelium of mammalian testes. They occur at basal junction complexes between neighboring Sertoli cells and at apical junctions between Sertoli cells and spermatids. They are proposed to internalize intercellular junctions during the translocation of spermatocytes from basal to adluminal compartments of the seminiferous epithelium, and during sperm release from Sertoli cells. Although TBCs are specific to the seminiferous epithelium, they morphologically resemble podosomes in osteoclasts. Previously, we have reported that a key group of proteins consisting of N-WASP, Arp2/3, cortactin and dynamin that occur at podosomes also is present at TBCs. Here we explore the prediction that zyxin, a focal adhesion protein known to be present at podosomes, also is present at apical TBCs. A rabbit polyclonal anti-zyxin antibody (B71) was used to label fixed fragments and frozen sections of testis. In both fragments and sections, B71 labeled tubular regions of TBCs at apical sites of attachment between Sertoli cells and spermatids, in addition to being co-distributed at tubulobulbar complexes. Despite their distinctive characteristics, tubulobulbar complexes resemble podosomes in some other systems. Podosomes formed by monocyte-derived cells, like osteoclasts, consist of a tubular plasma membrane core surrounded by a cuff of actin filaments. Rather than a projection of one cell into another, podosomes form at areas of cell/substrate attachment and consist of a single membrane invagination. In addition, osteoclast podosomes do not have bulbar regions nor are they associated with clathrin-coated pits or other vesicular elements of the endocytosis machinery. Previously, we have shown that tubulobulbar complexes contain similar actin-related components including N-WASP, Arp2/3, cortactin and dynamin 3 to those reported to be present at podosomes and we speculate that the two structures also may have other molecular components in common. One of these components is zyxin. Zyxin is an 82 kDa protein that is known to concentrate at focal adhesions. It has been described as a mechano-sensitive protein due to its ability to mobilize and relocate from focal adhesions to actin stress fibers in response to mechanical cues. Zyxin is reported to be present at smooth muscle podosomes.

In smooth muscle cells, phorbol ester triggers the conversion of focal adhesions into podosomes. At discrete microdomains on the ventral surface of the plasma membrane, podosomes form near the sites where stress fibers insert into adhesion plaques. These structures are reported to contain β-actinin, F-actin and vinculin.
and exhibit a tubular, column-like structure arising perpendicularly to the “bottom” of phorbol dibutyrate treated cells. The composition of smooth muscle podosomes has not been confirmed at the ultrastructural level. Despite this lack of morphological evidence, their structure has been included in the description of podosomes that have a central membrane invagination surrounded by an actin filament cuff that is lined by a larger, ring-shaped structure containing focal adhesion proteins such as α-actinin and vinculin. Also, podosome formation is dependent on Arp 2/3-dependent actin polymerization.

Smooth muscle cell focal adhesions contain structural proteins zyxin and vinculin. During the simultaneous events of focal adhesion disassembly and podosome formation, cytoskeletal rearrangement takes place. At later stages of podosome formation, zyxin and vinculin redistribute to podosomes and co-localize at these sites. Both zyxin and vinculin have previously been reported to be present at ESs in the seminiferous epithelium. Furthermore, vinculin has been reported to be present at tubulobulbar complexes.

In this study, we explore the prediction that these two major structural components, vinculin and zyxin, are co-distributed at tubulobulbar complexes. We immunologically probe cryosections and epithelial fragments of perfusion fixed testis for vinculin and zyxin. We use rat testis as the model system because tubulobulbar complexes are best characterized in this model.

As predicted, zyxin and vinculin co-distribute at apical tubulobulbar complexes. Rather than the staining of individual complexes that was observed with actin assembly proteins, vinculin and zyxin display a more diffuse, although specific association with tubulobulbar complexes.

**Results**

The zyxin antibody labels ectoplasmic specializations and tubulobulbar complexes. In sectioned tissue, the zyxin antibody reacted at tubulobulbar complexes, in addition to reacting with ectoplasmic specializations as previously reported. Interestingly, the staining varies according to the stage of the tubule that is observed. At stage V of spermatogenesis when spermatids are situated deep in apical Sertoli cell crypts, the zyxin antibody clearly labels ectoplasmic specializations associated with the crypts as indicated by intense staining with phalloidin (Fig. 1A). In stage VII tubules, staining is present adjacent to the convex or dorsal face of late spermatids where ectoplasmic specializations are known to occur, and around tubulobulbar complexes clustered adjacent to the convex face of the hook-shaped spermatid heads (Fig. 1B).

Fragments of seminiferous epithelium viewed at 100X reveal intense staining associated with the dorsal curvature of spermatid heads and diffuse staining around tubulobulbar complexes (Fig. 2) although staining of individual complexes also was observed. Phalloidin can be seen labeling the actin cuff around individual complexes (Fig. 2).

Zyxin is present with vinculin at tubulobulbar complexes. Zyxin and vinculin are present at tubulobulbar complexes. The staining pattern for both proteins appears diffuse, and in some cases outlines individual complexes that are adjacent to the concave surface of mature spermatid heads. A western blot of whole testis and seminiferous epithelium confirms the presence of vinculin (Fig. 3). Interestingly, vinculin stain is not concentrated with zyxin stain in relationship to the dorsal aspect of spermatid heads.

Tubulobulbar complexes are best resolved in fragmented tissue. Zyxin can be seen diffusely labeling the Sertoli cell cuff in regions where tubulobulbar complexes are present. These staining patterns are absent in controls (Fig. 4).

In testis, the zyxin antibody is specific for zyxin and splice variant HED-2. The zyxin antibody B71 (Beckerle Lab) that was used in these studies reacted on western blots of whole testis and seminiferous epithelium at the appropriate molecular weight of 64 Spermatogenesis Volume 2 Issue 1
Interestingly, a second, more intense band at 20 kDa was present (Fig. 4E). A band of this weight has been previously reported to be present in human seminiferous epithelium and represents zyxin splice variant HED-2. Similar gene splicing may be occurring in rat although this remains to be determined. Like full-length zyxin, HED-2 contains a proline rich domain and three LIM domains. HED-2 is present in Sertoli cells and other tissues. A band also is present at 66 kDa. Hoffman and coworkers have reported this band previously in platelets as a protein that may have similarity to zyxin. Although we believe zyxin is being immunolocalized in Figures 1–4, it is unclear which isoform is being expressed at each specific location.

**Discussion**

In this study we demonstrate that zyxin is present at tubulobulbar complexes in the seminiferous epithelium of the testis. Furthermore, zyxin co-localizes with vinculin. Zyxin is a significant component of focal adhesions. The protein contains a number of dynamic structural features including three C-terminal LIM domains involved in protein-protein interactions, a proline rich domain that can interact with Ena/VASP family members and a nuclear export signal that allows translocation between the nucleus and cytoplasm. Zyxin-null 3T3 cells display increased motility in comparison to wildtype cells that express zyxin. This phenotype is due to weaker stress fibers that result from a lack of zyxin expression. Re-expression of zyxin suppresses the migratory phenotype.

A number of experiments have shown that zyxin distribution in a cell is mechanosensitive. In mouse fibroblasts, changes in the actin cytoskeleton were observed upon the application of uniaxial cyclic stretch. Filamentous actin realigned itself to be perpendicular to the stretch vector. Zyxin mobilized from focal adhesions to stress fibers when cells were subject to this stretch assay. At stress fibers, zyxin acts to reinforce the actin cytoskeleton in response to mechanical stimulation.

At focal adhesions in mouse fibroblasts zyxin and vinculin are colocalized. During cell stretching assays, zyxin translocates from the focal adhesion to stress fibers; however, vinculin stays at the focal adhesion. This is in contrast to what we have observed at tubulobulbar complexes and what Gimona and coworkers (Gimona et al., 2003) observed at smooth muscle podosomes where zyxin and vinculin both relocate from sites of adhesion to the actin cytoskeleton.
Tubulobulbar complexes are testis-specific structures that we propose engage in a sort of clathrin-mediated bulk endocytosis of intercellular junctions during sperm release and translocation of spermatocytes. “Clathrin-mediated bulk endocytosis” is a description that was recently coined in reference to the behavior of reconstituted membranes in a cell free system. Upon fission disruption with a G-protein analog, clathrin-mediated tubulation was observed resulting in structures that morphologically resemble tubulobulbar complexes.

Tubulobulbar complexes that develop at junctions between Sertoli cells and spermatids consist of a long tubular protrusion of spermatid plasma membrane into a corresponding invagination of the adjacent Sertoli cell. A network of actin filaments cuffs the double-membrane tube and the entire structure ends in a clathrin-coated pit.

TBCs have features in common with podosomes. Podosomes are subcellular, actin-containing structures formed at cell/matrix contacts. Although the function of podosomes is not entirely clear, adhesion and matrix degradation are likely. The formation of podosomes is dependent on an actin assembly complex involving N-WASp, Arp2/3, cortactin and dynamin. During this formation, zyxin and vinculin redistribute from focal adhesions to podosomes. A similar sort of redistribution appears to occur during the formation of tubulobulbar complexes in regions of intercellular attachment in the seminiferous epithelium.

In apical regions of the seminiferous epithelium, tubulobulbar complexes form in regions where unique actin-related intercellular adherens-like junctions (ectoplasmic specializations) occur at sites of Sertoli cell attachment to spermatids. Vinculin and zyxin have both been reported to be present at these actin-related junctions. The actin assembly proteins N-WASp, Arp2/3, cortactin and dynamin 3 are present at tubulobulbar complexes and presumably are involved with their formation. Vinculin and zyxin appear to redistribute from ectoplasmic specializations to tubulobulbar complexes as the former structures disassemble. The exact role of zyxin and vinculin at TBCs remains to be identified.

Materials and Methods

Animals used in this study were reproducitively active male Sprague Dawley rats. They were obtained from Charles River animal colonies and were maintained according to the guidelines established by the Canadian Council on Animal Care. All experiments were done at least in duplicate using tissue from different animals.

Reagents. Unless indicated otherwise, all reagents used in the studies were obtained from Sigma-Aldrich Canada. The paraformaldehyde was obtained from Fisher Scientific (04042-500). Secondary antibodies and phalloidins conjugated to Alexa fluorochromes were obtained from Invitrogen (A11008, A11036).
and those conjugated to HRP were purchased from Jackson ImmunoResearch Laboratories, Inc. (111-000-003). Normal serum or immunoglobulins used in specificity controls were purchased from Jackson ImmunoResearch Laboratories, Inc. (011-000-120, 011-000-003).

Primary antibodies. Primary antibodies were obtained from the following sources and used at the following concentrations for immuno-staining and western blots: mouse-anti-vinculin (Sigma-Aldrich, V 9131) (1:2500) [WB 1:250] IF; B7 rabbit-anti-zyxin (gift from Beckerle Lab, Huntsman Cancer Institute) [1:1250] WB; [1:400] IF.

Immunofluorescence. Tissue preparation. All testicular material was perfusion fixed by organ perfusion. Animals were anesthetized with isoflurane, and their testes removed. The spermatic arteries were cannulated with 26-gauge needles and perfused with warm PBS (1.5 M NaCl, 0.2 M NaKPO4, and 1.5 M KCl, pH 7.3) for 2 min followed by warm fixative (3% paraformaldehyde, 1.5 M NaCl, 0.2 M NaKPO4, and 1.5 M KCl, pH 7.3) for 30 min. The perfusion was changed back to PBS for the last 5 min to complete the fixation.

Fixed tissue sections. Fixed tissues were placed on aluminium stubs in a pool of OCT (Optimal Cutting Temperature) Compound (Sakura Finetek, 4583) and then frozen with liquid nitrogen. Frozen sections (5 μm) were cut, collected on poly-L-lysine coated slides and immediately plunged into cold (-20°C) acetone. After 5 min, the slides were removed from the acetone, allowed to air dry, and then immunostained.

Dissected tissue. All testicular material was discarded in PBS, and the seminiferous tubules were minced into small pieces using scalpels. The material was gently aspirated, first through an 18-gauge needle and then a 21-gauge needle to fragment the seminiferous epithelium. Larger material was allowed to sediment for 10 min and then the supernatant, containing epithelial fragments, was collected and concentrated using low-speed centrifugation. The fragments were resuspended in a small amount of PBS, and drops of the material were placed on poly-L-lysine (Sigma, P8920) coated slides. Excess fluid was removed, and the slides were plunged into cold (-20°C) acetone. After 5 min, the slides were removed from the acetone, air-dried, and immunostained.

Staining protocols. Slides with attached sections or fragments were rehydrated with 5% normal goat serum (NGS) (Sigma, G9023) in TPBS-BSA (PBS containing 0.5% Tween-20 and 0.1% BSA) for 20 min. Primary antibodies were diluted with TPBS-BSA containing 1% NGS, added to the tissue, and then the slides were incubated overnight at 4°C. The slides were washed with TPBS-BSA, and the slides were incubated for 1 h at 37°C with secondary antibodies conjugated to Alexa 488 or 568 and diluted 1:1000 in TPBS-BSA. Slides were washed and coverslips mounted using Vectorshield [Vector Labs (H-1000)] or Vectorshield containing 4', 6-diamidino-2-phenylindole (DAPI) (H-1200). Staining was evaluated and photographed using a Zeiss Axioshot microscope fitted with appropriate filter sets for detecting fluorescence.

To double immunostain samples, the two primary antibodies were added together as a cocktail to the slides, as were the two secondary antibodies. To label filamentous actin, the slides were stained for 20 min at room temperature with Alexa 488 phalloidin made up in TPBS-BSA, and then extensively washed with TPBS-BSA.

Disclosure of Potential Conflicts of Interest

The authors have no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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