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Phenotyping the Claudin 11 Deficiency in Testis: From Histology to Immunohistochemistry

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Abstract

The testis is a heterogeneous organ that comprises a number of cell types, including germ cells at different stages in their maturation, differentiated neighbor nursing cells, and endocrine somatic cells. Despite such cellular heterogeneity the testis is highly organized, with germ cell development and differentiation being compartmentalized into the interconnected tubular network of the seminiferous epithelium. Intratesticular scaffolds rely heavily on the basement membrane of the seminiferous tubules while germ cell development inside the seminiferous epithelium is critically dependent on the Blood Testis Barrier (BTB). The BTB is a macromolecular tight junction complex generated by somatic Sertoli cells within the seminiferous epithelium. The BTB divides the seminiferous epithelium into two compartments: the basal compartment, which delineates a niche for the proliferation and renewal of spermatogonia; and the adluminal compartment, where differentiating germ cells undergo meiosis and spermiogenesis. The BTB is unique in mammalian tissues because it is cyclically reconstructed during the spermatogenic cycle as preleptotene spermatocytes migrate from the basal compartment to the adluminal compartment and enter meiosis. In mouse, the loss of the BTB in the absence of the claudin 11 protein causes azoospermia and leads to infertility. Specifically, *cldn11* deficiency results in sloughing of the cells of the seminiferous epithelium into the lumen. Understanding this pathophysiology has involved histological examination of the tissue defects as well as immunohistological characterization. Here, we present a comparative study of several modifications to the classical Hematoxylin–Eosin stain that may improve the diagnostic usefulness of this technique, as well as the use of several selective markers to identify testicular cell types.

Keywords

Claudin 11; Spermatogenesis; Sertoli cell; Blood testis barrier; Gene regulation

1. Introduction

The Claudin (Cldn) family of tight junction (TJ) proteins comprises tetraspan transmembrane proteins that are the major selective diffusion barrier of the paracellular pathway between epithelial cells. The overall sieving properties of TJs are determined by the combination of individual Cldn family members incorporated into the intramembranous strands. Inactivation of the *Cldn11* gene in mice (1) has clearly demonstrated the pivotal role of the Cldn family in TJ physiology, principally because *cldn11* is often the only family member incorporated into the intramembranous strands; thus without *cldn11*, TJs are absent and the paracellular pathway is open. The characterization of *Cldn11*^{−/−} mice has revealed the importance of *cldn11* for spermatogenesis, central nervous system function, and normal hearing (1). Indeed, these mutants exhibit neurological deficits and deafness (2, 3), and males are sterile because of the failure of spermatogenesis beyond the stage of spermatocyte

differentiation. The testes in these animals also exhibit a striking disorganization of the seminiferous tubules (1, 4).

Understanding the relationship between *cldn11* deficiency and male sterility requires a detailed examination of testis organization. The testis comprises multiple somatic cell types, including steroid-secreting Leydig cells located in the interstitial compartment and Sertoli cells in the seminiferous tubules, as well as germ cells located in the seminiferous tubules (5). Sertoli cells play several key roles in spermatogenesis: they are hormone target cells; they provide structural and nutritional support for developing germ cells; and more importantly, they synthesize the proteins that constitute the blood testis barrier (BTB), including *cldn11*. The BTB is assembled at the commencement of puberty between adjacent Sertoli cells and delineates the adluminal compartment of the seminiferous epithelium in which meiosis and spermiogenesis occur. The permeability of the BTB is hormonally regulated; thus, androgens regulate *Cldn11* gene expression (6) and gonadotropins maintain Sertoli cell TJ integrity, including the trafficking and localization of *cldn11* to functional TJs (7).

In the primary study of the testicular phenotype in *Cldn11*^{-/-} mice (1), cell clusters were observed in the lumen of many seminiferous tubules instead of spermatozoa. Although the cells in these clusters were not identified, histochemical analysis indicated that differentiated Sertoli cells were present. We hypothesized that the identity of the cells in these clusters would be instrumental to understanding the etiology and, to this end, we developed a methodology comprising a series of tests, including macroscopic observation, histology, and immunohistochemistry.

Our approach initially involved staining of paraffin-embedded sections from wild type and *Cldn11*^{-/-} mice with Hematoxylin–Eosin (H&E) and several other stains. We examined a broad range of ages throughout sexual development and in adults to develop a broad understanding of the tissue defects. Secondly, we used a battery of selective antibodies to identify different cell types and stages of differentiation of these cells, using immunohistochemistry. This paper provides an in-depth demonstration of the full methods we have employed as well as a description of how the loss of Sertoli cell epithelial phenotype and azoospermia stem from the absence of *cldn11* TJ integrity.

2. Materials

2.1. Tissue Processing and Sectioning

1. Phosphate-buffered saline (PBS).
2. Paraformaldehyde (Sigma, MO, USA) 4% in PBS, prepared immediately before use. Paraformaldehyde was dissolved by heating.
3. Modified Davidson's fluid: 30% formaldehyde (Sigma), 15% ethanol, 5% acetic acid in water.
4. Bouin's Fluid: 150 mL of saturated picric acid (Sigma), 50 mL of 37% formaldehyde, and 10 mL of glacial acetic acid.
5. Graded series of ethanols (70%, 95%, and 100%), xylene, and paraplast (Labonord).
6. Slides: Superfrost and polylysine-coated slides (Labonord).

2.2. Dewaxing, Rehydration, Dehydration, and Mounting

1. Graded series of ethanol (50%, 70%, 95%, and 100%), xylene, and paraplast (Labonord).
2. Methanol-H₂O₂ (Sigma) 3%.
3. Eukitt mounting medium (Labonord).

2.3. Histological Stains

1. Harris hematoxylin solution (#HHS16, Sigma).
2. Eosin Y solution 0.5% in water (#E4382, Sigma).
3. Periodic acid solution and Schiff's reagent (#395-132 and #395-2016, respectively, Sigma).
4. Aniline blue (#1275, MERCK), acetic acid, phosphomolybdic acid (#20612, PROLABO), acidic fuchsin (#F8129, Sigma), Orange G (#GEPCO L01-61, Eurobio).
5. Tuchmann's blue stock solution: 1% (w/v) aniline blue, 2% (w/v) orange G, 7% (v/v) acetic acid in water.

2.4. Immunohistochemistry

1. Bovine Serum Albumin (BSA) (Fraction V, Sigma) 10% in PBS.
2. Epitope retrieval solution:
Sodium citrate stock, 100 mM: Dissolve 29.4 g of tri-sodium citrate (dihydrate) in 1 L of distilled water. Store this solution at room temperature for 3 months or at 4°C for longer storage.
Citric acid stock, 100 mM: Dissolve 29.4 g of citric acid (.2H₂O) into 1 L of distilled water. Store this solution at room temperature for 3 months or at 4°C for longer storage.
Citrate buffer, 10 mM: Prepare immediately before use by mixing 18 mL of 100 mM citric acid with 82 mL of 100 mM sodium citrate, adjusting to pH 6, and increasing the volume to 1 L with deionized water.
3. Antibody diluent (Dako Cytomation, #S2022) and antibody diluent with reducing background (Dako #S3022).
4. Primary antibodies: Ddx4 (diluted 1:750; kindly provided by Dr. T. Noce, Mitsubishi Kagaku Institute of Life Sciences, Tokyo, Japan and diluted 1:500; #Ab13840, Abcam) (8–10), TRA98 and TRA369 (calmegin) (both diluted 1:1,000; kindly provided by Dr. H. Tanaka, Osaka University, Osaka, Japan) (11, 12), Vimentin (diluted 1:100; LN-6 clone; DakoCytomation), and Gata4 and claudin11 (both diluted 1:100; sc-1237X and sc-2571, respectively, SantaCruz Biotech).
5. Secondary antibodies: Biotinylated anti-rabbit (diluted 1:500; #E0432, DakoCytomation) for rabbit primary antibodies (claudin11, vimentin), multilink swine anti-goat, -mouse, -rat IgG, biotinylated (diluted 1:500; #E0453, DakoCytomation) for goat primary antibodies (Gata4), biotinylated anti-rat IgG (diluted 1:200; #BA-4001, Vector) for rat primary TRA98 and TRA369 antibodies, Vectastain ABC kit (PK6100, Vector), DAB tablets (#D4168, Sigma).

3. Methods

3.1. Preparation of Samples

The choice of tissue fixative is of prime importance for characterization of tissue sections and diagnostic assessments. Immersion of whole testis in Bouin's fixative provides excellent histology which is especially useful in visualizing germ cell chromatin to distinguish between different meiotic stages and permit a reliable assessment of the spermatogenic cycle in individual tubules (13, 14). Paraformaldehyde provides a milder fixation that typically preserves antigenicity and is preferred for immunohistochemistry with most antibodies that do not detect their target antigen in Bouin-fixed tissue. However, paraformaldehyde requires particular handling during testis fixation because it penetrates tissue slowly and because the testis is encapsulated by the dense connective tissue layer of the tunica albuginea. Thus, the testis is either rendered more permeable by puncturing the tunica albuginea once at each pole with an 18G needle or is fixed in situ using transcardiac perfusion. Finally, modified Davidson's fluid has been proposed for testis fixation because it exhibits most of the useful properties of the Bouin's and paraformaldehyde fixatives (15) (see Note 1).

1. For Bouin's fixation, wild type, *Cldn11*^{+/−} and *Cldn11*^{−/−} testes are dissected, immersed in Bouin's solution for at least 24 h and washed several times with 70% ethanol until the yellow color of the picric acid largely disappears. Tissues can be stored in 70% ethanol and fixation should not exceed 48 h. Tissues immersed in Bouin's fixative rapidly turn yellow.
2. For modified Davidson's fluid, *Cldn11*^{+/−} and *Cldn11*^{−/−} testes are dissected, immersed in modified Davidson's fluid for at least 24 h and washed several times with 70% ethanol. Tissues placed in Davidson's fixative rapidly turn white and opaque. Tissue fixation should not exceed 48 h and 24 h is optimum.
3. For paraformaldehyde fixation, superior penetration of the fixative through the tunica albuginea is achieved by puncturing each pole of the testis once with an 18G needle. These holes are sufficiently small to allow fixative to enter the tissue but do not result in extrusion of the seminiferous tubules through the tunica albuginea. Tissue is then immersed in paraformaldehyde overnight at 4°C.
4. Testes are dehydrated through a graded series of ethanol into xylene and embedded in paraffin. Tissue blocks are cut in transverse orientation into 6 µm-thick sections, floated in a 45°C water bath to eliminate compression artifacts and mounted on poly-lysine or superfrost glass slides.

¹*Choice of histological stains.* The H&E stain is a routine technique that is widely used for nuclear staining and general assessment of tissue morphology. Nuclei appear blue, and cytoplasm pink to red. However, H&E has limited use for characterizing complex organs, such as testis, particularly when attempting to distinguish between different stages of the spermatogenic cycle, because it only relies on the examination of chromatin.

Because Periodic Acid-Schiff (PAS) stains glycoproteins, it has become the stain of choice for visualizing acrosome development, and especially the shape and orientation of the acrosome that permits accurate assessment of spermatid differentiation and staging of the seminiferous epithelium (16). In the case of the pathology of the *Cldn11*^{−/−} testis, the PAS stain also reveals PAS-positive material within adluminal cell clusters (Fig. 1a, b, e). Periodic acid is a potent oxidant capable of breaking the covalent bond between two hydroxyl groups of glucopyranose. The two aldehyde groups generated reduce the leucoderivative of basic fuchsin Schiff, which becomes pink. Hematoxylin is used to enhance the contrast of nuclei. PAS stains in red fuschia aldehydes (sugars or polysaccharides) of the plasma membrane or nucleic acids, glycogen, and glycoproteins appear pink. Because PAS reacts with polysaccharides, glycoproteins, and acrosomes in the testis, we were able to identify acrosome remnants in the cell clusters (4).

The Tuchmann-Duplessis trichrome stain provides good quality contrast of cells in a manner similar to H&E, but the inclusion of aniline blue in this technique also reveals matrix glycoproteins of the basement membrane, acrosomes, and nucleoli in pink (Fig. 1c, d, f). In *Cldn11*^{−/−} testis, the particular flattened shape of cell nuclei in clusters is associated with a more intense blue staining of their cytoplasm, but the morphology remains difficult to interpret. Thus, the most useful stain for *Cldn11*^{−/−} testes is PAS-H because of the capacity to determine the stage of the spermatogenic cycle for each tubule and the labeling of small vesicles within the cell clusters.

3.2. Histological Stain

For histological analysis, Bouin's or Davidson's fluid fixed organs are preferred to enable the accurate identification of germ cells from the fine fixation of their chromatin. The choice of histological stain has been instrumental in characterizing testicular pathology. H&E is commonly used in most laboratories, but Periodic Acid-Schiff-hematoxylin (PAS-H) staining is superior for distinguishing germ cell types. Indeed, PAS-H labels nuclei in blue and basement membranes and acrosomes in fuchsia pink and is the method of choice for histological evaluation of testis because it reveals the shape and orientation of the maturing acrosomes for staging of the spermatogenic cycle (16). Trichrome techniques, such as the Tuchmann-Duplessis stain, are also commonly used for epithelial tissues because they highlight the edges of epithelial units (17) (see Note 2).

3.2.1—For all histological stains, tissue sections are heated at 37°C for 30 min, deparaffinized in xylene (2 changes, 10 min each), rehydrated in graded ethanols (100%, 100%, 95%, 70%, and 50%, 5 min each) and briefly washed in distilled water.

3.2.2. H&E Stain

1. Immerse slides in HHS (diluted 1/3) for 30–60 s;
2. Wash in running tap water for 15 min and rinse in distilled water;
3. Counterstain in eosin Y solution for 1–3 min;
4. Wash slides briefly in running water and dehydrate through 95% ethanol, 2 changes of 100% ethanol (5 min each), clear in xylene and coverslip in a xylene-based mounting medium (Eukitt).

3.2.3. Periodic Acid-Schiff-Hematoxylin Stain

1. Immerse slides in periodic acid for 5 min at 25°C, wash in running water, and rinse in distilled water;
2. Immerse slides in Schiff's reagent for 15 min at 25°C under a fume hood, then wash in running water for 5 min, and rinse in distilled water;
3. Counterstain sections with Harris hematoxylin diluted one-third for 30 s;
4. Wash slides in running water for 15 min, then dehydrate through 95% ethanol, 2 changes of 100% ethanol (5 min each), clear in xylene, and mount with Eukitt.

²*Choice of antibodies for immunohistochemistry.* Histological examination of the *Cldn11*^{−/−} testicular pathology provided the initial insights into tissue structure, cell composition, and cell health; however, the use of several specific antigenic markers for Sertoli cells and germ cells enabled us to monitor the fate of each cell type and has permitted indisputable identification of cells in the adluminal clusters. These cells are positive for GATA4 and vimentin and negative for H3 phosphohistone, DDX4, CGLN/TRA369, TRA98, vitronectin, and *cldn1* ((4) and Fig. 2). In the oldest animals, cell clusters mainly comprise Sertoli cells, but immature germ cells are occasionally observed. For Sertoli cell markers, the use of antibodies to label nuclear antigens provides more definitive identification than do cytoplasmic antigens (compare Fig. 2e–h) because cell shape is markedly variable as the seminiferous epithelium degenerates in older *Cldn11*^{−/−} mice. We also paid particular attention to Sertoli cell markers with stage-specific expression through the seminiferous cycle (19, 20). Numerous proteins exhibit such cyclic expression during the development and maturation of the seminiferous epithelium or in association with testicular pathology, such as germ cell loss (20). We selected markers that continue to be expressed by Sertoli cells even in disease states. Thus, our immunohistochemical approach (4) increased the accuracy of identifying cell clusters compared to the earlier histochemical approach (1). Given the rarity of current mouse models of male infertility in which Sertoli cell sloughing from the seminiferous epithelium is prevalent, we consider that characterization of the cellular mechanism of Sertoli cell sloughing in *Cldn11*^{−/−} mice is of prime importance.

Finally, immunohistochemistry of several important BTB components revealed persistent expression of ZO-1 and occludin (1). Our data supports these findings and indicates that the expression of these proteins is induced in response to the inactivation of the *Cldn11* gene (4).

3.2.4. Tuchmann-Duplessis Trichrome Stain

1. Stain sections using the H&E stain (Subheading 3.2.2) and after eosin Y staining, wash briefly in running water;
2. Immerse slides in 1% acid fuchsin in water for 1 min, wash briefly in 1% acetic acid;
3. Immerse slides in 2% orange G solution in water for 1 min; then directly in 5% phosphomolybdic acid in water for 30 s, and immerse five to six times in running water;
4. Immerse sections in Tuchmann's blue (diluted 1/4) for 4–10 min; then immerse five to six times in running water and quickly dehydrate, clear and mount. If the Tuchmann's blue stain is too intense, let the slides in water for several minutes while surveying the dilution of the blue.
5. Dehydrate through 95% ethanol, 2 changes of 100% ethanol (5 min each), clear in xylene, and mount with Eukitt.

3.3. Immunohistochemistry

Although histology can generally be used to identify germ cell types if special attention is given to choosing the fixative and stain, this classical technique has limited application for characterizing the pathology in *Cldn11*^{-/-} testes. In particular, identification of the cells sloughing from the seminiferous epithelium has not been possible. In the youngest animals, the composition of cell clusters is homogeneous, but the cells do not appear to be either germ cells or Sertoli cells (4). In older animals, cell clusters are heterogeneous, and identification of the various cell types has proven difficult (1). The choice of selective antibodies against a number of somatic and germ cell proteins, including Gata4 and vimentin (Vim) for Sertoli cells, Ddx4, TRA98, and TRA369 (Calmegin), has greatly improved our immunohistochemical characterization of cell clusters (see Note 3).

3.3.1. Dewaxing, Rehydration, and Inactivating Endogenous Peroxidase—

Sections are heated at 37°C for 30 min, deparaffinized in xylene (2 changes, 10 min each), rehydrated in a graded ethanol series (100%, 100%, 95%, 70%, and 50%, 5 min each), briefly washed in distilled water and rinsed in PBS. Endogenous peroxidases are inactivated by immersing slides in 0.3% H₂O₂ in methanol for 20 min following the rehydration step in 95% ethanol.

³*Choice of tissue fixation.* The use of testis tissue sections for both histological and immunohistochemical characterization requires that chemical fixation should simultaneously preserve morphological structure and protein antigenicity. A major problem in this effort is that high-quality preservation of chromatin architecture comes at the expense of preserving antibody epitopes.

For histology, good morphology is crucial for developing insights into testis physiology and fixatives such as Bouin's or modified Davidson's fluids that rapidly penetrate the testis and contain acetic acid to coagulate nucleic acids is preferred and recommended by the Society for Toxicologic Pathology (21). However, antigenicity is preserved to variable extents with these fixatives and may differ for each fixative. In Bouin's fixed tissue, Gata4 and Vimentin are barely detectable (not shown) while DDX4, TRA98, and CLDN11 are readily detected. On the other hand, DDX4, TRA98, and CGLN are detected in modified Davidson's fixed tissue (Fig. 3) but CLDN11 is not. Furthermore, immunohistochemical labeling of Bouin's fixed tissue often shows a decreasing gradient of labeling intensity toward the center of the tissue, a pronounced side-effect due to the rate of fixative penetration into the organ. There is a clear difference in the compactness and staining intensity between Bouin's and modified Davidson's fixed tissue that may be associated with shrinkage artifacts caused by the latter fixative (15).

Paraformaldehyde fixation is the first-choice fixative for many tissues because it is a mild fixative that preserves the antigenicity of many antibody epitopes. Indeed, all antibodies described in the current study labeled paraformaldehyde-fixed testes (Fig. 2c–h). However, paraformaldehyde performs relatively poorly for preserving testis morphology because of its slow rate of tissue penetration. To maintain the integrity of seminiferous tubules and interstitial tissue, testis must be fixed whole. Unfortunately, the tunica albuginea surrounding the tissue acts as a diffusion barrier which slows fixative penetration even when a needle is used to puncture this dense conjunctive tissue at the poles. A reasonable compromise for paraformaldehyde is to use intracardiac perfusion and subsequent postfixation.

3.3.2. Epitope Retrieval—A major adverse effect of formalin or other aldehyde fixatives is the masking of tissue epitopes by formaldehyde, which cross-links the amino side chains of proteins and results in weak or false-negative immunohistochemical detection of some proteins. The citrate-based unmasking buffer reverses the protein cross-links and increases the accessibility of epitopes in formalin-fixed, paraffin-embedded tissue sections for increased staining intensity by antibodies.

1. Slides are immersed in a Coplin jar containing the citrate buffer solution. The jar is covered and placed in a water bath, then microwaved twice for 2–3 min at maximum power with a 5 min delay between each treatment.
2. The jar is allowed to cool slowly for at least 30 min at room temperature and the slides are rinsed in PBS.

3.3.3. Blocking (Nonspecific) and Immunostaining—All steps are carried out at room temperature unless otherwise noted. Sections are blocked in PBS containing 10% BSA for at least 20 min in a moist chamber. After a rinse in PBS, sections are covered with primary antibody diluted appropriately in primary antibody diluent overnight at 4°C (optimal dilutions of primary antibodies are determined in pilot experiments). After several rinses in PBS, sections are incubated for at least 1 h with the appropriate secondary antibody diluted in PBS. After several rinses in PBS, sections are incubated with the avidin–biotin complex (ABC) for 30 min according to the manufacturer's instructions. After several rinses in PBS, sections are incubated in diaminobenzidine substrate (DAB) and stain intensity is monitored using a microscope. After staining, sections are washed in PBS, counterstained in Harris hematoxylin (diluted 1/15) for 1 min, washed in water, dehydrated in a graded ethanol series, cleared in xylene, and mounted with Eukitt.

3.4. Conclusion

We have developed a step-by-step battery of histological and immunohistochemical tests that have enabled an accurate characterization of the testicular phenotype in *Cldn11*^{−/−} mice. However, a deeper understanding of pathophysiology in the absence of the BTB will require ultrastructural analysis beyond that which has been performed (1), including transmission electronic microscopy and freeze-fracture, as well as immunocytochemistry to localize other TJ components. In this vein, signaling mechanisms that underlie the transient opening of the TJs and the cyclical expression of TJ components during migration of spermatogonia into the adluminal compartment are poorly understood (18). Determining the full extent of BTB permeability in *Cldn11*^{−/−} mice will require injection of a biotin tracer into the testicular artery and immunohistochemical analysis with antibodies against additional TJ components (6). Microarray analysis likely will be complementary to such in situ approaches for determining the mechanisms that regulate expression of the genes encoding BTB components (4).

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Fig. 1. Comparison of the PAS-Hematoxylin and Tuchmann-Duplessis trichrome stains for testis histology. Transverse sections of P20 testis from control (**a, c**) and *Cldn11*^{-/-} (**b, d-f**) mice were processed for either PAS-Hematoxylin histology (**a, b, e**) or Tuchmann-Duplessis trichrome stain (**c, d, f**). scale bars: 100 μ m (**a-d**) and 50 μ m (**e, f**).

Fig. 2.

Immunohistochemical analysis of cell clusters in the adluminal compartment of *Cldn11*^{-/-} mice. Immunolabeling for germ cell markers DDX4 (**a, b**) and calmegin (CGLN) recognized by the TRA369 antibody (**c, d**); Sertoli cell markers GATA4 (**e, f**) and Vimentin (VIM) (**g, h**). Sections from P20 (**a, b**) or P28 (**c-h**) control (**a, c, e, g**) and *Cldn11*^{-/-} (**b, d, f, h**) testes show that cell clusters observed in the adluminal compartment of *Cldn11*^{-/-} seminiferous tubules (*arrow*) comprise Sertoli cells. Scale bars: 100 μ m.

Fig. 3. Comparison of immunocytochemical labeling using different methods of tissue fixation. Testes were fixed with either Bouin's fixative (**a, c, e, g**) or modified Davidson's fixative (**b, d, f, h**) and processed for immunohistochemistry with anti-cln11 (CLDN11, **a, b**), anti-DDX4 (**c, d**), anti-TRA98 (**e, f**), or anti-CGLN antibodies recognized by the TRA369 antibody (**g, h**). Scale bars: 100 μ m.