THE ROLE OF TIGHT JUNCTION PROTEINS CLAUDIN-3 AND CLAUDIN-7 IN URETERIC BUD BRANCHING

Nicholas Haddad

Department of Human Genetics
McGill University, Montreal
June, 2009

A thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements of the degree of Master’s of Science.

© Nicholas Haddad, 2009
# TABLE OF CONTENTS

TABLE OF CONTENTS .................................................................................................................. 1  
ABSTRACT .................................................................................................................................. 3  
RÉSUMÉ ........................................................................................................................................ 5  
ACKNOWLEDGEMENTS .............................................................................................................. 7  
CONTRIBUTION OF CO-AUTHORS .......................................................................................... 10  
LIST OF FIGURES ...................................................................................................................... 11  
ABBREVIATIONS ....................................................................................................................... 12  
CHAPTER I: Introduction .............................................................................................................. 14  
1. Overview of kidney function .................................................................................................. 15  
2. The kidney: three stages of development ............................................................................ 16  
   2.1 The pronephros and the mesonephros ........................................................................... 16  
   2.2 The metanephros ........................................................................................................... 17  
3. The genetics of branching morphogenesis ......................................................................... 18  
   3.1 The Gdnf/Ret/Gfrc1 pathway and Pax2 ......................................................................... 19  
   3.2 Adhesion molecules ....................................................................................................... 20  
4. Tight junctions and claudins .................................................................................................. 21  
   4.1 Tight junctions are composed of various proteins .......................................................... 21  
   4.2 Claudins are essential components of tight junctions .................................................... 22  
   4.3 Claudin structure and function ...................................................................................... 23  
5. Claudins in the kidney ............................................................................................................ 24  
6. Claudins and human disease ................................................................................................. 25  
7. The function of Claudin-3 and Claudin-7 ............................................................................ 26  
   7.1 Claudin-3 ......................................................................................................................... 26  
   7.2 Claudin-7 ......................................................................................................................... 27  
8. Summary of objectives ........................................................................................................... 28  

CHAPTER II: The tight junction proteins Cldn3 and Cldn7 are expressed in the ureteric bud and promote tubulogenesis *in vitro* ......................................................................................... 30  
1. Abstract .................................................................................................................................. 31  
2. Introduction ........................................................................................................................... 32
## References

### APPENDIX B: Permission to reprint

### APPENDIX A: Ethics approval and certificates

## Chapter IV: Future Experiments

1. Introduction .......................................................................................................................... 57
2. Materials and Methods ......................................................................................................... 58
3. Results ................................................................................................................................. 58
4. Discussion ........................................................................................................................... 58

## Chapter III: Electroporation of embryonic kidney explants

1. Abstract ............................................................................................................................... 48
2. Introduction .......................................................................................................................... 49
3. Procedure ............................................................................................................................. 50
   3.1 Mouse embryonic kidney cultures ............................................................................... 50
   3.2 DNA constructs .............................................................................................................. 51
   3.3 Microinjection and electroporation .............................................................................. 51
4. Results ................................................................................................................................. 52
   4.1 Establishing parameters for microinjection and electroporation ............................... 52
   4.2 Tissue-specific expression ............................................................................................ 53
5. Comments ........................................................................................................................... 54

## Chapter II: Electroporation

3. Materials and methods ......................................................................................................... 35
   3.1 Animal care ................................................................................................................... 35
   3.2 Electron microscopy ....................................................................................................... 35
   3.3 Whole mount in situ hybridization .............................................................................. 36
   3.4 Double immunofluorescence ...................................................................................... 36
   3.5 Cell lines and constructs ............................................................................................... 37
   3.6 Immunoblotting ............................................................................................................ 38
   3.7 Collagen gel assays ....................................................................................................... 38
4. Results ................................................................................................................................. 39
   4.1 Tight junction structures are present on the apical domain of UB cells ...................... 39
   4.2 Cldn3 and Cldn7 mRNA are expressed in the UB throughout branching morphogenesis .................................................................................................................. 40
   4.3 Cldn3 protein localizes to the apical domain of the UB, while Cldn7 is expressed primarily on the basolateral domain .................................................................................. 40
   4.4 Overexpressing Cldn3 or Cldn7 in mIMCD-3 cells leads to increased tubulogenesis in vitro ............................................................................................................................................ 41
5. Discussion ........................................................................................................................... 42

## Chapter I: Materials and methods

3. Materials and methods ......................................................................................................... 26
   3.1 Animal care ................................................................................................................... 26
   3.2 Electron microscopy ....................................................................................................... 26
   3.3 Whole mount in situ hybridization .............................................................................. 27
   3.4 Double immunofluorescence ...................................................................................... 27
   3.5 Cell lines and constructs ............................................................................................... 28
   3.6 Immunoblotting ............................................................................................................ 28
   3.7 Collagen gel assays ....................................................................................................... 28
4. Results ................................................................................................................................. 29
   4.1 Tight junction structures are present on the apical domain of UB cells ...................... 29
   4.2 Cldn3 and Cldn7 mRNA are expressed in the UB throughout branching morphogenesis .................................................................................................................. 30
   4.3 Cldn3 protein localizes to the apical domain of the UB, while Cldn7 is expressed primarily on the basolateral domain .................................................................................. 30
   4.4 Overexpressing Cldn3 or Cldn7 in mIMCD-3 cells leads to increased tubulogenesis in vitro ............................................................................................................................................ 31
5. Discussion ........................................................................................................................... 32

## Table

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
</tr>
<tr>
<td>35</td>
</tr>
<tr>
<td>35</td>
</tr>
<tr>
<td>36</td>
</tr>
<tr>
<td>36</td>
</tr>
<tr>
<td>37</td>
</tr>
<tr>
<td>38</td>
</tr>
<tr>
<td>38</td>
</tr>
<tr>
<td>39</td>
</tr>
<tr>
<td>39</td>
</tr>
<tr>
<td>40</td>
</tr>
<tr>
<td>40</td>
</tr>
<tr>
<td>41</td>
</tr>
<tr>
<td>42</td>
</tr>
<tr>
<td>47</td>
</tr>
<tr>
<td>48</td>
</tr>
<tr>
<td>49</td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>51</td>
</tr>
<tr>
<td>51</td>
</tr>
<tr>
<td>52</td>
</tr>
<tr>
<td>52</td>
</tr>
<tr>
<td>53</td>
</tr>
<tr>
<td>54</td>
</tr>
<tr>
<td>57</td>
</tr>
<tr>
<td>62</td>
</tr>
<tr>
<td>72</td>
</tr>
<tr>
<td>75</td>
</tr>
</tbody>
</table>
ABSTRACT

The claudin family of proteins is required for the formation of tight junctions between epithelial cells. Tight junctions form uninterrupted paracellular barriers on the apical surface linking adjacent epithelial cells. As a result, they promote cell-cell adhesion and regulate the paracellular flow of soluble ions. During kidney development, an epithelial outgrowth of the nephric duct called the ureteric bud (UB) emerges and invades the neighboring metanephric mesenchyme where it undergoes a series of branching events in a process known as branching morphogenesis. It has been shown that claudin-3 (Cldn3) and claudin-7 (Cldn7) transcripts are upregulated in the ureteric bud (UB) versus the metanephric mesenchyme (MM) during kidney development. We hypothesize that if Cldn3 and Cldn7 form tight junctions in the epithelial UB, they will determine the pattern of UB branching.

Using transmission electron microscopy, we have established that tight junctions are situated between epithelial cells of the UB that undergo branching. Whole-mount in situ hybridization assays established that Cldn3 and Cldn7 transcripts are expressed in the UB at embryonic day (E)10.5, 13.5 and 16.5. Double immunofluorescence experiments revealed that CLDN3 is localized to tight junctions at the apical domain of UB cells, while CLDN7 is predominately expressed on the basolateral membrane. To determine the functional role of these claudins, we took advantage of the mIMCD-3 cell culture model of tubulogenesis. The mIMCD-3 cell line is derived from the embryonic UB, and when placed in a type-I collagen matrix these cells undergo tubulogenesis and branching in a manner morphologically similar to the UB. Double immunofluorescence and Z-stacking
showed that mIMCD-3 cells express both CLDN3 and CLDN7 at the tight junction. Stable cell lines expressing either CLDN3 or CLDN7 fused at the N-terminus to the red fluorescent protein (RFP) mCherry were isolated and seeded in type-I collagen matrix. Quantification of branching following 48 hours in culture revealed that cell lines overexpressing either Cldn3 or Cldn7 underwent significantly increased branching compared to mCherry controls. This data suggests that both Cldn3 and Cldn7 may promote branching morphogenesis in the cells of the ureteric bud.

Explant cultures of murine embryonic kidneys provide an important ex vivo model to study the molecular and cellular processes that govern kidney development including branching morphogenesis and nephrogenesis. To study the role of claudin-3 and claudin-7 during branching morphogenesis, we developed a method to target the ureteric bud lineage of mouse embryonic kidneys by microinjecting plasmid DNA into the UB lumen followed by electroporation. An expression vector encoding the RFP protein mCherry was microinjected and electroporated into the UB lineage, and its expression persisted for up to 96 hours. This improved method allows both gain-of-function and loss-of-function experiments to be performed and is currently being applied to perturb claudin expression in the UB lineage.
**RÉSUMÉ**

La famille de protéines claudine est nécessaire pour la formation des jonctions serrées entre les cellules épithéliales. Les jonctions serrées forment des obstacles paracellulaires ininterrompus sur la surface apicale entre les cellules épithéliales adjacentes. En conséquence, ils favorisent l'adhérence cellule-cellule et régularisent le transport des ions solubles paracellulaire. Au cours du développement du rein, une excroissance épithéliale du canal nephric appelée urétérale bourgeon (UB) se dégage et envahit le mésenchyme métanephrique (MM) voisins où il subit une série de manifestations de branchement dans un processus connu sous le nom de la morphogenèse de ramification. Il a été démontré que claudin-3 et claudin-7 transcriptions sont augmentées dans l’UB par rapport au MM au cours du développement du rein. Nous faisons l'hypothèse que, si Cldn3 et Cldn7 forment les jonctions serrées dans l’épithéliales UB, ils détermineront le type de ramification UB.

En utilisant la microscopie électronique en transmission, nous avons établi que des jonctions serrées sont situées entre les cellules épithéliales de l'UB qui subissent ramification. L’hybridation *in situ* établi que claudin-3 et claudin-7 sont exprimés en UB à jour embryonnaire (E) 10,5, 13,5 et 16,5. Double immunofluorescence a révélée que la protéine Cldn3 est localisée à des jonctions serrées au domaine apical de l'UB, tandis que Cldn7 est surtout exprimé sur la membrane basolatérale. Pour déterminer le rôle fonctionnel de ces claudins, nous avons profité de la mIMCD-3 modèle de la culturecellulaire de la formation de tubules. Le mIMCD-3 lignée cellulaire est issue de l'embryon de UB, et lorsqu'il est placé dans un type-I matrice collagène, ces cellules
commence à former des tubules et une ramification d'une manière morphologiquement semblables à l'UB. Double immunofluorescence et des images Z-stack a montré que ces cellules expriment les deux Cldn3 et Cldn7 protéines à la jonction serrée. De lignées cellulaires stables exprimant Cldn3 ou Cldn7 en fusion à l'extrémité N-terminale de la protéine fluorescente rouge (RFP) mCherry ont été isolées et ensemencées sur du collagène de type-I. Une quantification de ramification après 48 heures dans la culture a révélé que les lignées cellulaires surexprimant la Claudin-3 a subi une augmentation significative par rapport à la ramification des contrôles. Ces données suggèrent que Cldn3 peuvent promouvoir une morphogenèse de ramification dans les cellules du bourgeois urétéral.

Les cultures des reins embryonnaires explants fourni un important modèle ex vivo pour étudier les processus cellulaires et moléculaires qui régissent les reins et la morphogenèse de ramification. Pour étudier les fonctions de claudin-3 et claudin-7 au cours de la morphogenèse de ramification, nous avons développé une méthode pour cibler les cellules du bourgeois urétéral en microinjectant les reins par l'ADN plasmidique dans le lumen d’UB suivie par électroporation. Un vecteur d'expression codant pour la protéine mCherry a été microinjecté et électroporé en UB, et son expression s'est maintenue pendant et jusqu'à 96 heures. Cette méthode permet à la fois de gain de fonction et la perte de fonction des expériences à réaliser et est actuellement appliquée pour perturber l’expression du claudine dans l’UB.
ACKNOWLEDGEMENTS

I am forever indebted to my supervisor, Dr. Indra Gupta, for giving me the opportunity to join her laboratory two and a half years ago. I thank her for supporting me through the difficulties of starting as an international student, and for having faith in my ability to overcome the obstacles I faced in the lab. I could not have asked for a more trusting supervisor, passionate teacher and knowledgeable mentor. Dr. Gupta always believed in me, and I hope I lived up to her expectations.

I would like to thank the members of my supervisory committee, Dr. Aimee Ryan and Dr. John Hanrahan, for letting me get the most out of my graduate education. They treated me like I was student in their own laboratory and were always available when I had questions or needed advice. I always looked forward to meeting with them, and I hope we cross paths again in the future.

My time in Dr. Gupta’s lab was an exceptional experience largely due to the friendship and camaraderie of my fellow lab members, both past and present. Mrs. Inga Murawski was always ready to go out of her way to help whenever I needed it. She never held back when things needed to get done, and a lot of my accomplishments in the lab are because I followed her example. She asked me to describe her as “smart and fun”, and that is the most accurate description I can think of. Thanks Inga, and I am sure those scissors will turn up somewhere. Mr. Dave Myburgh and Mr. Bruno St. Jacques were a pleasure to work with and I have much to thank them for. Dave for his patience and expertise in all things molecular, and Bruno for getting the stable cell lines off the ground. I hope they have both found their calling, and I wish them the best. Summers in
the lab would not have been the same without the cheer and good humor of Ms. Rita Maina. Thanks for putting up with us Rita.

I have everyone to thank at the institute for this work. The Ryan lab: Michelle, Erminia and Annie, were always willing to entertain my questions and random thoughts. They were always willing to lend a protocol or reagent…many reagents. In the Goodyer I have everyone to thank. Lee Lee, Diana, Reyhan, Michelle and Murielle made me feel at home (sometimes a little too at home) and if was not for their kindness and understanding, much of the work in this thesis could not have been possible. I would also like to acknowledge Dr. Loydie Majewska and her student Ms. Didem Sakaraya for helping me with the last few experiments, making this thesis complete. Last but not least I would like to recognize everyone at the institute for all the good times. If I had to do this again I would not hesitate to come back to the MCH-RI. No work environment is perfect, but I think we were pretty close. Beyond the institute I would like to extend my deepest appreciation to Mr. Daniel Houle and Dr. Pierre LeSimple. Daniel was generous, kind and extremely helpful and I count myself lucky to have him as not just a collaborator but a friend as well. Pierre was an invaluable resource, and his advice helped shape the focus of this thesis. I am happy to consider him a friend first, and a colleague second. I want to thank all my friends, in Montreal and beyond, for believing in me and never doubting that I could get this far.

Most importantly, none of my academic successes could have been possible without the support of my parents, Michel and Antoinette, my brother Jade, and sister
Rouba. They are the source of my strength and confidence, and I would not have made it this far without their faith. I am eternally grateful.
CONTRIBUTION OF CO-AUTHORS

This work was written in accordance with the guidelines provided by the Faculty of Graduate and Post-Doctoral Studies, McGill University. All mouse studies were performed in accordance with the rules and regulations of the Canadian Council of Animal Care. The candidate was responsible for the planning and execution of all experiments presented. Data analysis, writing of drafts and editing was performed in conjunction with Dr. Indra Gupta. Mr. Bruno St. Jacques assisted in generating stable cell lines. Ms. Melissa Yu assisted in double immunofluorescence experiments as part of her independent studies project. Mr. Daniel Houle provided training in the use of the Leitz® microscope and micromanipulators for the microinjection and electroporation experiments. Dr. Indra Gupta supervised the work which was carried out in her laboratory.
LIST OF FIGURES

Figure 1.1  The nephron is divided into several segments
Figure 1.2  Claudins are essential components of tight junctions
Figure 1.3  Kidney development and renal branching morphogenesis in the mouse
Figure 1.4  Nephron formation in metanephric kidney development is a sequential process
Figure 1.5  The expression of claudins in the adult kidney varies in each nephron segment
Figure 2.1  Tight junction structures are present on the apical surface of ureteric bud cells
Figure 2.2  Claudin-3 and claudin-7 transcripts are expressed in the ureteric bud (UB) during branching morphogenesis
Figure 2.3  The subcellular localization of claudin-3 and claudin-7 differs in the ureteric bud lineage
Figure 2.4  Cldn3 and Cldn7 proteins colocalize with ZO-1 in mIMCD-3 cells
Figure 2.5  Ectopic expression of claudin-3 or claudin-7 in mIMCD-3 cells leads to an increase in tubulogenesis
Figure 3.1  Early stages of mouse metanephric kidney development used in microinjection and electroporation experiments
Figure 3.2  The method of microinjection and electroporation in embryonic kidney explants
Figure 3.3  Tissue-specific expression is achieved by targeted microinjection and electroporation of DNA constructs into different cell populations within mouse embryonic kidney explants
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>μs</td>
<td>Microsecond</td>
</tr>
<tr>
<td>Cdh1</td>
<td>E-Cadherin</td>
</tr>
<tr>
<td>ClDN3/7</td>
<td>Claudin-3/-7</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>EpCAM</td>
<td>Epithelial cell Adhesion Molecule</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FHHNC</td>
<td>Familial Hypercalciuric Hypomagnesemia with Nephrocalcinosis</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial-Derived Neurotrophic Factor</td>
</tr>
<tr>
<td>Gfrα1</td>
<td>Gdnf family receptor α1</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>HEK 293</td>
<td>Human Embryonic Kidney 293 cells</td>
</tr>
<tr>
<td>JAM-A</td>
<td>Junctional Adhesion Molecule A</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madine-Darby Canine Kidney cells</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal to Epithelial Transition</td>
</tr>
<tr>
<td>MM</td>
<td>Metanephric Mesenchyme</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Matrix Metalloproteinase-2</td>
</tr>
<tr>
<td>Ms</td>
<td>Millisecond</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>Membrane Type – Matrix Metalloproteinase</td>
</tr>
<tr>
<td>MUPP1</td>
<td>Multi-PDZ Domain Protein 1</td>
</tr>
<tr>
<td>NISCH</td>
<td>Neonatal Ichthyosis and Sclerosing Cholangitis</td>
</tr>
<tr>
<td>Pax2</td>
<td>Paired Box Gene</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PKD1</td>
<td>Polycystin-1</td>
</tr>
<tr>
<td>P/S</td>
<td>Penicillin and Streptomycin</td>
</tr>
<tr>
<td>Ret</td>
<td>Rearranged during Transfection</td>
</tr>
<tr>
<td>RFP</td>
<td>Red Fluorescent Protein</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscope</td>
</tr>
<tr>
<td>TXR</td>
<td>Texas Red</td>
</tr>
<tr>
<td>UB</td>
<td>Ureteric Bud</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>WNK4</td>
<td>Protein kinase, lysine-deficient 4</td>
</tr>
</tbody>
</table>
ZO-1/-2/-3  Zona Occludens-1/-2/-3
CHAPTER I

Introduction
1. Overview of kidney function

The kidney is a highly organized and intricate organ that maintains the volume and composition of body fluids in the context of variable salt and water intake (1). The complex functions of the kidney have evolved from an evolutionary need of land-dwelling organisms to continuously adapt to an environment in which access to salt and water is severely restricted (2). The kidney regulates body fluid osmolality and volume, electrolyte and acid-base balance, and the excretion of metabolic substances and foreign chemicals. In addition, it produces and secretes hormones (1). The functional unit of the kidney, known as the nephron, is composed of a glomerulus where blood is filtered, and a long convoluted epithelial tubule where inorganic ions, amino acids and carbohydrates are re-absorbed or secreted into the filtrate (Fig. 1.1). The renal tubule consists of the proximal tubule, followed by the loop of Henle and the distal tubule. Each nephron segment is composed of highly specialized epithelial cells that have evolved to perform specific transport functions. The filtrate is modified along the nephron and then drains into the collecting duct system where it is concentrated further prior to its passage to the bladder via the ureter (1).

Solutes within the filtrate pass across the renal tubular epithelium either by transcellular or paracellular transport. Transcellular transport is characterized by the passage of solutes through the cytoplasm of the renal tubular cell as a result of specialized proteins located on both the apical and basolateral membranes. In contrast, paracellular transport is characterized by the passage of solutes through the space separating adjacent epithelial cells. Paracellular transport occurs through
Figure 1.1 - The nephron is divided into several segments. In humans, each kidney contains anywhere from 300,000 to 1 million nephrons. The nephron begins with the glomerulus which contains glomerular capillaries and Bowman’s capsule. The glomerular capillaries are fed by the afferent arteriole (aa) and drained by the efferent arteriole (ea). Blood is filtered across the glomerular basement membrane and the filtrate is collected in Bowman’s capsule which then leads into the proximal tubule. The proximal tubule is initially convoluted and then straightens and connects to the Loop of Henle. The Loop of Henle is divided into the thin ascending limb and the thick ascending limb. The distal tubule begins after the thick ascending limb and is connected to the collecting duct. Each segment of the nephron consists of cells that are adapted to perform specific reabsorption and transport functions.
Figure 1.2 - Claudins are essential components of tight junctions. (A) Tight junctions form a paracellular seal at the apical membrane of adjacent epithelial cells. (B) Tight junction proteins span the paracellular space to interact with proteins on the opposing cell membrane. As a result, the paracellular distance between adjacent epithelial cells is minimized, forming a paracellular barrier perforated by aqueous pores. (C) Claudins are the major transmembrane proteins present in tight junctions. They contain four transmembrane domains and two extracellular loops that mediate homo- and heterotypic interactions with claudins on neighbouring cells. The first extracellular loop contains charged residues that line the aqueous pores to regulate the electrostatic transport of soluble ions. Both the N- and C-terminus are located intracellularly. The C-terminus contains a PDZ-binding motif that allows claudins to bind to tight junction-associated scaffolding proteins, linking the tight junction to the actin cytoskeleton.
structures called tight junctions that separate the luminal and basolateral compartments of all epithelial tissues. Tight junctions form aqueous pores that are size and charge selective and restrict the passive transport of soluble ions in a manner that is specific to each nephron segment (1) (Fig. 1.2A,B).

2. **The kidney: three stages of development**

2.1 **The pronephros and the mesonephros**

During the embryonic process known as gastrulation, three germ layers are formed and then separate: the ectoderm, the endoderm and the mesoderm. The mesoderm, found in between the ectoderm and endoderm, further differentiates into the paraxial, the lateral plate and the intermediate mesoderm. It is the intermediate mesoderm that gives rise to the urogenital system (2). During mammalian kidney development, three serial kidneys are formed: the pro-, the meso- and the metanephros. All three kidneys originate from two tissues that are derived from the intermediate mesoderm: the nephric duct and the nephric cord. The nephric duct is an epithelial tubule that extends caudally alongside a mesenchymal tissue known as the nephric cord (3). In the mouse, the pronephros develops at embryonic day (E)8 at the level of the forelimbs. At this point, the anterior portion of the nephric duct induces the adjacent mesenchyme of the nephric cord to undergo mesenchymal-to-epithelial transition (MET), forming tubules that open into the nephric duct. Although the pronephros serves as a
functional excretory organ in lower vertebrates such as amphibians and fish, in mammals, it is a transitory tissue. The pronephros degenerates by E9, leaving the more caudal regions of both the nephric duct and the nephric cord to form the mesonephros at E9.5. This second primitive kidney forms near the mid-portion of the embryo, and also arises as a result of the induction of tubulogenesis in the adjacent mesenchyme. While the mesonephros is also transitory, the mesonephric tubules do produce a filtrate during embryogenesis (4).

2.2  The metanephros

Metanephric kidney development begins at E10.5 in the mouse after both the nephric duct and nephric cord have elongated caudally to the level of the hindlimbs (Fig. 1.3) (3). At this point, a structure called the ureteric bud (UB) emerges out of the nephric duct and invades the neighboring undifferentiated mesenchymal cells contained within the caudal end of the nephric cord termed the metanephric mesenchyme (MM). As a result of reciprocal signaling and induction between the MM and the UB, the UB proceeds to elongate and divide in a process known as branching morphogenesis. The UB is a highly dynamic structure, rapidly expanding within the MM through a series of repeated bifurcations at the UB tips (5). This process leads to the formation of a UB tree, which eventually becomes the collecting duct system of the functional adult kidney. In the mouse, branching morphogenesis involves about 10-11 rounds of bifurcations producing approximately 1600 branches within each developing kidney (6).
Figure 1.3 - Kidney development and renal branching morphogenesis in the mouse.
(A) The pronephros (pn) forms at embryonic day (E)8.0 in the mouse as a result of reciprocal induction between the nephric duct and neighboring nephrogenic cord. Caudal elongation of the nephric duct leads to the formation of the mesonephros (mn) at approximately E9.5 from a similar inductive process between the nephric duct and the adjacent mesenchymal cells. By E10.5, both the pronephros and the mesonephros have degenerated, and the ureteric bud (UB) has emerged out of the nephric duct to invade the metanephric mesenchyme (MM) at the level of the hindlimbs. (B) Metanephric kidney development begins at E10.5 when the ureteric bud first enters the MM. As a result of reciprocal signaling and induction by the MM, the UB proceeds to elongate and divide in a process known as branching morphogenesis. The first branching event within the MM becomes evident at E11.5, splitting the UB into a T-like structure which contains two groups of cells: tip-cells located within the ampullae at the leading edges of the branches (red), and trunk cells that comprise the UB stalks. Branching morphogenesis is driven by a surge of proliferation in the ampullae concomitant with transient UB trunk elongation. The UB undergoes multiple rounds of branching as the metanephric kidney grows, inducing nephron formation in undifferentiated mesenchymal cells surrounding each UB tip.
As the ureteric bud branches, it induces nephron formation in undifferentiated mesenchymal cells surrounding each UB tip (2). With each round of dichotomous branching, the number of UB tips available for nephrogenesis doubles such that nephron formation expands exponentially during kidney organogenesis (5). The efficiency of branching morphogenesis is therefore directly linked to final nephron number in the adult kidney. In fact, a 2% decrease in UB branching during development can lead to a 50% decrease in nephron number (7). The formation of nephrons is a sequential process driven by MET that occurs as a result of reciprocal signaling events between the UB and the MM. Initially, undifferentiated mesenchymal cells are induced to condense around the UB tip where they polarize to form the renal vesicle (Fig. 1.4). The cells of the renal vesicle go on to form a comma-shaped body, then an S-shaped body. The distal end of the S-shaped body fuses with the ureteric bud to form a continuous lumen, whereas the proximal end is invaded by endothelial cells that induce glomerulogenesis (2).

3. The genetics of branching morphogenesis

The ureteric bud consists of two cell types: tip cells that are located at the leading edges of the branches, and trunk cells that are located in the UB stalks (8, 9). Branching morphogenesis is thought to be driven by a surge of proliferation at the UB tips concomitant with transient UB trunk elongation. It has recently been shown that tip cells give rise to much of the trunk epithelium, as a number of tip
Figure 1.4 - Nephron formation during metanephric kidney development is a sequential process. (A) At each ureteric bud (UB) tip, mesenchymal cells are induced to aggregate and condense. The condensed cells of the metanephric mesenchyme (MM) undergo mesenchymal-to-epithelial transition (MET) and become polarized to form a renal vesicle. (B) The newly polarized cells form a comma-shaped body with a luminal space that fuses with that of the UB. (C) The epithelialized cells of the MM continue to proliferate and rearrange to form an S-shaped body. At this stage, endothelial cells (red) invade the proximal cleft of the S-shaped body to begin glomerulogenesis. (D) The fully developed nephron contains a vascularized glomerulus that leads to a tubular segment that ultimately connects to the collecting duct at the distal end.
daughter cells are left behind in the trunks during the early stages of branching morphogenesis (8).

3.1 The Gdnf/Ret/Gfrα1 pathway and Pax2

Various classes of genes, including transcription factors, soluble growth factors, extracellular matrix and cell-adhesion molecules, have all been implicated in renal branching morphogenesis (10). One pathway critical to initial UB emergence and expansion involves signaling through the receptor tyrosine kinase, Ret. The MM-secreted growth factor glial-derived neurotrophic factor (GDNF) serves as the main ligand for Ret and its co-receptor GDNF family receptor (Gfr) α1. Ret and Gfrα1 are exclusively expressed in UB tip cells, and following GDNF binding, these cells are induced to proliferate in the direction of the GDNF-secreting MM cells. GDNF/Gfrα1/Ret signaling in the UB is crucial for kidney development, as the absence of any of these genes will lead to defective branching morphogenesis (11-13). Besides its role in Ret signaling, GDNF is a direct transcriptional target for other key proteins in kidney development such as Pax2, a transcription factor expressed in both the UB and the MM (3). Mice lacking Pax2 fail to develop a metanephros and genital tract (14). Pax2 has further been shown to promote the expression of other genes crucial for kidney development such as the Wnt family of secreted proteins, as well as E-cadherin (Cdh1), an important modulator of cell-adhesion (3, 15, 16).
3.2  Adhesion molecules

Given the epithelial nature of the ureteric bud, proteins involved in cell adhesion and cell polarity are likely to be involved in patterning the UB. In order for the UB to undergo branching morphogenesis, epithelial cell migration as a result of both cell-cell and cell-matrix interactions, is important (17). For example, αβ-integrins, cell surface receptors that mediate cell binding to the extracellular matrix, have been shown to be important for UB branching. Mice deficient in α3-integrin have fewer collecting ducts suggesting decreased branching during development, while those lacking both α3- and α6-integrin do not develop ureters (18, 19). A deficiency in α8-integrin also results in severe renal malformations from defective branching morphogenesis (20). Polycystin-1 (PKD1) is a membrane-bound protein that has been shown to be important in branching morphogenesis due to its role in cell-adhesion (21). Mutations in PKD1 are associated with autosomal dominant polycystic kidney disease, a common renal disease characterized by the formation of macroscopic cysts that arise from the renal tubular epithelium (22). During kidney development, PKD1 is expressed in the UB in focal adhesions and adherens junctions (23, 24). Inhibition of PKD1 activity in embryonic kidney explants results in disrupted branching morphogenesis such that there is a reduction in the number of branch tips, in addition to a decrease in overall UB length, volume and area (21).

In a study investigating the mechanics of UB branching, it was suggested that branching is mediated by the formation of outpouches at the tips of the
ureteric bud. These outpouches consist of wedge-shaped epithelial cells with abundant levels of actin, myosin-2 and ezrin found on the apical domain of the UB epithelium. It was proposed that this high concentration of actin and myosin would promote cytoskeletal tightening along the apical domain, creating a “purse-string” effect. Apical tightening would induce an increase in the surface area of the basolateral membrane and bulging at the basolateral surface of the epithelium. This process would in turn drive the protrusion of the epithelium into the extracellular matrix and possibly promote the exocytotic release of morphogens important for branching morphogenesis. The high proliferation rates at UB tips would then drive the elongation of the new bud and by extension the growth of the UB network as a whole (25). The authors further suggested that this phenomenon could be due to the upregulation of tight junction proteins in the apical domain of the UB epithelium. Indeed, in one microarray analysis comparing the expression profile of UB tip cells with those of the trunk, as well as the MM, it was found that the tight junction protein claudin-3 (Cldn3) was upregulated in UB tip cells along with another claudin family member, claudin-7 (Cldn7) (26).

4. **Tight junctions and claudins**

4.1 **Tight junctions are composed of various proteins**

Tight junctions are the most apical of cell-cell junctions located between epithelial cells and form a continuous paracellular barrier. As a result, they
promote cell-cell adhesion and regulate passive transepithelial ion transport through the paracellular space (27, 28). Tight junctions consist of transmembrane proteins that include the claudin family of proteins, occludin, tricellulin, junctional adhesion molecules (JAM), scaffolding proteins such as ZO-1/-2/-3, and various cytosolic transcription factors and kinases (29). Electron microscopy is commonly used to identify tight junctions, and depending on how a particular tissue is processed and then imaged, the morphology of the tight junction can appear quite different. When transverse, ultra-thin sections of an epithelial tissue are viewed under a transmission electron microscope, tight junctions appear as a fusion of opposing cell membranes (30). In contrast, using freeze fracture electron microscopy which permits the visualization of the surface of cells, tight junctions appear as continuous, intramembranous strands (31).

4.2 Claudins are essential components of tight junctions

The barrier and cell adhesion functions of tight junctions are mediated by transmembrane proteins such as claudins, occludin and JAMs. These proteins contain extracellular domains that occupy the intercellular space and interact with proteins on the opposing cell membranes as well as solutes that permeate through the paracellular cleft (32). Of all these membrane-bound proteins, the claudin family is the only one whose expression is sufficient for the formation of tight junction strands (27, 33). Mice lacking occludin do not display altered tight junction morphology, and its downregulation in cultured cell lines does not affect paracellular ion permeability (34, 35). In contrast to claudins, when occludin or
JAM-A is overexpressed in fibroblasts, tight junctions are not established (36, 37).

4.3 **Claudin structure and function**

In mammals, the claudin family of proteins is composed of 24 members that range in size from 20 to 27kDa (32). Claudins contain four transmembrane domains, two extracellular loops, and an N- and C-terminus that are both located intracellularly (30) (Fig. 1.2C). The extracellular loops mediate barrier and adhesive functions through the formation of homo- or heterotypic interactions with claudins in opposing cell membranes. The first extracellular loop consists of approximately 50 amino acids that include two conserved cysteines which promote barrier function, and several charged residues which determine the electrostatic selectivity of the paracellular pore (30, 38, 39). The second extracellular loop (16-31 amino acids) has not been well studied, but recent evidence suggests it may be required for interaction with claudins on opposing membranes (32, 40). The C-terminal tail displays the greatest amount of variation among claudin species in terms of length, ranging from 22-55 amino acids (30). The C-terminal tail contains a PDZ-binding motif (except for claudin-12) that permits claudins to bind to tight junction associated scaffolding proteins such as ZO-1/-2/-3 and MUPP1 (30). These interactions link membrane-bound claudins to the actin cytoskeleton, possibly stabilizing them at the tight junction to maintain the integrity of the epithelial barrier (41, 32). Claudin localization and function has also been shown to be affected by phosphorylation of the C-terminal tail by
serine/threonine and tyrosine kinases (e.g. WNK4, PKC) (42, 43). For example, mutating the phosphorylation site of claudin-1 \((\text{Cldn1})\) was shown to eliminate its ability to mediate the normal, leftward direction of heart-looping during chick embryogenesis (43). Furthermore, phosphorylation can either increase the barrier function of tight junctions or decrease it making the epithelial barrier more permeable (44, 45).

5. **Claudins in the kidney**

The expression pattern of over 12 claudin species has been established in the mouse adult kidney (32) (Fig. 1.5). Most localize to tight junctions in the epithelial cells of the renal tubules such that each nephron segment expresses a distinct combination of claudins. The specific combination of claudins is thought to shape the paracellular ion transport characteristics of each segment (32, 30). For example claudin-8, which functions mainly as a cation barrier, is expressed primarily in the distal portion of the nephron where high ion concentrations in the filtrate must be maintained, and the flow of ions across the epithelial barrier is limited (46, 47). In contrast, claudin-2 forms a highly permeable cation pore, and is expressed in the proximal tubule where it contributes to the significant amount of sodium reabsorption that occurs in this segment (48, 49). Claudins can also be found in structures besides the tubular epithelium. Claudin-5 is expressed exclusively in endothelial cells, including renal blood vessels while claudin-6 has been shown to localize to tight junction-like structures in podocytes (50, 51).
Figure 1.5 - The expression of claudins in the adult kidney varies in each segment of the nephron. The expression of selected claudins is summarized from several studies (32). Claudin-5 is expressed in the endothelial cells of the glomerular capillaries and is therefore not expressed in the epithelial cells of the nephron. Claudin-1, -4, -6, -9, -10 and -11 are also expressed along the nephron but are not shown. The expression pattern of over 12 claudins has been characterized in the kidney (32). The specific expression of claudins reflects the unique transport functions of each nephron segment. Cldn-12, -18, -20, -21, -22, -23 and -24 expression has not yet been charaterized in the kidney.
6. **Claudins and human disease**

The role of claudins in human disease has been extensively investigated over the past decade. Familial hypercalcuiric hypomagnesemia with nephrocalcinosis (FHHNC) is an autosomal recessive disease characterized by severe magnesium and calcium wasting in the urine leading to calcium deposition in the renal tubules (52). In 1999, FHHNC was shown to be associated with loss-of-function mutations in claudin-16 (Cldn16) (53). Cldn16 is expressed in the thick ascending limb of Henle, the nephron segment responsible for the majority of Mg$^{2+}$ reabsorption in the kidney (1). To date, more than 30 loss-of-function mutations in Cldn16 have been reported in families with FHHNC (32). In 2006, mutations in the claudin-19 (Cldn19) locus were found in families with FHHNC who also had severe ocular abnormalities (54). Cldn19 protein colocalizes with CLDN16 in the thick ascending limb of Henle, and is also highly expressed in the retina (54). It appears that CLDN16 and CLDN19 form heteromers in the cells of the thick ascending limb of Henle, and together they promote magnesium reabsorption (55). How loss-of-function mutations in Cldn19 lead to ocular abnormalities has not yet been described.

Claudins have also been implicated in nonrenal diseases. Mutations in Cldn1 were found in patients with neonatal ichthyosis and sclerosing cholangitis (NISCH), a syndrome characterized by scaly skin, sparse hair and inflammation of the bile ducts. Loss of CLDN1 from tight junctions may lead to increased permeability of the skin and bile ducts, leading to dehydration and biliary leakage.
(56). Human hereditary deafness has been attributed to loss-of-function mutations in claudin-14 (Cldn14), which is normally expressed in the inner ear. Cldn14 is expressed in the organ of Corti where it maintains the high cation gradient between the endolymph and perilymph. Loss of this gradient prevents the depolarization of sensory hair cells and inhibits signal transduction leading to hearing loss (57, 58). In addition, claudin expression has been shown to be either up- or downregulated in a variety of cancers, potentially modulating processes such as epithelial-to-mesenchymal transition and metastasis (30, 59).

7. The function of Claudin-3 and Claudin-7

7.1 Claudin-3

In the adult mouse kidney, Cldn3 (23kDa) is expressed on the apical surfaces of epithelial cells in the distal nephron and collecting ducts, where ion flow is tightly regulated (50). Overexpressing Cldn3 in an epithelial ovarian cancer cell line reduced tight junction-mediated ion permeability, suggesting that one of its functions may be to “tighten” paracellular channels making them less permeable to ions and other larger molecules (45). In addition to its role in paracellular transport, Cldn3 also plays a role in cell motility and tumor formation. Claudin-3 is one of the most highly expressed genes in ovarian tumors and it is expressed in nearly 90% of ovarian cancers. This overexpression has been associated with increased cellular motility and survival suggesting a role for Cldn3 in promoting metastasis and malignancy (60). Indeed, downregulation of
*Cldn3* in ovarian tumors resulted in a significant reduction in cell proliferation concomitant with decreased tumor growth (61).

### 7.2 Claudin-7

*Cldn7* (22kDa) is also expressed in the distal nephron and collecting ducts as well as the descending limb of Henle, where ion exchange through the paracellular pathway is more prominent (47). Overexpressing *Cldn7* alone in the porcine epithelial cell line LLC-PK1 resulted in decreased paracellular permeability of chloride ions, and an increase in sodium transport (62). When studied in MDCK type II cells however, *Cldn7* was found to act as a chloride channel (49). Indeed, when studying the ion permeability functions of claudins, results have varied based on the cell line used (30).

An unusual feature of *Cldn7* protein is that in epithelial tissues it localizes primarily to the basolateral membrane, away from the conventional apical location of tight junctions (47). The function of CLDN7 on the basolateral membrane is not known, but recent evidence shows that it binds directly to the adhesion molecule EpCAM (32, 63). The EpCAM-CLDN7 complex was found to promote cell proliferation and tumorigenicity when both proteins were expressed in the human kidney cell line HEK293 (64).

Both *Cldn3* and *Cldn7* appear to play important and separate roles in the terminally differentiated epithelial cells of the adult kidney. However, their function during UB branching morphogenesis has not been characterized. Given the importance of cell-adhesion proteins in regulating branching morphogenesis,
and the presence of apically driven mechanical forces that could promote UB branching at the tips, tight junction proteins may play a key role (18-20). Microarray analysis has shown that Cldn3 and Cldn7 are upregulated in the UB during embryonic kidney development (26). When studied in cancer cell models, both claudins are associated with an increase in cell proliferation and migration, which are processes that are also important in branching morphogenesis (60, 61, 64). If Cldn3 and Cldn7 are expressed in the epithelial ureteric bud, they may therefore function as important modulators of UB branching during kidney development.

8. **Summary of objectives**

The objectives of my research are to characterize the expression patterns of Cldn3 and Cldn7 during metanephric kidney development and to describe their function during renal branching morphogenesis. The expression of both claudin species will be assayed at the transcript and protein levels at critical stages of metanephric kidney development. The functional role of claudins in the branching UB will be investigated through gain-of-function assays in a UB derived kidney cell line that undergoes branching *in vitro*. We hypothesize that both claudin species will be highly expressed in the ureteric bud epithelium, where they will function to promote the process of branching morphogenesis.

This thesis contains two manuscript-based chapters. The first chapter describes the expression patterns of Cldn3 and Cldn7 at critical stages of
metanephric kidney development, and examines the functional role of *Cldn3* and *Cldn7* when overexpressed in a UB-derived epithelial cell line. The second chapter describes the development of a model to study genetic perturbations in the UB lineage in embryonic kidneys explants. Rodent embryonic kidneys can be dissected and cultured as explants such that branching morphogenesis and nephrogenesis can be observed *ex vivo*. We describe a new method in which DNA constructs can be targeted to the UB lineage in cultured embryonic kidney explants using microinjection and electroporation. The development of this model is critical to our future goal of perturbing claudin expression in the UB lineage of the developing mouse kidney.
CHAPTER II

The tight junction proteins Cldn3 and Cldn7 are expressed in the ureteric bud and promote tubulogenesis in vitro

Nicholas Haddad, Bruno St. Jacques, Melissa Yu, Indra R. Gupta

Manuscript to be submitted
1. **Abstract**

The claudin family of proteins is required for the formation of tight junctions (TJs) between epithelial cells. It has been shown that *Cldn3* and *Cldn7* are upregulated in the ureteric bud (UB) versus the metanephric mesenchyme (MM) during kidney development. We hypothesize that if *Cldn3* and *Cldn7* form TJs in the epithelial UB, they will determine the pattern of UB branching. Using transmission electron microscopy, we have established that TJs are situated between epithelial cells of the UB that undergo branching. Whole-mount *in situ* hybridization has shown that *Cldn3* and *Cldn7* transcripts are expressed in the UB at E10.5, E13.5 and E16.5. Double immunofluorescence experiments revealed that CLDN3 localizes to tight junctions at the apical domain of UB cells, while CLDN7 is predominately expressed on the basolateral membrane. To determine the functional role of these claudins, we used the mIMCD-3 cell line which is derived from the embryonic UB. These cells undergo tubulogenesis when placed in type-I collagen matrix in a manner morphologically similar to the UB. Double immunofluorescence and Z-stacking showed that these cells express both *Cldn3* and *Cldn7* at the tight junction. Stable cell lines expressing CLDN3 or CLDN7 fused at the N-terminus to the red fluorescent protein (RFP) mCherry were isolated and seeded in collagen matrix. Quantification of branching following 48 hours in culture revealed that cell lines overexpressing either *Cldn3* or *Cldn7* underwent significantly increased branching compared to mCherry controls. *Cldn3* and *Cldn7* may therefore promote branching morphogenesis in the cells of the ureteric bud.
2. *Introduction*

The claudin family of proteins, of which there are 24 members in mammals, are required for the formation of tight junctions between epithelial cells (30, 33). Tight junctions form uninterrupted paracellular barriers on the apical surface linking adjacent epithelial cells. They promote cell-cell adhesion and regulating passive transepithelial ion transport through the paracellular pathway (28, 27). Tight junctions consist of transmembrane proteins that include the claudin family of proteins, occludin, tricellulin, junctional adhesion molecules (JAM), scaffolding proteins such as ZO-1/-2/-3, and various cytosolic transcription factors and kinases (29). Of all these membrane bound proteins, the claudin family is the only one whose expression is sufficient for the formation of tight junction strands (33).

Claudins mediate both paracellular ion transport and cell adhesion (27, 30). They have specific expression patterns that determine the different permeability properties of epithelial tissues. In the adult kidney for example, over 12 different claudins are expressed along the length of the nephron, and each one contributes to the specific permeability properties of each nephron segment (32). With regards to cell adhesion, it has been shown that expressing claudin-1, -2 or -3 in L-fibroblasts will lead to *de novo* formation of tight junctions and epithelial strands of cells, suggesting claudin expression is a key determinant in the development of an epithelial tissue (27). During embryogenesis, claudins are crucial for the development of various structures by maintaining tissue integrity and by separating fluids of different compositions. Claudin-4 and claudin-6 have been found to be critical for blastocyst formation during early
embryogenesis, while claudin-1 is important for epithelial stratification during skin development (66, 67).

Mammalian kidney development involves the formation of three serial kidneys: the pro-, meso- and metanephros. The metanephros forms the definitive adult kidney. All three kidneys originate from two primordial tissues that are derived from the intermediate mesoderm: the nephric duct and the nephric cord (2, 3). Metanephric kidney development begins at embryonic day (E)10.5 after both the nephric duct and nephric cord have elongated caudally to the level of the hindlimbs. At this point, an epithelial structure known as the ureteric bud (UB) emerges out of the nephric duct and invades the neighboring undifferentiated mesenchymal cells termed the metanephric mesenchyme (MM) (2). As a result of reciprocal signaling and induction by the MM, the UB proceeds to elongate and divide through repeated bifurcations in a process known as branching morphogenesis, eventually forming the adult collecting duct system (2, 8). Branching morphogenesis in the mouse involves 10-11 rounds of bifurcations which produce approximately 1600 branches within each developing kidney (6). It is thought that branching morphogenesis is largely driven by a surge of proliferation at the UB tips followed by bifurcation, concomitant with transient UB trunk elongation (8). To date, many transcription factors and growth factors have been reported to participate in branching morphogenesis (3). Given the epithelial nature of the ureteric bud, proteins involved in cell-cell adhesion and cell polarity are also likely to be involved in this process. Indeed, in one microarray analysis comparing the expression profile of the cells of UB tips with those of the MM, it was found that claudin-3 (Cldn3) was upregulated in the UB tip cells along with another tight junction protein, claudin-7 (Cldn7) (26).
\textit{Cldn3} (23kDa) is expressed on the apical surfaces of cells in the distal nephron and collecting ducts, where ion flow is highly regulated (50). Overexpressing \textit{Cldn3} in an epithelial ovarian cancer cell line reduced tight junction-mediated ion permeability, suggesting that one of its functions may be to “tighten” paracellular channels making them less permeable to ions and other larger molecules (45). \textit{Cldn7} (22kDa) is also expressed in the distal nephron and collecting ducts as well as the descending limb of Henle where ion exchange is more essential (47). Overexpressing \textit{Cldn7} in the porcine epithelial cell line LLC-PK1 resulted in decreased paracellular permeability of chloride ions, and an increase in sodium transport (62). When studied in MDCK type II cells however, \textit{Cldn7} was found to act as a chloride channel (49). Indeed, when studying the ion permeability functions of claudins, results have varied based on the cell line used (30). Both \textit{Cldn3} and \textit{Cldn7} appear to play important and separate physiological roles in the terminally differentiated epithelial cells of the adult kidney. However, their function during ureteric bud branching morphogenesis has not been characterized.

Here we show that \textit{Cldn3} and \textit{Cldn7} are expressed in the ureteric bud during critical stages of branching morphogenesis. Using a cell line derived from renal collecting ducts, we generated stable clones overexpressing either \textit{Cldn3} or \textit{Cldn7} and found that both claudins stimulate branching morphogenesis \textit{in vitro}. 
3. Materials and methods

3.1 Animal care

Timed-pregnant CD1 mice (Charles River Laboratories, St. Constant, QC, Canada) were sacrificed at embryonic day (E) 10.5, 12.5, 13.5, or 16.5 to retrieve embryonic tissue as previously described (68). Whole embryos or metanephric kidneys were dissected in phosphate buffered saline (PBS) using an M5A stereomicroscope (Wild Leitz®, Willowdale, ON, Canada. The mouse studies were performed in accordance with the rules and regulations of the Canadian Council of Animal Care.

3.2 Electron microscopy

Embryonic kidneys aged E12.5 were dissected and fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for 24 hours, then washed in cacodylate buffer, and then immersed in 1% osmium tetroxide for 2 hours. Samples were then dehydrated in acetone and embedded in EPON®812. Sections were taken at 0.5μm thickness, stained with 1% toluidine blue, and then imaged using light microscopy. For transmission electron microscopy (TEM), ultrathin sections were collected on 200 mesh copper grids (Electron Microscopy Sciences, Hatfield, PA, USA) and stained with uranyl acetate and lead citrate. Images were obtained using a FEI Tecnai 12 transmission electron microscope (FEI/Philips Electron Optics, Eindhoven, The Netherlands) that was attached to a Gatan Bioscan CCD Camera Model 792.
3.3 Whole mount in situ hybridization

In situ hybridization was performed on partially eviscerated whole embryos at E10.5 and on dissected kidneys at E13.5 and E16.5. All samples were fixed overnight in 4% PFA in PBS at 4°C. Coding sequences for mouse Cldn3, Cldn7, and Ret were subcloned in the PCRII-Topo vector (Invitrogen, Carlsbad, CA, USA) and then linearized using XhoI, XbaI, and BamHI restriction enzymes respectively. DIG-labeled UTP probes were generated in vitro using the RNA polymerases SP6 for Cldn3 and Cldn7, and T3 for Ret. Anti-DIG antibody conjugated to alkaline-phosphatase was used to detect target RNA sequences. Treated samples were developed using NBT/BCIP substrate in NTMT. Cryosections of treated embryonic kidneys aged E13.5 and E16.5 were obtained at a thickness of 20μm and counterstained with eosin.

3.4 Double immunofluorescence

Whole embryos were dissected at E13.5 and E16.5 and fixed in a mixture of ethanol, water, and 37% formaldehyde in a ratio of 6:3:1, prior to dehydration and paraffin embedding. Transverse sections were obtained at the level of the kidneys at a thickness of 7μm. Following rehydration, samples underwent antigen retrieval with heated 10mM citrate buffer (pH 6.0), followed by blocking with 10% goat serum in PBS and 0.3% TritonX. Double primary antibody incubations were performed with polyclonal rabbit anti-CLDN3 (Spring Bioscience, Fremont, CA, USA) at a dilution of 1:100, or rabbit anti-CLDN7 (Spring Bioscience, Fremont, CA, USA) at 1:200 together with
monoclonal mouse anti-ZO-1 (Invitrogen, Camarillo, CA, USA) antibody at 1:100 in PBS with 5% goat serum and 0.3% TritonX at 4°C overnight. Secondary antibodies were goat anti-rabbit IgG (Invitrogen, Camarillo, CA, USA) conjugated to Alexa Fluor 594 (TXR) and goat anti-mouse IgG (Invitrogen, Camarillo, CA, USA) conjugated to Alexa Fluor 488 (FITC), both at a dilution of 1:500. The secondary antibodies were incubated with the samples for 1 hour at room temperature. For double immunofluorescence performed on mIMCD-3 cells, the same reagents and conditions were used with the exception that mIMCD-3 cells were grown in monolayer to confluence on glass coverslips and then fixed with 4% paraformaldehyde at 4°C for 10 minutes. All slides and coverslips were mounted with Slowfade Gold antifade reagent (Invitrogen, Eugene, OR, USA).

3.5 Cell lines and constructs

mIMCD-3 cells were grown in DMEM/F12 medium containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S) in a humidified atmosphere at 37°C with 5% CO₂. The pcDNA3 expression vector containing the coding sequence of mCherry red fluorescent protein RFP downstream of a CMV promoter was kindly provided by Aimee K. Ryan (McGill University Health Centre, Montreal, QC, Canada). Complete coding sequences of Cldn3 and Cldn7 were subcloned upstream of mCherry, in frame, to produce a fusion protein in which the mCherry reporter tag was fused to the N-terminus of either CLDN3 or CLDN7. Vectors expressing mCherry-CLDN3, mCherry-CLDN7 or mCherry alone were stably transfected into mIMCD-3 cells using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA). The selection drug G418 was added after 48
hours in culture and colonies expressing high levels of RFP were selected. Stable clones were maintained in DMEM/F12 containing 500μg/ml of G418.

3.6 Immunoblotting

Confluent wildtype or stably transfected mIMCD-3 cells were dissolved in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NO-40, 1.5mM MgCl₂, 10% glycerol, 1mM EDTA, 25mM NaF, 1mM Na₃VO₄ and protease inhibitor cocktail; Roche, Mannheim, Germany). Total protein extracts were separated on SDS-PAGE and transferred to a nitrocellulose membrane. Blocking was performed in 5% milk in PBST, followed by incubation with polyclonal rabbit anti-CLDN3 antibody (Invitrogen, Camarillo, CA, USA) at a 1:500 dilution at 4°C overnight. This was followed by incubation with peroxidase-labeled anti-rabbit IgG secondary antibody (Cell Signaling Technology Inc, Denvers, MA, USA) at a 1:2500 dilution for 1 hour at room temperature. Antigen-antibody binding was detected using the ECL Western Blot Detection System (Amersham, Buckinghamshire, UK). Molecular mass was determined relative to a defined protein ladder (Fermentas, Burlington, ON, Canada).

3.7 Collagen gel assays

100,000 mIMCD-3 cells from either wild-type or stable transfectants expressing mCherry-CLDN3, mCherry-CLDN7, or mCherry alone were seeded in a mixture of rat-tail type I collagen (BD Biosciences, Mississauga, ON, Canada) containing HEPES, NaHCO₃ and DMEM-F12 (69). Cells were distributed in a 96-well plate such that 10,000 cells were seeded per well. Gels were left to solidify then 100μl of DMEM-F12
containing 10% FBS and 1% P/S was added to each well. Cultures were grown in a humidified atmosphere at 37°C with 5% CO₂ and observed over 48h. At 24h intervals, gels were removed from culture and fixed in 4% paraformaldehyde, rinsed with PBS and mounted on slides. The number of branches in a standardized area was determined for each cell line by first randomizing the images collected such that quantification was performed in a blinded manner. ImageJ Software (NIH, rsb.info.nih.gov/ij/) was used to count the number of tubular structures. Statistical analysis was performed using the Student’s t-test.

4. Results

4.1 Tight junction structures are present on the apical domain of UB cells

To establish the presence of tight junctions in the cells of the branching ureteric bud (UB), transmission electron microscopy (TEM) was performed on ultra-thin sections taken from embryonic kidneys at E12.5. At this time point the UB has already gone several branching events and is the only epithelial tissue present in the developing kidney. TEM confirmed that tight junctions are present at the most apical regions of cell-cell contacts (Fig. 2.1). The presence of tight junctions in the ureteric bud suggests that the claudin family of proteins may be important in the process of branching morphogenesis.
Figure 2.1 - Tight junction structures are present on the apical surface of ureteric bud cells. (A) An embryonic kidney at E12.5 was sectioned and the ureteric bud (UB) network was imaged. A UB-tip in cross-section is highlighted (black box) (scale bar = 50μm). (B) Ultra-thin sections were examined using transmission electron microscopy. Polarized epithelial cells of the UB lineage and their most apical lateral surfaces (black box) are shown (scale bar = 5μm). (C) Upon closer examination of apical cell-cell contacts, tight junctions (TJ) are detected at several points where the opposing cell membranes appear to touch (black arrows) (scale bar = 200nm).
4.2  *Cldn3 and Cldn7 mRNA are expressed in the UB throughout branching morphogenesis*

To characterize the expression patterns of *Cldn3* and *Cldn7* mRNA in the branching UB, whole mount *in situ* hybridization was performed at three stages of kidney development: E10.5, E13.5 and E16.5. At E10.5, there is the initial outgrowth of the UB, at E13.5 the UB has undergone several branching events, and at E16.5 the UB continues branching against a backdrop of terminally differentiated mesenchymal tissue (i.e. nephrons) and UB derivatives (i.e. collecting ducts). Both *Cldn3* and *Cldn7* transcripts are expressed in the UB and its derivatives at all three time points (Fig. 2.2). The expression of both *Cldn3* and *Cldn7* mRNA is detected in the nephric duct and the emerging ureteric bud at E10.5. By E13.5, expression is found throughout the ureteric bud lineage, but not in the differentiating mesenchymal cells of the MM. At E16.5, both *Cldn3* and *Cldn7* are strongly expressed in the UB tips, but expression weakens in the stalks. The UB tip marker *Ret* was used as a positive control to recognize the branching ureteric bud. These hybridization assays established that the UB cell lineage expresses the mRNA of both claudin species throughout kidney development.

4.3  *Cldn3 protein localizes to the apical domain of the UB, while Cldn7 is expressed primarily on the basolateral domain*

To determine the subcellular localization of *Cldn3* and *Cldn7* protein, double immunofluorescence was performed at E13.5 and E16.5 with the tight junction marker *zona occludens-1* (ZO-1) and the adherens junction and basolateral domain marker *E-cadherin* (Cdh1) (Fig. 2.3). *Cldn3* is expressed in the apical domain of the UB at both
Figure 2.2 - Cldn3 and Cldn7 transcripts are expressed in the ureteric bud (UB) during branching morphogenesis. Whole mount in situ hybridization was performed on whole embryos (Embryonic day (E)10.5) or dissected embryonic kidneys (E13.5, E16.5) which were then cryosectioned. At the start of branching morphogenesis at E10.5, Cldn3 and Cldn7 mRNA was expressed throughout the nephric duct (nd) and the emerging ureteric bud (UB, black box) in a pattern similar to the UB marker, Ret. At E13.5, Cldn3 and Cldn7 mRNA continues to be expressed in the UB lineage (upper row), and following cryosection both claudin species, are expressed throughout the UB and not in the differentiating mesenchyme (lower row). Ret expression is restricted to the tips of the UB. At E16.5, Cldn3 and Cldn7 expression persists exclusively in the UB, with the strongest levels found at the UB tips of the UB with weak to no signal in the stalks (upper, lower rows). Ret expression remains in the UB tips. White scale bar = 0.5mm, black scale bar =50mm.
Figure 2.3 - The subcellular localization of CLDN3 and CLDN7 differs in the ureteric bud lineage. Double immunoflourescence was performed using antibodies to CLDN3 or CLDN7 (2° TXR) with either the tight junction marker zona occludens-1 (ZO-1, 2° FITC) or basolateral membrane marker E-cadherin (CDH1). CLDN3 was found to colocalize with ZO-1 at both embryonic day (E)13.5 and E16.5, indicating that CLDN3 is localized to tight junctions in the UB lineage (arrows). CLDN7 is found primarily at the basolateral surface (arrowheads) of UB cells and largely overlaps with CDH1. Insets represent a higher magnification of the UB at E16.5. Scale bar = 50μm.
E13.5 and E16.5, consistent with the known expression pattern of ZO-1. This suggests that *Cldn3* functions within tight junctions during branching morphogenesis. *Cldn7* protein is expressed primarily on the basolateral domain of UB cells, with only limited colocalization with ZO-1 at both E13.5 and E16.5. In contrast, CLDN7 colocalizes with CDH1 at both embryonic stages. *Cldn7* protein may therefore associate with different membrane linked structures in the basolateral domain of the ureteric bud.

4.4 Overexpressing Cldn3 or Cldn7 in mIMCD-3 cells leads to increased tubulogenesis in vitro

The mIMCD-3 cell line is derived from adult mouse inner medullary collecting ducts, an epithelial tissue that is a descendant of the embryonic ureteric bud (70). When placed in type-I collagen matrix, these cells undergo tubulogenesis and branching in a manner morphologically similar to the UB. Using double immunofluorescence, we have established that *Cldn3* and *Cldn7* proteins colocalize with ZO-1 at the cell membrane of mIMCD-3 cells (Fig. 2.4). Z-stacks confirm co-localization of CLDN3 and CLDN7 to tight junctions at the apical membrane.

To determine the function of *Cldn3* and *Cldn7* during branching, stable mIMCD-3 clones expressing mCherry red fluorescent protein (RFP) fused to CLDN3 or CLDN7 (not shown), and unfused mCherry, were isolated. Western Blot analysis confirms mCherry-CLDN3 expression in stably transfected clones (Fig. 2.5A). Endogenous expression of *Cldn3* is detected in wild-type mIMCD-3 cells and in empty vector mCherry clones, and is also present in the mCherry-CLDN3 clones albeit at a lower level.
Figure 2.4 - Cldn3 and Cldn7 proteins colocalize with ZO-1 in mIMCD-3 cells. mIMCD-3 cells were grown in monolayer and double immunofluorescence was performed using antibodies to Cldn3 and Cldn7 (2° FITC), and ZO-1 (2° TXR). Cldn3, Cldn7 and ZO-1 are expressed at the cell membrane of mIMCD-3 cells. The merged images show the overlapping expression domains of CLDN-3 and -7 with ZO-1. Z-stacks indicate that both claudin species localize with ZO-1 to tight junctions in mIMCD-3 cells. Scale bar = 10μm
Figure 2.5 - Ectopic expression of Cldn3 or Cldn7 in mIMCD-3 cells leads to an increase in tubulogenesis. (A) Protein extracts were obtained from wild-type mIMCD-3 cells and clones stably transfected with empty vector mCherry or mCherry-CLDN3 and Western Blot was performed. The fusion protein is approximately 45kDa in size and is expressed in the clones overexpressing Cldn3. Endogenous CLDN3 was detected in all three cell lines, but was diminished in mCherry-CLDN3 cell line. (B) mIMCD-3 cell lines expressing mCherry-CLDN3 and mCherry-CLDN7 fusion constructs, as well as mCherry alone, were seeded in type I collagen matrix at a concentration of 10,000 cells per well. Cells were grown for 48 hours then fixed and imaged at 24 hour intervals under light microscopy. A qualitative increase in tubule formation was first observed at 24 hours in cells overexpressing Cldn3 or Cldn7 (data not shown) when compared to cells expressing the empty vector alone. (C) Quantification of branching after 48 hours revealed that cell lines overexpressing either Cldn3 or Cldn7 had significantly increased branching when compared to mCherry controls. (Student’s t-test, *P<0.001). Data is presented as mean ± s.e. from a representative experiment, out of three that were performed, such that n is the number of wells per cell line for that experiment.
To evaluate the effect of Cldn3 overexpression in mIMCD-3 cells, overexpressing clones were seeded in type-1 collagen matrix and *in vitro* tubulogenesis was evaluated over 48 hours in culture (Fig. 2.5B, C). Starting at 24 hours, a qualitative increase in tubule formation is observed in cells lines ectopically expressing either mCherry-CLDN3 or mCherry-CLDN7 (data not shown). The tubules are longer and more branched as compared to controls. Quantification following 48 hours in culture revealed that cell lines overexpressing either claudin species have significantly increased tubule formation compared to mCherry controls (P<0.001). Cldn3 and Cldn7 therefore promote branching in an *in vitro* model of tubulogenesis, suggesting tight junction proteins may contribute to the process of branching morphogenesis in the cells of the ureteric bud.

5. **Discussion**

We have characterized the expression patterns of Cldn3 and Cldn7 throughout embryonic kidney development and found that they are restricted to the epithelial UB lineage. Although they are expressed in the same cell lineage during kidney organogenesis, their subcellular localization is different. Cldn3 protein is expressed on the apical surface of UB cells, while Cldn7 is primarily found on the basolateral surface. We overexpressed Cldn3 and Cldn7 in a renal epithelial cell line that is a descendant of the embryonic UB, and found that both promoted increased formation of tubular structures. Our data suggests that tight junctions play a role in promoting branching morphogenesis of the UB *in vivo*.

Studies on isolated UB cultures *in vitro* suggest that tight junction proteins may generate the mechanical forces that guide UB branching. Meyer *et al.* showed that UB
branching proceeds through swelling at the tips of the ureteric bud caused by the formation of “outpouches” (25). These outpouches share a continuous lumen with the UB trunks and are composed of wedge-shaped epithelial cells with a concentration of actin and myosin-2 on the apical domain of the cytoplasm. The authors proposed that an apically driven “purse-string” mechanism could push the formation of outpouches, as the surface area of the apical membrane decreases and that of the basolateral membrane increases, changing the UB cells from columnar to wedge-shaped, and thereby propagating the UB luminal network (25). In light of the essential role of claudins in establishing tight junctions, the apical expression of Cldn3 suggests that this claudin member may contribute to the “purse-string” mechanism that drives branching morphogenesis.

Our results show that Cldn7 localizes to the basolateral domain of UB cells as opposed to the traditional subcellular localization of tight junction proteins at the apical domain. This unconventional localization of Cldn7 has been reported in the adult kidney, as well as other epithelial tissues such as the distal colon, the epididymus and the respiratory epithelium (47, 71-73). The function of Cldn7 in the basolateral membrane is still not fully known, but it has been suggested that basolateral claudins may serve as a storage pool that can be quickly recruited to tight junctions should there be a functional need (47). Recently, Cldn7 protein has been shown to form a complex with the epithelial cell adhesion marker (EpCAM) (63). The EpCAM-CLDN7 complex has been shown to localize to areas of the cell membrane called tetraspanin-enriched microdomains where it promotes cell proliferation, drug resistance and cell migration. The signaling pathways triggered by localization of EpCAM-CLDN7 to these microdomains are unknown, but
their identification may shed new light on the signaling functions of claudins with regards to proliferation and cell migration, which are essential processes in the progression of branching morphogenesis (17, 64). EpCAM is also expressed in the ureteric bud, therefore further characterization of the EpCAM-CLDN7 complex could provide new insight into the mechanisms that induce UB branching (74).

The overexpression of \textit{Cldn3} and \textit{Cldn7} in mIMCD-3 cells promoted tubule formation when these cells were seeded in a collagen matrix. Wild-type mIMCD-3 cells exposed to hepatocyte growth factor (HGF) display increased cell proliferation and migration and form more tubules when seeded in collagen (75). Interestingly, increased proliferation and cell migration have also been observed in ovarian epithelial cells with increased expression of \textit{Cldn3} (60, 61). In fact, \textit{Cldn3} is one of the most highly expressed genes in ovarian tumors, and is also overexpressed in prostate, colorectal and breast cancer (60, 76-79). Additionally, siRNA-mediated knockdown of \textit{Cldn3} in ovarian cancer cells reduced migration (60). Moreover, when \textit{Cldn3} was downregulated in mice with ovarian cancer xenografts there was a significant reduction in tumor cell proliferation and growth with an increase in the number of apoptotic cells (61). Experiments are currently underway to evaluate the proliferative and migratory response of mIMCD-3 cells showing \textit{Cldn3} overexpression.

Although considerable evidence has been generated demonstrating the effects of claudin-3 upregulation on cell migration and proliferation, it has also been associated with other mechanisms that may promote branching morphogenesis such as degradation of the extracellular matrix. Studies have shown that the overexpression of \textit{Cldn3} led to
increased matrix metalloproteinase-2 (MMP-2) activity (60, 80). Pro-MMP-2 activation is mediated by a subtype of membrane-bound MMPs, so-called membrane-type (MT)-MMPs. Claudins-1, -2, -3 and -5 stimulate the transformation of pro-MMP-2 into its active form in cooperation with MT-MMPs. In fact, claudin-1 and MT1-MMP proteins have been shown to interact directly in human embryonic kidney (HEK293) cells through immunoprecipitation (80). Claudins may recruit MT1-MMP to the cell surface where the activation of pro-MMP-2 occurs, thereby facilitating degradation of the extracellular matrix and promoting UB branching. The cells of the UB express MMP-2, and the inhibition of MMP activity has been shown to disrupt branching morphogenesis in kidney organ culture, suggesting that CLDN3 and MMP-2 interaction may constitute a novel mechanism for the regulation renal branching morphogenesis (81, 82).

Claudins form homo- and heteromeric interactions with other claudin species that are either next to them or on the opposing cell membrane (30). Cldn3 protein specifically interacts with Cldn-1, -2 and -5, but it is not known to interact with Cldn7 (83). The specific combination of claudins expressed in an epithelial tissue has been shown to affect the permeability properties of those cells (30). Heteromeric claudin interactions may also influence other claudin-mediated molecular processes. Assessing the ability of the mCherry-CLDN3 fusion protein to form heteromers will provide important information as to whether it activates MMP-2 alone or in combination with other claudin species. It will also provide insight into the signaling events that are mediating tubulogenesis in mIMCD-3 cells.
We show that when *Cldn3* or *Cldn7* is overexpressed in mIMCD-3 cells, they both promote an increase in tubulogenesis. *In vivo*, we also show that *Cldn3* and *Cldn7* proteins localize to different subcellular domains within the cells of the UB, suggesting they may function differently, at least in the developing kidney. Studies on the role of *Cldn3* suggest it can influence cell proliferation and migration as well as MMP activity, processes that are crucial for branching morphogenesis. The precise mechanism through which *Cldn3* influences these processes is currently under investigation through further characterization of the stable cell lines and by assessing its function in cultured embryonic kidneys.
CHAPTER III

Electroporation of embryonic kidney explants

Nicholas Haddad, Daniel Houle, Indra R. Gupta
1. **Abstract**

Explant cultures of embryonic kidneys provide an important *ex vivo* model to study the molecular and cellular processes that govern kidney development. We have developed a protocol in which we can successfully target the metanephric mesenchyme or the ureteric bud lineages of embryonic kidneys by microinjecting plasmid DNA into these tissue types followed by electroporation. Expression of a fluorochrome reporter gene encoded in the vector persisted in the targeted cell population for up to 96 hours. Microinjection and electroporation of embryonic mouse kidney explants is therefore an effective technique to study the function of genes and their roles in ureteric bud branching and nephrogenesis during kidney formation.
2. *Introduction*

Metanephric kidney development in the mouse begins at embryonic day (E) 10.5, when the ureteric bud, an outgrowth of the epithelial nephric duct, invades the neighboring metanephric mesenchyme. The ureteric bud then undergoes a series of branching events to form the collecting duct network of the adult kidney (Fig. 3.1). As each ureteric bud tip forms, the adjacent undifferentiated mesenchyme is induced to epithelialize and form a nephron, the functional unit of the adult kidney that filters waste. Rodent embryonic kidneys can be dissected and cultured as explants such that branching morphogenesis and nephrogenesis can be observed *ex vivo* (69, 84-86).

The roles of signaling molecules and transcription factors during kidney development have been studied in explant cultures by introducing small interfering RNA, by treating with pharmaceutical agents, or by using viral transduction to overexpress mutant proteins (87-89). More recently, microinjection followed by electroporation has been used to successfully express plasmid DNA encoding proteins important for ureteric bud induction, branching morphogenesis, and nephrogenesis in the developing kidney (68, 90, 91). This approach allows for both gain-of-function and loss-of-function experiments in a high-throughput, cost-effective manner. Tissue-specific effects can also be analyzed: DNA constructs can be delivered to either the metanephric mesenchyme or the ureteric bud lineages to evaluate the primary effects of genetic perturbation within a target cell population. This method can be used to evaluate the function of any gene of interest to determine whether it is then feasible to invest in the creation of more labour-intensive conditional knock-out or knock-in mouse lines. From expression profiling
Figure 3.1 - Early stages of mouse metanephric kidney development used in microinjection and electroporation experiments. The Hoxb7/GFP\textsuperscript{+/-} transgenic mouse can be used to monitor the morphological changes undergone by the ureteric bud during branching. The Hoxb7 promoter drives the expression of green fluorescent protein (GFP) within the nephric duct (nd), the ureteric bud (UB), and the branched derivatives. The metanephric kidney arises at E10.5 (top panel, left) when the UB emerges from the nephric duct and invades the neighboring metanephric mesenchyme (MM), demarcated by the dotted white line. As a result of reciprocal signaling and induction by the adjacent MM, the UB proceeds to elongate and divide repeatedly in a process termed branching morphogenesis (bottom panel, left and right). The undifferentiated mesenchymal cells surrounding each UB tip undergo mesenchymal to epithelial transformation (MET), which is the first step in nephrogenesis. Microinjection and electroporation has been used to study the process of UB budding at E10.5-11.5 (top row) as well as nephrogenesis and branching from E12.5-14 (bottom row).
technologies such as microarrays and SAGE, a large number of genes have been implicated in kidney development, yet for most of these genes, their function in organogenesis remains uncharacterized (25, 26). Microinjection with electroporation therefore provides an economical means to rapidly evaluate the function of previously described genes, and to characterize the role of genes more recently implicated in kidney development.

3. **Procedure**

The method is based on microinjection of a DNA construct into specific regions of the developing kidney. The kidney is then electroporated by exposing the tissue to an electric field. This permeabilizes cell membranes by creating pores that permit DNA constructs to enter targeted cells.

3.1 **Mouse embryonic kidney cultures**

Timed-pregnant CD1 mice (Charles River Laboratories, Wilmington, MA, USA) were sacrificed at embryonic day (E) 12, 13, or 14 to retrieve the embryos. *Hoxb7/GFP*<sup>+/−</sup> males (kindly provided by F. Costantini) were mated with CD1 females to obtain heterozygous offspring that expressed green fluorescent protein (GFP) in the ureteric bud and its branched derivatives. The mouse studies were performed in accordance with the rules and regulations of the Canadian Council of Animal Care. Using a M5A stereomicroscope (Wild Leitz ®, Willowdale, ON, Canada), metanephric kidneys were dissected from the embryos, suspended in 20μl of ViaSpan® (Barr Pharmaceuticals, Pomona, NY, USA), and cooled to 4°C prior to and during
microinjection by placing them on a stage with a cooling system (20/20 technology inc.,
model BC-100, USA).

3.2 DNA constructs

The expression vectors used in these experiments were pEGFP-C1 (Clontech
Laboratories Inc., Palo Alto, CA, USA), containing enhanced green fluorescent protein
(EGFP) as a reporter tag, and pcDNA3 containing mCherry red fluorescent protein (RFP)
as a reporter tag. In both vectors, the human cytomegalovirus (CMV) promoter drives
gene expression.

3.3 Microinjection and electroporation

Microinjection was performed using an inverted microscope (Leitz Labovert FS,
Foster City, CA, USA) with Nomarksi differential interference optics and a Leitz®
micromanipulator. Microinjection needles were prepared from Pyrex® capillary tubing
containing Omega Dot fiber (FHC Inc., Bowdoinham, ME, USA) and were pulled using
the KOPF 720 needle puller (Narashige Co., Tokyo, Japan) and cut at the tips. Glass
needles were connected via plastic tubing to an IM 300 microinjector (Narishige) which
delivered the DNA expression vector at a concentration of either 2 or 5μg/μl diluted in
ddH2O. The DNA was mixed with 1% FastGreen FCF dye (Fisher Scientific Inc.,
Ottawa, ON, Canada) in PBS in a 50:1 ratio. Each kidney was injected at a single site,
receiving an average total volume of 0.025μl. To target the metanephric mesenchyme, the
needle was inserted into the cortex of the explanted kidney (Fig. 3.2). To target cells of
the ureteric bud, the needle was inserted into the lumen of the ureter such that after
Figure 3.2 - The method of microinjection and electroporation in embryonic kidney explants. To target cells of the metanephric mesenchyme (MM) (left panel, top row), the needle is inserted in the cortex of the kidney and DNA is injected into a single injection site. After electroporation, the transgene is expressed within the region of injection. For ureteric bud (UB) targeting (left panel, bottom row), the needle is inserted into the lumen of the severed ureter to ensure that the plasmid DNA is dispersed throughout the branching UB. Microinjection of DNA and FastGreen dye into the ureter of an embryonic day 12 kidney is shown (right panel). The dye is seen dispersed throughout the ureteric bud and its branches. scale bar: 0.5 mm
microinjection, the DNA solution was dispersed throughout the branching derivatives of the ureteric bud (Fig. 3.2). Following microinjection, kidneys were placed on a platinum plate Petri dish-base electrode (CUY701-P2E, Protech International Inc., San Antonio, TX, USA) and covered with an L-shaped platinum plate covered electrode (CUY701-P2L, Protech International). A BTX square wave electroporator (ECM830, Genetronics Inc., San Diego, CA, USA) was used to deliver 5 pulses of 175V for 75μs with a 1s delay between pulses. Following microinjection and electroporation, kidney explants were grown on 0.4μm pore size PET track-etched membranes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in DMEM/F-12 with 10% FBS and 0.1% penicillin/streptomycin (Wisent Inc., St.Bruno, QC, Canada). Explants were cultured in 6-well plates at 37ºC for up to 96 hours.

4. Results

4.1 Establishing parameters for microinjection and electroporation

Most electroporation protocols in mammalian tissues have used conditions that were originally described in the chick consisting of a low voltage of 25V applied in 1-5 pulses of 50ms each, which is considered a long pulse time. Microinjection and electroporation of the anterior tibialis muscle of adult mice has demonstrated that the parameters of high voltage, 900V, combined with a short pulse, 100μs, lead to higher and more sustained transgene expression (92). Therefore, we screened a range of electroporation parameters to identify the optimal conditions for widespread gene expression and to minimize tissue necrosis in mouse embryonic kidney explants collected from E12-14 (68). After microinjecting the expression vector pEGFP-C1, we showed
that kidney explants electroporated with a high voltage (250V) and a long pulse time (100ms) had high levels of EGFP expression, yet grew poorly with excessive cell death. Kidneys electroporated using a low voltage (25V) and a long pulse time (50ms) grew as well in culture as non-electroporated kidneys, however EGFP expression was significantly lower and more focal than in kidneys electroporated with a high voltage. The number of pulses was also important for gene expression: kidneys that received at least 5 pulses displayed the highest EGFP expression. The conditions for electroporation were therefore established such that kidneys were electroporated with 5 short pulses, 75μs each, using a high voltage of 175V. Kidneys that were microinjected and electroporated with these parameters grew well and underwent branching morphogenesis and nephrogenesis. The kidneys expressed the reporter gene as early as 2 hours after electroporation, and maintained high expression for 96 hours in culture.

4.2 Tissue-specific expression

Adjusting the site of microinjection allowed us to target either the metanephric mesenchyme or the ureteric bud within the developing kidney. Peak levels of reporter gene expression, mCherry RFP, were detected 24 hours after electroporation within the metanephric mesenchyme (Fig. 3.3). The metanephric mesenchyme was successfully targeted 95% of the time (n=19/20 explants), by manually inserting the needle within the explant. The ureteric bud injections required more precision and were successfully targeted 75% (n=30/40) of the time. Prior to microinjection, the ureter was severed at its connection to the renal pelvis, generating a short ureteral stump. Plasmid DNA was injected directly into the lumen of the cut ureter using a micromanipulator which
Figure 3.3 - Tissue-specific expression is achieved by targeted microinjection and electroporation of DNA constructs into different cell populations within mouse embryonic kidney explants. Embryonic day (E) 14 kidneys were dissected and then microinjected in either the metanephric mesenchyme (MM, top and bottom rows) or ureteric bud (UB, middle row) lineages with a plasmid encoding the mCherry RFP (red fluorescent protein) fluorochrome, and subsequently electroporated. Peak tissue-specific expression in the explants was detected after 24 hours in culture. Kidneys derived from the Hoxb7/GFP+/- transgenic mouse line can also be used for microinjection and electroporation experiments (bottom row). An E12 GFP-expressing kidney was microinjected in the MM with mCherry RFP and then electroporated. The Hoxb7/GFP+/- explants can be used to monitor primary effects on UB branching by targeting the UB itself or the MM. scale bar: 0.5 mm
facilitated the distribution of DNA throughout the branching ureteric bud via the lumen of the renal pelvis (Fig. 3.2). E14 kidneys were examined 24 hours later, and reporter gene expression was detected within the ureteric bud branching network of the kidney explant (Fig. 3.3). We were also able to successfully target the metanephric mesenchyme or the ureteric bud when E12 or E13 kidneys were microinjected and electroporated.

Localized gene expression after microinjection and electroporation allows researchers to compare a transgene’s effect in regions of expression versus regions of no expression within the same kidney (Fig. 3.3). In this way, each kidney serves as its own control. Alternatively, two different kidneys with similar amounts of fluorochrome expression can be paired for comparative analyses (68). Kidneys can be analyzed as whole mounts or they can be fixed and cryosectioned.

5. **Comments**

Microinjection and electroporation has been applied to a number of embryonic stages beginning at E10.5 when the UB first emerges, and continuing up to E14 when nephrogenesis is well established. Kidney explants taken at later stages of development are more difficult to study because they fail to grow adequately in culture. Several measures, that have been shown to be beneficial in other systems, were applied to this model with the objective to reduce tissue necrosis and to improve transgene expression.

During dissection, kidney explants were suspended in ViaSpan®, a solution originally designed to preserve organs that have been harvested for transplantation. When cells are suspended in this solution and then electroporated with DNA, there is less
toxicity (93). ViaSpan is enriched in potassium and magnesium, and it is postulated this may reduce the loss of these ions from the intracellular compartment during electroporation, thereby reducing cytotoxicity.

When electroporation is performed at 4°C, cell viability is enhanced and the duration of cell permeabilization is increased, permitting greater DNA entry and higher expression (94). We therefore performed microinjection and electroporation at 4°C to minimize cytotoxicity and improve gene expression. Expression levels of reporter genes have also been enhanced when plasmid DNA is linearized prior to electroporation (95). We investigated this, but found no significant difference in expression when linearized as opposed to circularized DNA was microinjected and electroporated in embryonic kidney explants (data not shown).

Although our current protocol leads to high levels of gene expression with minimal toxicity, additional measures may be beneficial. Transgene expression may be significantly increased if microinjected kidneys are exposed sequentially to two different electric fields: the first one containing a high voltage with a short pulse time followed by the second one containing a low voltage and long pulse time (96). The initial high voltage permits a greater cell surface area to be permeabilized, while the subsequent long pulse time allows for increased DNA transport across the membrane (97). Gene transfer and expression is also enhanced by increasing the proportion of the cell membrane that interacts with DNA. DNA travels in the direction of the anode when an electric field is applied. The area of the cell membrane that interacts with DNA can be increased when electric pulses are applied in different orientations and polarities (98). It is therefore
possible to augment transgene expression in mouse kidney explants if the orientation of
the kidneys is changed during electroporation, or if the polarities of the paddle and plate
electrodes are reversed between pulses. Treatment with the enzyme hyaluronidase, which
is known to degrade components of the extracellular matrix (ECM), has been shown to
improve the expression levels of plasmid DNA injected into muscle fibers \textit{in vivo} (99). Degradation of the ECM enhanced cellular exposure to plasmid DNA and permitted the
use of lower voltages which decreased cytotoxicity (100). It is possible that pre-treating
kidney explants with this enzyme may also improve transgene expression and permit the
use of lower voltages.

In our experiments, reporter gene expression began to weaken as early as 48 hours
post-electroporation, and was significantly diminished after 5 days in culture (68). This
may be due to the high level of cell proliferation and differentiation taking place in the
developing kidney. Microinjection and electroporation of embryonic mouse brain \textit{in
utero} has shown that this technique can generate long-term expression, up to 6 weeks
after treatment, when transfected DNA is not diluted by cell division (101). Longer
transgene expression may therefore be possible if terminally differentiated cells are
targeted within kidneys at later stages of development (102, 103).
CHAPTER IV

Future Experiments
The stably transfected mIMCD-3 cells expressing CLDN3 and CLDN7 fused to mCherry RFP have been informative in our analysis of claudin function. With time however, RFP expression in the stable cell lines has faded, hampering our efforts to further evaluate the subcellular localization of the fusion protein and its effects on tubulogenesis in vitro. Currently, we are isolating new colonies that express enhanced green fluorescent protein (EGFP) at the N-terminus as EGFP-CLDN3 or EGFP-CLDN7. The use of GFP for N-terminal fusions of other claudins has not prevented the protein’s ability to localize to the cell membrane (62, 104). These new clones will be used to confirm our data and to complete our analysis of the tubulogenesis phenotype.

Our results show that Cldn3 and Cldn7 are expressed throughout the UB during branching morphogenesis, and that the overexpression of Cldn3 or Cldn7 promotes tubulogenesis in vitro. The tubules observed were considerably longer and more branched in our overexpressing clones when compared to mCherry controls. What remains to be determined is the precise mechanism that drives the increase in tubule formation. Three relevant cellular mechanisms will be examined: proliferation, migration and adhesion. Cell proliferation may promote tubule formation due to an increase in the number of available cells, which then favors the growth of longer tubules and perhaps increases the number of branch points per tubule. A change in the proliferation rate of stable cell lines overexpressing Cldn3 or Cldn7 will be assessed using a Bromodeoxyuridine (BrdU) labeling assay, in which BrdU is incorporated in cells undergoing replication. These results will be evaluated in conjunction with a more detailed analysis of the tubulogenesis phenotype, such that the average tubule length and the number of branch points per tubule will be quantified.
Increased tubule formation in collagen matrix has been observed when mIMCD-3 cells are treated with hepatocyte growth factor, HGF, and this has been attributed to an increase in cell migration shown by scattering assays from cells grown in subconfluent monolayers (75). Enhanced cell migration has been observed in cancer cell lines from elevated Cldn3 and Cldn7 protein expression (60, 64). It is therefore possible that an increased migratory response is contributing to increased tubule formation in our stable transfectants. We will assess cell migration in our overexpressing clones using a transwell migration assay to assess cellular movement across a porous membrane.

Tight junctions contribute to cell-cell adhesion, a cellular process that may affect the behavior of mIMCD-3 cells once seeded in collagen. Increased levels of claudins may promote cell adhesion leading to more tubule formation. To assess the effect of Cldn3 or Cldn7 overexpression on cell adhesion, we will perform cell dissociation and re-aggregation assays. Briefly, these assays are based on counting “clumps” of cells following trypsinization and removal of Ca^{2+} from the culture medium (27). Calcium is required for E-cadherin-mediated cell adhesion, and its removal allows us to evaluate claudin-mediated cell adhesion which is Ca^{2+}-independent. The more cells found in clumps following trypsinization the more adherent the cells are. For re-aggregation assays, the adherence of cells is measured by the time required for cells to form clumps when cultured on a rotating platform.

Within tight junctions, claudins form both homo- and heteromeric interactions with other claudin species that are either next to them or on the opposing cell membrane (30). CLDN3 specifically interacts with CLDN1, 2, and 5 in addition to forming
homodimers, but it is unknown if it interacts with CLDN7 (83). The ability of Cldn3 and Cldn7 proteins to interact with each other will be examined by transfecting cells that do not endogenously express claudins (e.g. HEK293 cells) with mCherry-CLDN3 or GFP-CLDN7 (or vice-versa). The cell lines can then be co-cultured to explore the potential colocalization of fluorescent signals. Additionally, co-immunoprecipitation experiments will be performed to confirm any CLDN3-CLDN7 interaction, as well as to “pull-down” any other relevant proteins they may interact with to mediate proliferation and migration. Assessing the ability of the CLDN3 and CLDN7 fusion proteins to form both homo- and heteromers will provide important insight into the signaling events at the tight junction that are mediating tubulogenesis.

The development of microinjection and electroporation to target DNA constructs to the UB lineage of the embryonic mouse kidney is crucial for our goal of studying claudin function in branching morphogenesis. We aim to microinject and electroporate DNA constructs encoding either mCherry-CLDN3 or mCherry-CLDN7 into the UB lineage, and to compare the effects with our results using the mIMCD-3 tubulogenesis assay. To visualize UB branching in culture in real-time, we will perform these experiments using dissected kidneys from the Hoxb7/GFP+/- transgenic mouse. In this mouse, GFP expression is driven by the UB-specific Hoxb7 promoter, such that the emerging UB and its derivatives express GFP (8). UB-targeted microinjections will be performed on kidneys harvested at E11.5. At this early stage in kidney development, genetic perturbation to the UB is likely to have the greatest effect. We expect to see significant changes in branching morphology with regards to trunk length, number of
branching events and the number of UB tips in the cultured embryonic kidney explants as a result of the increased levels of Cldn3 and Cldn7.
REFERENCES


47. Li WY, Huey CL, Yu AS. Expression of claudin-7 and -8 along the mouse nephron. *American journal of physiology* 2004; **286**: F1063-1071.


59. Soini Y. Expression of claudins 1, 2, 3, 4, 5 and 7 in various types of tumours. *Histopathology* 2005; **46**: 551-560.

60. Agarwal R, D'Souza T, Morin PJ. Claudin-3 and claudin-4 expression in ovarian epithelial cells enhances invasion and is associated with increased matrix metalloproteinase-2 activity. *Cancer research* 2005; **65**: 7378-7385.


62. Alexandre MD, Lu Q, Chen YH. Overexpression of claudin-7 decreases the paracellular Cl- conductance and increases the paracellular Na+ conductance in LLC-PK1 cells. *Journal of cell science* 2005; **118**: 2683-2693.


76. Zhu Y, Brannstrom M, Janson PO, Sundfeldt K. Differences in expression patterns of the tight junction proteins, claudin 1, 3, 4 and 5, in human ovarian surface epithelium as compared to epithelia in inclusion cysts and epithelial ovarian tumours. *Int J Cancer* 2006; **118**: 1884-1891.


APPENDIX A: Ethics approval and certificates

THE RESEARCH INSTITUTE OF THE McGill UNIVERSITY HEALTH CENTRE
ENVIRONMENTAL HEALTH AND SAFETY CERTIFICATE

This is to certify that Nicholas Haddad from the Department of Human Genetics, McGill University successfully completed training in Biosafety on April 25, 2007.

Pubalee Bera, Environmental Health & Safety Officer
23rd May 2007

Jean-Marie Chavannes, Technical Support Manager
2007/05/23

Date

Certification is valid until April 25, 2010
McGill University
Animal Use Protocol – Research

Title: Genetic Determinants of Ureteric Budding: Impact on Vesico-ureteric Reflex and Renal Malformations

1. Investigator Data:
Principal Investigator: Indra Gupta
Unit/Department: Pediatrics
Address: 4060 Ste. Catherine's St. West, Room PT.413-2, Montreal
Phone #: 514-412-4400 ext 23539
Fax #: 514-412-4478
Email: indra.gupta@muhc.mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.
Name: Inga Murawski
Work #: 23322
Emergency #: (514) 939-7776
Name: Nicholas Haddad
Work #: 23322
Emergency #: (514) 561-0565

3. Funding Source:
External
Source(s): Kidney Foundation of Canada
Peer Reviewed for the project proposed in this Animal Use Protocol: YES
Status: Awarded
Funding period: July 1, 2007-June 30, 2009

Investigator’s Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee’s approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator’s signature: ___________________________ Date: July 9, 2008

Chair, Facility Animal Care Committee: ___________________________ Date: July 23, 2008

Approved Animal Use Period: START = June 1, 2008 END = June 30, 2008
Renewal next year requires submission of full Animal Use Protocol form

4. Research Personnel and Qualifications

List the names of the Principal Investigator and of all individuals who will be in contact with animals in this study and their employment classification (investigator, technician, research assistant, undergraduate/graduate student, fellow). Indicate if Principal Investigator is not handling animals. If an undergraduate student is involved, the role of the student and the supervision received must be described. Training is mandatory for all personnel listed here. Refer to www.animalcare.mcgill.ca for details. Each person listed in this section must sign to indicate that s/he has read this protocol. (Space will expand as needed)

<table>
<thead>
<tr>
<th>Name</th>
<th>Classification</th>
<th>Animal Related Training Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inga Murawski</td>
<td>Graduate Student</td>
<td>Animal Workshop and online training, McGill U</td>
</tr>
<tr>
<td>Bruno St-Jacques</td>
<td>Research Assistant</td>
<td>Animal Workshop and online training, McGill U</td>
</tr>
<tr>
<td>Nicholas Haddad</td>
<td>Graduate Student</td>
<td>Animal Workshop and online training, McGill U</td>
</tr>
<tr>
<td>Rita MAina</td>
<td>Workstudy student</td>
<td>Animal Workshop and online training, McGill U</td>
</tr>
<tr>
<td>Indra Gupta</td>
<td>Principal Investigator</td>
<td>Animal Workshop and online training, McGill U</td>
</tr>
</tbody>
</table>

will be directly supervised by Inga Murawski

Signature

* Indicate for each person, if participating in the local Occupational Health Program, see www.mcgill.ca/research/compliance/animal/occupational for details.

5. Summary (in language that will be understood by members of the general public)

5 a) AIMS AND BENEFITS: Describe, in a short paragraph, the overall aim of the study and its potential benefit to human/animal health or to the advancement of scientific knowledge.

40% of childhood endstage kidney disease can be attributed to congenital renal maldevelopment that arises in utero. Kidney malformations frequently occur with urinary tract malformations since the embryological development of these two systems are tightly linked. We are studying the events that control the formation of the kidney and the urinary tract using mouse models of development.

5 b) SPECIFIC OBJECTIVES OF THE STUDY: Summarize in point form the primary objectives of this study.

1. To study urinary tract development in refluxing and nonrefluxing mouse strains.
2. To determine if the position of ureteric budding can be altered in vitro using cultured urogenital explants and then to assess the effect on kidney and urinary tract development.
3. To identify genes that predispose to the development of vesico-ureteric reflux and renal malformations by screening a panel of inbred mouse strains and a panel of recombinant inbred mouse strains.

5 c) Indicate if and how the current goals differ from those in last year’s application.

The current protocol is essentially unchanged from last year’s application in terms of the types of procedures and the types of tissues we will be harvesting from the mouse. The difference in this year’s protocol is that we are now testing other inbred mouse strains with the final objective to identify genes that predispose to vesico-ureteric reflux and/or renal malformations in humans. We are also testing BXH recombinant inbred strains for vesico-ureteric reflux and/or renal malformations.

5 d) List the section / subsection numbers where significant changes have been made
APPENDIX B: Permission to reprint

From: Wart van der, Neil, Springer SBM NL [Neil.vanderwet@springer.com] On Behalf Of Permissions Europe/NL [Permissions.Dordrecht@springer.com]
Sent: Tuesday, May 26, 2009 10:32 AM
To: Nicholas Haddad
Subject: RE: Customer question from springer.com

Dear Sir,

With reference to your request (copy herewith) to reprint material on which Springer Science and Business Media controls the copyright, our permission is granted, free of charge, for the use indicated in your enquiry.

This permission:
- allows you non-exclusive reproduction rights throughout the World
- permission includes use in an electronic form, provided that content is password protected;
- at intranet;
- excludes use in any other electronic form. Should you have a specific project in mind, please reapply for permission.
- requires a full credit (Springer/Kluwer Academic Publishers book/journal title, volume, year of publication, page, chapter/article title, name(s) of author(s), figure number(s), original copyright notice) to the publication in which the material was originally published, by adding: with kind permission of Springer Science and Business Media. The material can only be used for the purpose of defending your dissertation, and with a minimum of 30 extra copies in paper.

Permission free of charge on this occasion does not prejudice any rights we might have to charge for reproduction of our copyrighted material in the future.

Best regards,

Neil van der Wart (Ms)
Rights and Permissions/Springer

Van Godewickstraat 30 | P.O. Box 17
3300 AA Dordrecht | The Netherlands
tel +31 (0) 78 5576 298

fax +31 (0)78 5576-300
Neil.vanderwet@springer.com
www.springer.com

-----Original Message-----
From: Eichhorn, Katja, Springer DE On Behalf Of Permissions Heidelberg, Springer DE
Sent: dinsdag 26 mei 2009 13:48
To: Permissions.Europe@nl
Subject: FW: Customer question from springer.com
-----Original Message-----
From: SpringerAlerts@springeronline.com [mailto: SpringerAlerts@springeronline.com]
Sent: Monday, May 25, 2009 8:51 PM
To: Permissions Heidelberg, Springer DE
Subject: Customer question from springer.com

To whom it may concern,

My name is Nicholas Haddad and I am writing to request permission to include a chapter, for which I am an author, in my Master's thesis submission to McGill University, Montreal, Canada.

The details of the material requested are as follows:
ISBN: 978-4-431-09426-5
Book title: Electroporation and Sonoporation in Developmental Biology
-All material from Chapter 19: Electroporation of embryonic kidney explants
Nicholas Haddad, Daniel Houle and Indra R. Gupta
pp. 219-227
Editor: Haruka Nakamura
Year of publication: 2009

This content will only be used as a segment in my Master's thesis submission to McGill University. If any additional information is required please contact me at the following coordinates. Thank you.

Nicholas Haddad
4060 St. Catherine's Street West
Room PT413.2
Montreal, Quebec
Canada
H3Z 2Z3
Tel: (514) 412-4400 x23322
Fax: (514) 412-4479
nicholas.haddad@mail.mcgill.ca

[sender name: Nicholas Haddad
sender email: nicholas.haddad@mail.mcgill.ca
INTERNAL NAME: Rights and Permissions Department - Springer Verlag Heidelberg
ORIGINAL URL: ]