Biology of the Nephron Progenitor Cell and the Origins of Wilms Tumour

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August 2020

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy.

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Abstract

The nature of nephron progenitor cells (NPCs) in embryonic kidney has intrigued biologists for nearly half a century, following the seminal observations of Grobstein who demonstrated differentiation of the metanephric mesenchyme in response to contact with spinal cord and other inducing tissues. Herzlinger later showed the inducing substance was a secreted WNT molecule and Carroll confirmed that NPC differentiation is activated by WNT9B released by the ingrowing ureteric bud. While the nature of the inducing substance has been studied in detail, the molecular mechanisms that prime NPCs to respond to WNT9B remain enigmatic. The pathways that transduce WNT signalling are complex (19 WNTS, 10 FZD receptors, 2 LRP co-receptors and a long list of downstream effectors), reflecting the need for specific regulation in distinct progenitor cell lineages and the need for exquisite focus in targeting local developmental events within large complex organs.

In mammals, nephrogenesis comes to an end in the perinatal period when the rate of nephron induction exceeds signals maintaining the NPC pool. Although, adult mammals are unable to form new nephrons if the kidneys are injured, recent evidence indicates that a small set of scattered NPC-like cells are retained within each mature nephron and these can re-populate segments of the damaged renal tubule. Interestingly, Lazzeri showed that when primed NPCs from an embryonic kidney are infused into adult mice with acute kidney injury, the exogenous cells are incorporated directly into damaged renal tubules. Our lab has shown that this phenomenon requires NPCs carrying molecular components that can activate the canonical β-catenin signaling pathway in response to WNT signals. Thus, the mechanisms by which NPCs respond to WNTs are of considerable interest to understanding the prospect for therapeutic use of NPCs in treating acute kidney injury.

Unfortunately, Wilms tumour may also arise from the NPC. Insight into these embryonal tumours came with the discovery that hereditary Wilms tumors could be attributed to heritable heterozygous mutations of the *WT1* gene. WT1 is a transcription factor that sets the cell fate of NPC in the intermediate mesoderm; subsequent somatic loss of *WT1* in a single NPC precludes

the response to WNT9B and leads to a clone of developmental arrested cells. The mechanisms that subsequently drive malignant transformation are poorly understood but often involve acquisition of secondary activating mutations in *CTNNB1* allowing uncheck cell growth. Thus, the role of WT1 in priming the NPC for normal nephrogenesis may be of considerable interest for understanding the pathogenesis of Wilms tumor.

In this thesis, we first analyzed the molecular components of the primed NPC that allow it to respond to the WNT9B signal during development. We used the M15 cell line (representative of an early NPC precursor) to analyze the FZD and LRP receptor molecules conferring responsiveness to WNT9B. In a systematic way, we identified FZD5 and LRP6, in addition to a WNT-signalling agonist RSPO1, as essential for the WNT9B response. We also showed that experimental priming of M15 cells with recombinant RSPO1 protein increased the ability of cells to integrate into the damaged nephrons of mice with glycerol-induced renal injury. Secondly, we addressed the problem of how *WT1*-gene loss puts the NPC at increased risk for malignant transformation into a Wilms tumour. We identified that a large panel of DNA-repair enzymes are activated in embryonic kidney versus adult. One repair enzyme, *Neil3*, was activated 15-fold higher in NPCs than in the rest of the embryonic kidney. We showed that *Neil3* expression is WT1-dependent and that WT1 binds directly to the *Neil3* promoter to activate transcription. The loss of *Neil3* in *WT1*-negative NPCs could contribute to the apparent genomic instability in *WT1*-negative nephrogenic rests and the increased incidence of activating *CTNNB1* mutations in Wilms tumour.

Resume

La nature des cellules progénitrices du néphron (NPC) dans le rein embryonnaire a intrigué les biologistes depuis près d'un demi-siècle. Carroll a confirmé que la différenciation des NPC est activée par le WNT9B du bourgeon urétéral en croissance. Bien que la nature de la substance inductrice ait été étudiée en détail, les mécanismes moléculaires qui amènent les NPC à répondre au WNT9B restent énigmatiques. Les voies de signalisation de WNT sont complexes (19 WNTS, 10 récepteurs FZD, 2 corécepteurs LRP et une longue liste d'effecteurs en aval), reflétant la nécessité d'une régulation spécifique dans des lignées de cellules progénitrices distinctes et la nécessité d'une attention particulière dans le ciblage local des événements développementaux au sein de grands organes complexes.

Chez les mammifères, la néphrogénèse prend fin dans la période périnatale, lorsque le taux d'induction du néphron dépasse les signaux maintenant le pool de NPC. Bien que les mammifères adultes soient incapables de former de nouveaux néphrons si les reins sont blessés, des preuves récentes indiquent qu'un petit ensemble de cellules de type NPC dispersées est retenu dans chaque néphron mature et que celles-ci peuvent repeupler les segments du tubule rénal endommagé. Lazzeri a montré que lorsque les NPC amorcées d'un rein embryonnaire sont infusées dans des souris adultes atteintes de lésions rénales aiguës, les cellules exogènes sont incorporées directement dans les tubules rénaux endommagés. Notre laboratoire a montré que ce phénomène nécessite des NPC porteuses de composants moléculaires capables d'activer la voie de signalisation canonique de la béta-caténine en réponse aux signaux WNT. Ainsi, les mécanismes par lesquels les NPC répondent aux WNT sont d'un intérêt considérable pour comprendre la perspective d'une utilisation thérapeutique des NPC dans le traitement des lésions rénales aiguës.

Malheureusement, tumeur de Wilms peuvent également provenir des NPC. Un aperçu de ces tumeurs embryonnaires est venu avec la découverte que les tumeurs héréditaires de Wilms pouvaient être attribuées à des mutations hétérozygotes héréditaires du gène WT1. WT1 est un facteur de transcription qui définit le sort cellulaire des NPC dans le mésoderme intermédiaire.

La perte somatique ultérieure de *WT1* dans une seule NPC exclut la réponse à WNT9B et conduit à un clone de cellules au développement arrêté. Les mécanismes qui conduisent par la suite la transformation maligne sont mal compris mais impliquent souvent l'acquisition de mutations activatrices secondaires dans *CTNNB1* permettant une croissance cellulaire incontrôlée. Ainsi, le rôle du WT1 dans l'amorçage des NPC pour la néphrogénèse normale peut être d'un intérêt considérable pour comprendre la pathogenèse de la tumeur de Wilms.

Dans cette thèse, nous avons d'abord analysé les composants moléculaires des NPC amorcées qui leur permettent de répondre au signal WNT9B pendant le développement. Nous avons utilisé la lignée cellulaire M15 pour analyser les molécules des récepteurs FZD et LRP conférant la réactivité au WNT9B. De manière systématique, nous avons identifié FZD5 et LRP6, en plus d'un agoniste de signalisation WNT, RSPO1 comme étants essentiels pour la réponse à WNT9B. Nous avons également montré que l'amorçage expérimental des cellules M15 avec la protéine RSPO1 recombinante augmentait la capacité des cellules à s'intégrer dans les néphrons endommagés de souris souffrant de lésions rénales induites par le glycérol. Deuxièmement, nous avons abordé le problème de la façon dont la perte du gène WT1 expose les NPC à un risque accru de transformation maligne en une tumeur de Wilms. Une enzyme de réparation, *Neil3*, a été activée 15 fois plus dans les NPC que dans le reste du rein embryonnaire. Nous avons montré que l'expression de *Neil3* dépend de WT1 et que WT1 se lie directement au promoteur *Neil3* pour activer la transcription.

Acknowledgements

First and foremost, I would like to thank my supervisor, Dr. Paul Goodyer. Your support and guidance helped drive this project to completion. You were always there to bounce ideas off of and also gave me the freedom to experiment with new ideas whenever I reached a roadblock. I will always be grateful for being given the opportunity to work in your lab and on this project.

Secondly, I would like to thank the members of my supervisory committee, Dr. Janusz Rak, Dr, Jerry Pelletier and Dr. Michael Witcher. Your input and suggestions helped strengthen the foundation of this project and helped keep me pushing in the right direction.

To my fellow lab members, past and present, thank you for always making it fun to come to work every single day. Thank you to Dr. Murielle Akpa for taking me under your wings for a seamless orientation when I first joined the lab. You took time out of your day to help train me while you were near the end of your Ph.D. and I cannot thank you enough for that. To Lee Lee Chu, thank you for always being there to talk to and helping troubleshoot experiments and ensuring the lab ran smoothly. The lab would be lost without you. And to the rest of my fellow lab members over the years (Melanie Cosgrove, Emma Brasell, Fatima Tokhmafshan, Leah Hammond, Sarah Park, Jung Hwa Seo, Zhao Zhang and Rachel Corsini), thank you for being amazing colleagues and keeping the lab an exciting place to be.

I would like to give a special thanks to members of the core facilities at the MUHC that have helped throughout the years, in particular Ekaterina Iourtchenko (Immunophenotyping), Suzy Loucao (Animal Resource Division) and Dr. Min Fu (Molecular Imaging).

Last but not least, I would like to thank my family. Thank you for your support, not only during my studies, but my entire life. Your love, support and patience are the reason for all of my successes. I would not be in this position without you. Thank you from the bottom of my heart.

Abbreviations

6-4PP	6-4 pyrimidine-pyrimidone photoproduct
AGO2	Protein argonaute-2
AKI	Acute kidney injury
AMER1	APC Membrane Recruitment Protein 1
amMSC	Amniotic fluid derived mesenchymal stem cell
APC	Adenomatosis polyposis coli
ARPC	Adult renal progenitor cells
BER	Base excision repair
BMI1	BMI1 Proto-Oncogene, Polycomb Ring Finger
BRCA	Breast And Ovarian Cancer Susceptibility Protein
CD133	Prominin 1
CHEK1	Checkpoint Kinase 1
ChIP	Chromatin immunoprecipitation
CITED1	Cbp/P300 Interacting Transactivator With Glu/Asp Rich Carboxy-Terminal Domain 1
CK1α	Casein kinase 1 alpha
CK1γ	Casein kinase 1 gamma
CKD	Chronic kidney disease
CPD	Cyclobutane-pyrimidine dimers
CRD	Cysteine-rich domain
CS	Cockayne syndrome
CTNNB1	Catenin Beta 1/ B-catenin
DGCR8	DiGeorge Syndrome Critical Region Gene 8
DSB	Double strand break
DVL	Dishevelled
E	Embryonic day
ER	Endoplasmic reticulum
ERCC	Excision Repair Cross-Complementation
ESC	Embryonic stem cell
EZH2	Enhancer of zeste homolog 2
FACS	Fluorescence-activated cell sorting
FZD	Frizzled
G2	Gap 2
GDNF	Glial-derived neurotrophic factor
GFR	Glomerular filtration rate
GG-NER	Global genome NER
GSK3β	Glycogen synthase kinase 3 beta
H3K27	Histone H3 lysine 27
HEK293T	Human embryonic kidney 293 T
HOXB7	Homeobox B7
HR	Homologous recombination
HSC	Hematopoietic stem cell

IDL	Insertion-deletion loop
IGF2	Insulin like growth factor 2
IM	Intermediate mesoderm
JNK	c-Jun N-terminal kinases
KU70/80	X-Ray Repair Cross Complementing 5 and 6
LEF	Lymphoid enhancer-binding factor
LGR	Leucine-rich repeat-containing G-protein coupled receptor
LIG4	DNA ligase IV
LRP	Low density lipoprotein receptor related protein
LS	Lynch Syndrome
MBD5	Methyl-CpG-binding domain protein 4
miRNA	Micro RNA
MLH	MutL homolog
MM	Metanephric mesenchyme
MMR	Mismatch repair
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
MSH	MutS Homolog
MSI	Microsatellite instability
MUTYH	MutY DNA glycosylase
MYCN	MYCN Proto-Oncogene
NEIL1	Nei Like DNA Glycosylase 1
NEIL3	Nei Like DNA Glycosylase 3
NER	Nucleotide excision repair
NFAT	Nuclear factor of activated T-cells
NHEJ	Non-homologous end joining
NPC	Nephron progenitor cell
OCT4	Octamer-binding transcription factor 4
OSR1	Odd-skipped-related 1
P	Postnatal day
PAX2/8	Paired box 2/8
PBS	Phosphate-buffered saline
PCP	Planar cell polarity
PLC	Phospholipase C
PMS2	Mismatch repair endonuclease PMS2
PRC	Polycomb repressive complex
PTA	Pre-tubular aggregate
RAC1	Ras-related C3 botulinum toxin substrate 1
RAD51	RAD51 Recombinase
RB	Retinoblastoma
RHOA	Ras homolog family member A
RISC	RNA-induced silencing complex
RNF43	Ring Finger Protein 43
ROR	RAR-related orphan receptors

RSPO	R-spondin
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
RYK	Receptor Tyr kinase
S	Synthesis
SCID	Severe combined immunodeficiency
siRNA	Small interfering RNA
SIX2	Sine Oculis Homeobox Homolog 2
SNP	Single nucleotide polymorphisms
ssDNA	Single stranded DNA
TARBP2	Trans-Activation Responsive RNA-Binding Protein
TC-NER	Transcription coupled NER
TCF	T-cell factor
TP53	Tumor protein p53
TRIM28	Tripartite Motif Containing 28
TSR	Thrombospondin type I repeat
UB	Ureteric bud
UD-NPC	Urine-derived nephron progenitor cell
UNG	Uracil DNA Glycosylase
V(D)J	variable (V), diversity (D) and joining (J)
WAGR	Wilms tumour, Aniridia, Genitourinary abnormalities and mental Retardation
Wg	Wingless
WNT	Homolog of the Drosophila wingless-type gene
WT	Wilms tumour
WT1	Wilms tumour 1
WTX	Wilms tumour on the X
XP	Xeroderma Pigmentosum
XPA	Xeroderma pigmentosum, complementation group A
XPO5	Exportin 5
ZNRF3	Zinc and Ring Finger 3

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Preface

This thesis is formatted in concordance with the guidelines set by the Faculty of Graduate and Postdoctoral studies at McGill University. Chapter 1 contains an introduction including a literature review supporting the rationale for the research. Chapter 2 contains a published manuscript accepted by PLoS One in 2019. Chapter 3 contains a manuscript to be submitted for publication in September 2020. Chapter 4 is a comprehensive discussion of all chapters and includes a brief discussion of future directions. Appendix I describes unpublished data. Appendix II contains a list of additional publications including reviews and conference abstracts.

Contribution to Original Knowledge

Key findings described in my thesis:

1. WT1 expression in nephron progenitor cells (NPCs) is necessary but not sufficient to render cells responsive to the inductive WNT9B signal from the ureteric bud (UB).
2. NPCs must express *Rspo1*, *Fzd5* and *Lrp6* prior to the arrival of the UB to optimally respond to the WNT9B signal. Expression of these components was confirmed in bona fide *Cited1*(+) NPCs isolated from embryonic kidney.
3. NPCs can be pre-treated with recombinant RSPO1 protein prior to infusion to increase the amount of cell integration into damaged renal tubules in a mouse with acute kidney injury.
4. DNA-repair genes are differentially expressed in embryonic kidney versus adult kidney.
5. Lineage specific expression of DNA-repair genes was detected in NPCs (*Neil3*) and UB cells (*Rad51b*, *Ung* and *Brca1*) of the developing kidney.
6. Enrichment of *Neil3* in cap mesenchyme NPCs is dependent on WT1, highlighted by the observation that *Wt1* knockdown in NPCs results in reduced *Neil3* expression.
7. WT1 directly binds to the *Neil3* promoter to activate transcription.

Contribution of Authors

Leah Hammond performed immunofluorescence imaging of Cited1/Tom kidneys (Figure 2.5A) and experiments described in Figures 3.1-3.4. Courtney Karner performed Frizzled in situ hybridization (Figure 2.2A). Nicolas Hastie donated the M15 cell line used in our studies as a model of an early nephron progenitor cell. Thomas Carroll was the supervisor of Courtney Karner when he performed the experiment included in Chapter 2. Murielle Akpa and Lee Lee Chu assisted with experimental design and analysis of results in Chapter 3. Caleb Tse Lalonde and Alexandre Goumba helped harvest embryonic kidneys from embryos in some experiments described in Chapter 3.

Chapter 1: Introduction

Kidneys are the filtration units of the body and play a pivotal role in removing metabolic waste from the bloodstream and maintaining the volume of fluids in the body. The nephron is the functional unit of the kidney which performs the filtration process and is made of a highly specialized structure which allows kidneys to adapt to changes in blood composition to maintain homeostasis (Lote. 2012). The nephron consists of two primary components: the renal corpuscle – composed of the glomerulus and the Bowman's capsule – and the renal tubule. The glomerulus is a network of capillaries delivering blood to the nephron. Pressure in the capillaries filters fluid and small molecules from the blood vessels into the Bowman's capsule where the filtrate continues into the renal tubules (Scott and Quaggin. 2015). The tubular portion of the nephron can be broken down into several segments; the proximal tubule, Loop of Henle, distal tubule and the collecting duct, with each segment having a highly specialized role in regulating the final composition of the filtrate (Lote. 2012). The structure allows for the reabsorption of nutrients, elimination of waste, maintenance of body pH and osmolality and regulation of blood pressure (McMahon. 2016). On average, humans have approximately one million nephrons per kidney and total nephron number in a mammalian kidney is established shortly after birth (Bertram et al., 2011). Unlike the well-studied Zebrafish model which has the ability to regenerate new nephrons during adult life (Reimschuessel. 2001), mammals cannot add new nephrons after kidney development has completed and have a very limited capacity to repair themselves from injury (Davidson. 2011). This highlights the importance of understanding the biology of nephron progenitor cells (NPCs) to ensure the developmental program proceeds normally to generate an optimal number of nephrons to sustain an individual throughout their lifetime.

1.1 Origins of Nephron progenitor cells

At the blastocyst stage of embryonic development, stem cell genes within the inner cell mass are highly expressed and genes responsible for tissue specificity and commitment to certain cell fates are downregulated. The downregulation of transcription occurs at the epigenetic level due to the activity of the polycomb repressive complex (PRC) (Surface et al., 2010). The PRC is made up of a number of proteins with the primary function of transferring methyl groups to histone H3 in gene promoters which marks transcription to become suppressed (Surface et al., 2010). In mouse

embryonic development, the endoderm, mesoderm and ectoderm begin to form around embryonic day (E) 6.5 (Kojima et al., 2014). At this stage, stem cells begin differentiation and are pushed towards specific cell fates. The mesoderm differentiates into three distinctive layers: the lateral plate mesoderm (forms the heart, blood vessels, lymphatic system, adipose), paraxial mesoderm (forms bone, muscle, cartilage and skin), and the intermediate mesoderm (forms kidneys, lower urinary tract and the reproductive system) (Gilbert. 2000).

1.1.1 Metanephric Mesenchyme the Nephron Progenitor Cell

Transcription factor Odd-skipped-related 1 (*Osr1*) marks the mesoderm around E6.5 but becomes restricted to the intermediate mesoderm due to gradients of growth factors inhibiting expression in the lateral plate and paraxial mesodermal layers (Di Giovanni et al., 2011; Wang et al., 2005). *Osr1* knockout mice fail to develop organs derived from the intermediate mesoderm including kidneys and gonads (James et al., 2006; Lan et al., 2011). On E8.5, a subset of OSR1(+) cells in the cranial section of the intermediate mesoderm (IM) differentiate into PAX2(+)/PAX8(+)/GATA3(+) epithelial cells of the paired nephric ducts (Bouchard et al., 2002; Grote et al., 2006). While a portion of the IM differentiates into the nephric duct, around E9 another compartment of the OSR1(+) IM begins to express Wilms tumour 1 (WT1) (Rackley et al., 1993). Similar to *Osr1* knockout mice, a *Wt1* knockout mouse also failed to develop kidneys (Schedl and Hastie. 1998). The OSR1(+)/WT1(+) IM goes on to form the metanephric mesenchyme (MM), a cell compartment containing the NPC population. Fate mapping studies demonstrated that the metanephric mesenchyme is solely derived from OSR1 expressing cells labeled at E7.5 (Mugford et al., 2008). As the nephric duct extends caudally, it reaches the MM which secretes glial-derived neurotrophic factor (GDNF). The GDNF signal stimulates an outgrowth from the nephric duct, the ureteric bud (UB), which invades the MM (Towers et al., 1998). Upon UB invasion, the MM begins to condense and form a “cap” of cells around the UB.

At this stage, cells within the cap mesenchyme begin to express a number of genes including *Cited1* followed by *Six2* (Boyle et al., 2008; Boyle et al., 2007; Lovvorn et al., 2007; Kobayashi et al., 2008; Self et al., 2006). *Cited1* has been accepted as the marker of a “bona fide” NPC as it appears to mark a population of uncommitted/uninduced NPCs (Mugford et al., 2009; Park et al.,

2012). Surprisingly, *Cited1* knockout does not affect nephrogenesis and mature kidneys form normally (Boyle et al., 2007). However, *Six2* knockout results in premature loss of the NPC pool and renal hypoplasia (Self et al., 2006). Lineage tracing studies determined all cell types of the mature nephron tubule except the collecting duct, were derived from both *Cited1*(+) or *Six2*(+) cells of the cap mesenchyme (Boyle et al., 2008; Kobayashi et al., 2008). Additionally, both *Cited1*(+) and *Six2*(+) cells have the ability to self-renew, allowing for the maintenance of the NPC pool as the kidney grows (Kobayashi et al., 2008; Boyle et al., 2008). Around E11.5 the UB branches to form a T-shape and it is at this stage where nephrogenesis begins (Fig 1.1A) (Dressler. 2009).

1.1.2 Differentiation of Nephron Progenitor Cells

Induction of nephrogenesis is dependent on secretion of WNT9B from the UB (Carroll et al., 2005). As the NPCs differentiate, they begin to form polarized epithelia in both a proximal-distal axis, extending the length of the nephron and also an apical-basal axis which forms the lumen of the tubules (Kobayashi et al., 2005; Kopan et al., 2007). The inductive WNT9B signal induces differentiation and migration of NPCs around the UB tip where they form WNT4/PAX8 expressing pre-tubular aggregates (PTAs) (Stark et al., 1994). In a stepwise fashion, the PTAs go on to form the renal vesicle followed by the S-shaped body. As development continues, the distal end of the S-shaped body fuses with the UB branch which forms the collecting duct of the mature nephron (Fig 1.1B) (Kobayashi et al., 2005). The medial portion of the S-shaped body matures into the proximal tubule and loop of Henle. Lastly, the proximal segment of the S-shaped body goes on to form the Bowman's capsule and podocytes of the glomerulus (McMahon. 2016). In mouse kidney development, UBs undergo twelve branching events and this process terminates on postnatal day 2 (P2) where on average 15,000 nephrons are formed and the population of NPCs can no longer be detected (Short et al., 2014).

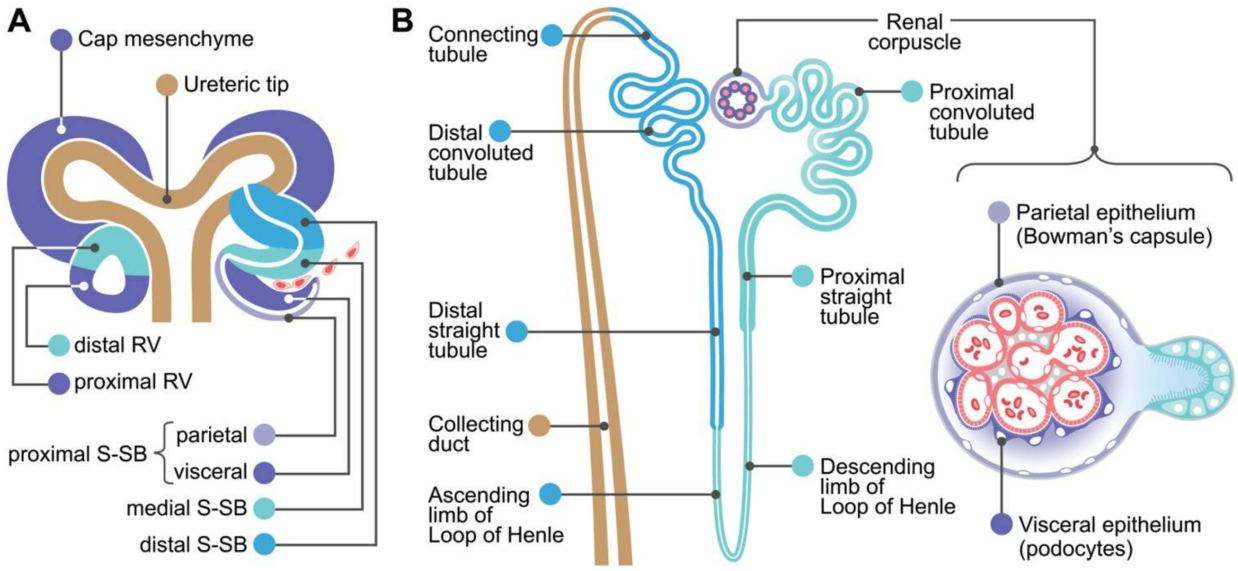

Figure 1.1. Schematic of nephrogenesis and nephron patterning.
A) Illustration of NPCs of the cap mesenchyme surrounding the ureteric bud (UB) tip, differentiating and migrating around the UB trunk to go on to form the renal vesicle (RV) (left half) and eventually the S-shaped body (S-SB) (right half). B) Illustration of a mature nephron, colour coded to represent the early compartments they are derived from. The collecting duct arises from the UB, the distal convoluted tubule arises from the distal S-SB, the proximal straight tubule and descending limb of the Loop of Henle arise from the medial S-SB and the renal corpuscle arises from the proximal S-SB. Image adapted from McMahon (2016) Current Topics in Developmental Biology (McMahon. 2016).

1.2 WNT-signalling overview

The WNT-signalling pathway can be divided into two major branches: the canonical signalling pathway and non-canonical signalling pathway, which both play an essential role in embryonic development and adult tissues. During development, the WNT-signalling pathway is involved in not only maintaining self-renewal of stem/progenitor cells but also inducing stem/progenitor cells to differentiate. It also plays a role in cell polarity and convergent extension during embryogenesis via the non-canonical/planar cell polarity pathway (Andre et al., 2015). WNT-signalling also maintains an important role in adult tissues, including maintaining the stem cells population in the epithelial crypts of the small intestine (Korinek et al., 1998; Fevr et al., 2007), generating osteoblasts in bone (Bennett et al., 2005; Day et al., 2005) and also hair follicles stem

cells (Huelsken et al., 2001; Andl et al., 2002). In humans, there are a total of 19 WNT ligands, which are glycoproteins secreted into the extracellular matrix. Due to the addition of palmitoleic acids in the endoplasmic reticulum (ER) (Takada et al., 2006; Willert et al., 2003), WNTs become hydrophobic and have a limited capacity to diffuse in aqueous environments. Therefore, they bind to cell surface receptors in an autocrine or paracrine manner (Bhanot et al., 1996; Wehrli et al., 2000). The primary WNT-receptor is a heterodimeric complex consisting of one Frizzled (FZD) and one Low density lipoprotein receptor related protein (LRP). In humans, there are a total of 10 FZD receptors and 2 LRP receptors involved in WNT-signalling. The co-receptor combination determines WNT-ligand specificity and also the downstream effector molecules that are activated.

1.2.1 Canonical WNT-signalling pathway

The canonical WNT-pathway is also referred to as the β-catenin (CTNNB1) pathway. β-catenin is a multifunctional protein involved in maintaining cell structure integrity through interaction with E-cadherin (Ozawa et al., 1989) and also functions as a transcriptional co-activator via interaction with the TCF/LEF family of transcription factors (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996). The ability of β-catenin to mediate cell signalling was discovered due to the observation of high homology between β-catenin and Drosophila protein, Armadillo. In Drosophila, the Wingless (Wg) protein is homologous to WNT, and Armadillo was shown to be essential in mediating Wg signalling (Siegfried et al., 1994). Further studies demonstrated that Wg signalling resulted in accumulation of Armadillo in the cytoplasm (Peifer et al., 1994). This observation was validated in a vertebrate model where Wnt1 expression resulted in cytoplasmic β-catenin accumulation. In the absence of a WNT ligand, β-catenin is continuously ubiquitinated and targeted for degradation by the β-catenin destruction complex. The destruction complex is made up of Axin, adenomatosis polyposis coli (APC), casein kinase 1 alpha (CK1α), and glycogen synthase kinase 3 beta (GSK3β) (Behrens et al., 1998; Hart et al., 1998; Munemitsu et al., 1995; Rubinfeld et al., 1996; Rubinfeld et al., 1993; Amit et al., 2002; Liu et al., 2002). GSK3β phosphorylates serine/threonine residues of β-catenin which targets it for proteasomal degradation. Upon WNT binding, cytoplasmic protein Dishevelled (DVL) is recruited to the plasma membrane and interacts with the intracellular domain of the FZD receptor (Cong et al., 2004;

Tauriello et al., 2012). DVL then interacts with Axin of the destruction complex and sequesters it at the plasma membrane (Cliffe et al., 2003). This process brings kinases GSK3β and CK1γ in close proximity to the LRP receptor which subsequently gets phosphorylated (Tamai et al., 2004; Zeng et al., 2005; Davidson et al., 2005). Phosphorylated LRP inhibits GSK3β activity and prevents β-catenin phosphorylation, allowing for cytoplasmic accumulation and nuclear translocation to activated transcription of WNT target genes (Fig 1.2) (Cselenyi et al., 2008; Piao et al., 2008; Stamos et al., 2014).

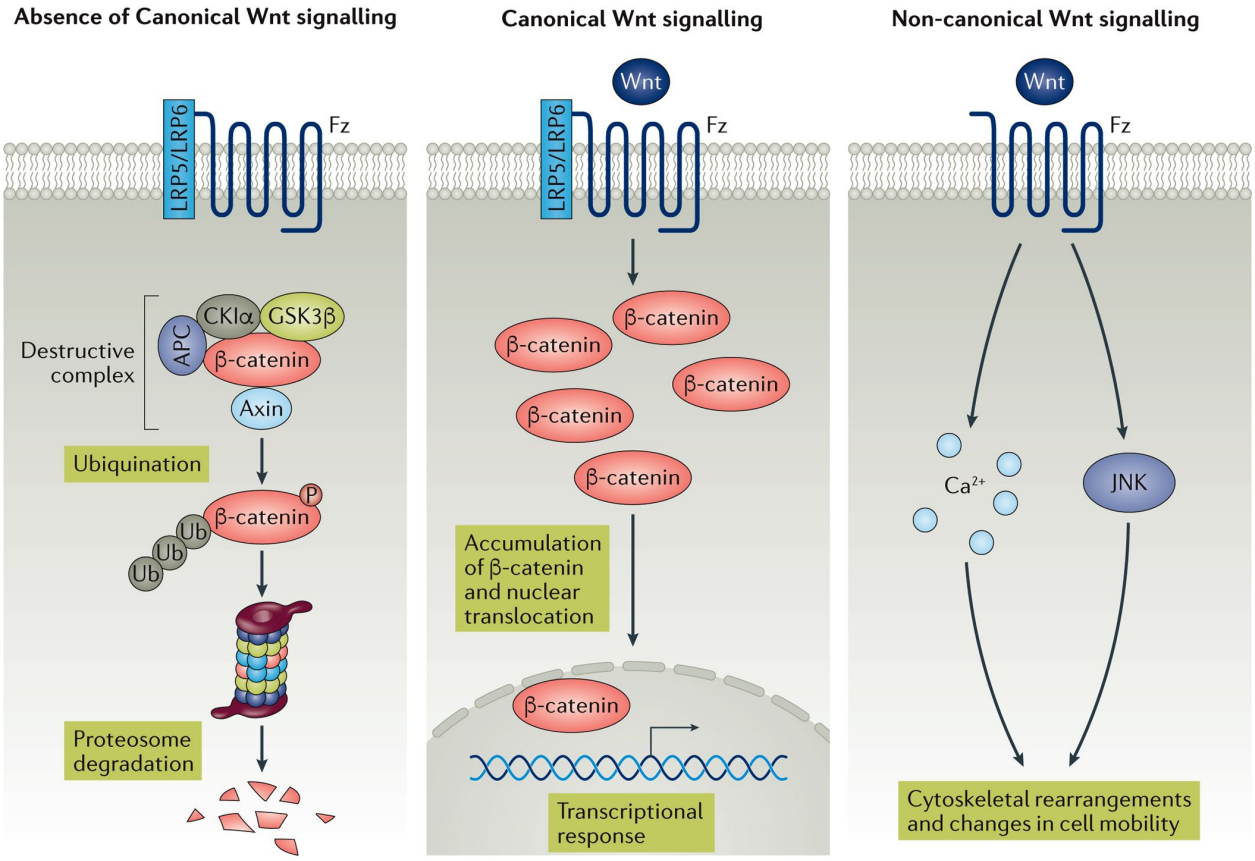

Figure 1.2. Overview of canonical and non-canonical WNT-signalling pathways.
For canonical WNT-signalling, in the absence of a WNT ligand, the destruction complex ubiquitinates B-catenin and targets it for proteasomal degradation (left). Upon binding of a WNT-ligand, the destruction complex is inactivated and allows B-catenin to accumulate in the cytoplasm and translocated into the nucleus to upregulate transcription of target genes (middle). Non-canonical WNT-signalling activation can either result in increased intracellular calcium levels

or activation of small GTPases including JNK (right). Image adapted from Monteagudo and Lories (2017) Nature Review Rheumatology (Monteagudo and Lories. 2017).

1.2.2 Canonical-WNT signalling modulation

The canonical WNT-signalling pathway can also be modulated in the presence of agonists. R-spondins (RSPO), a recently discovered family of WNT-agonists, has been the focus of many groups investigating canonical WNT-signalling. Genetic screening identified a novel gene expressed in the developing roof plate containing a thrombospondin type I repeat (TSR) domain (Kamata et al., 2004). Three other members of the family were discovered (Chen et al., 2002; Kazanskaya et al., 2004; Kim et al., 2005) and found to have conserved features including: an N-terminal signal sequence, important for a secreted protein, a TSR domain and also two adjacent furin repeats (Kim et al., 2005). RSPOs have been shown to play a role in female sex determination (Parma et al., 2006), limb (Bell et al., 2008) and facial bone development (Jin et al., 2011), cardiac development (Cambier et al., 2014) and nail development (Blaydon et al., 2006).

The ability of R-spondins to modulate canonical WNT-signalling activity was shown when Rspo2 and Wnt3a were transfected into HEK293T cells expressing a β-catenin luciferase reporter. The presence of Rspo2 stabilized β-catenin and increased luciferase activity compared to baseline levels (Kazanskaya et al., 2004). Knockout of either furin repeat, but not the TSR domain, reduced luciferase signal to baseline levels. Subsequent studies identified the Leucine-rich repeat-containing G-protein coupled receptor (LGR) family, specifically LGR4/5/6 as the receptor for RSPO ligands (Carmon et al., 2011; Carmon et al., 2012; de Lau et al., 2011; Ruffner et al., 2012; Glinka et al., 2011). The furin domains in RSPO1 and RSPO3 were shown to be essential for binding to the extracellular domain of LGR4/5 receptors (Glinka et al., 2011; Peng et al., 2013). Knockdown of LGR4 using small interfering RNA (siRNA) in HEK293T cells diminished the RSPO mediated increase in WNT-signalling (de Lau et al., 2011). Prior to identifying the ability of RSPOs to bind to LGR receptors, an association was already established in the epithelial crypts of the small intestine. Rspo1 knock-in mice exhibited hyperplasia within the intestinal crypt (Kim et al., 2005). A few years later, LGR5 was identified as a marker for the stem cell population within the

intestinal crypt as single sorted LGR5(+) cells were able to generate properly oriented crypt-villus structures in vitro (Sato et al., 2009).

Although RSPOs do not directly bind the FZD/LRP co-receptor, they can modulate canonical WNT-signalling through several mechanisms. The primary mechanism involves interaction with canonical WNT-signalling antagonists Zinc and Ring Finger 3 (ZNRF3) and Ring Finger Protein 43 (RNF43). Both proteins are E3-ubiquitin ligases that localize at the plasma membrane (Hao et al., 2012). Overexpression of ZNRF3 reduced activation of the canonical WNT-signalling pathway (Hao et al., 2012). Further analysis demonstrated that ZNRF3 knockout resulted in increased LRP6 phosphorylation and increased levels of FZD4/5/6/8 in the plasma membrane. ZNRF3 inhibits WNT signalling by ubiquitylation of FZD receptors, targeting them for lysosomal degradation. Results of adding RSPO1 to these cells mirrored the results of ZNRF3 knockdown (Hao et al., 2012). This also resulted in decreased plasma membrane levels of ZNRF3 and this process was determined to be dependent on the presence of LGR4. Membrane clearance of ZNRF3 resulted in decreased ubiquitylation of FZD receptors, ultimately leading to increased levels of the WNT co-receptor in the plasma membrane. Crystal structure analysis showed that the first furin domain within RSPO2 has high affinity to the extracellular domains of ZNRF3 and RNF43, while the second furin domain binds to the LGR receptor (Zebisch et al., 2013). Additionally, a binding assay studying the interaction between RSPO1, LGR5 and RNF43 identified that RSPO1 bound to LGR5 has higher affinity to RNF43 than RSPO1 alone (Chen et al., 2013). This highlights the important role the LGR receptor plays in stimulating agonist activity of RSPOs. However, a recent study identified an LGR-independent mechanism of RSPO agonist activity. Specifically, furin domains of RSPO2 and RSPO3 were able to directly bind to ZNRF3 and RNF43 to increase WNT-signalling activity. RSPO3 was also shown to bind to heparan sulfate proteoglycans via the TSR domain to increase WNT activity (Lebensohn and Rohatgi. 2018). The redundancy in activation of RSPO agonist activity highlights the importance of this pathway. In other words, there is a built-in fail-safe in the event that one mechanism is faulty.

1.2.3 Non-canonical WNT-signalling pathway

The non-canonical WNT-signalling pathway can be further broken down into two main branches: the planar cell polarity (PCP) and the WNT-Ca^{2+} pathway. Activation of the PCP pathway involves a WNT ligand binding to a FZD or RAR-related orphan receptors (ROR) receptor and activating a number of small GTPases, primarily Ras-related C3 botulinum toxin substrate 1 (RAC1), Ras homolog family member A (RHOA), and c-Jun N-terminal kinases (JNK) (Gómez-Orte et al., 2013). The PCP pathway plays an essential role in regulating gastrulation and neural tube closure during development (Wallingford and Harland. 2001; Wallingford et al., 2000; Heisenberg et al., 2000). The WNT-Ca^{2+} pathway is activated when a WNT binds to either a FZD or a receptor Tyr kinase (RYK) receptor which results in downstream activation of phospholipase C (PLC) and ultimately the release of calcium from intracellular stores and activation of nuclear factor of activated T-cells (NFAT) (Niehrs. 2012). This pathway plays a role in determining cell fate during development and also in inflammation, cancer and neurodegeneration in adult tissues (De. 2011). In the developing kidney, non-canonical WNTs play an important role as demonstrated by mouse knockout models. Mouse Wnt5a knockout resulted in a defect in the positioning of the metanephric mesenchyme which altered the normal developmental process, inducing ureteric bud formation in the wrong location and also interfered with ureteric bud branching (Nishita et al., 2014; Pietilä et al., 2016). Wnt11 knockout results in hypoplastic kidneys due to defects in ureteric bud branching (Nagy et al., 2016; Majumdar et al., 2003). While the non-canonical WNT pathway is essential for normal kidney development, the impact on survival and proliferation of NPCs in the metanephric mesenchyme appears to be minimal.

1.2.4 WNT-ligand specificity

FZD receptors contain N-terminal cysteine-rich domains (CRDs) which determine the specificity to WNT ligands. Crystal structure analysis of a WNT8/FZD8-CRD receptor complex identified a unique “hand” structure with two finger-like projections gripping the FZD8-CRD domain at two distinct sites (Janda et al., 2012). Amino acid residues in these two binding sites appear to be highly conserved in WNTs and FZD receptors, explaining the ability of WNT ligands to bind to several FZD receptors. However, phylogenetic analysis of human FZD receptors identify five subgroups: 1) Fzd1, Fzd2 and Fzd7; 2) Fzd3 and Fzd6; 3) Fzd5 and Fzd8; 4) Fzd9 and Fzd10; 5) Fzd4

(MacDonald and He. 2012). A subsequent study investigated the binding affinity of WNT ligands to various FZD receptors. For example, WNT3A was shown to have high binding affinity to FZD4/5/7/8 and intermediate binding affinity to FZD1/2. In contrast, WNT4 only bound to FZD8 with high affinity and had weak binding affinity to FZD1/2/4/5/7 (Dijksterhuis et al., 2015). Although FZDs and WNTs have highly conserved regions allowing cross-reactivity between several members of each family, receptor specificity still exist in the context of canonical WNT-signalling.

LRP5/6, the second component of the canonical WNT co-receptor complex also has the ability to bind to WNT-ligands. Multiple WNT binding sites have been identified within the N-terminal domain of LRP6 and have different binding affinities for each WNT ligand. For example, WNT5A and 5B were unable to bind to LRP6 in contrast to WNT9B which had extremely high binding affinity (Bourhis et al., 2010). It was also shown that both WNT9B and WNT3A were able to bind LRP6 simultaneously, indicating there are multiple binding sites within the LRP receptor. Blocking sites using specific antibodies revealed groups of WNTs that preferentially bind to one site over the other (Gong et al., 2010).

Given the complex nature of the binding of WNT ligands to their various receptors, it is possible for specific WNTs to activate both canonical and non-canonical pathways depending on the cell surface environment. WNT5a for example, has been well established as a strong non-canonical WNT, however, studies have also demonstrated its ability to activate the canonical pathway in the presence of FZD4 and LRP5/6 in the target cell plasma membrane (Fu et al., 2016; Ring et al., 2014).

1.2.5 WNT-signalling in the developing kidney

A seminal discovery from the 1950s demonstrated the ability of isolated metanephric mesenchyme to form renal tubules when cultured with embryonic spinal cord (Grobstein. 1956). This result showed that the renal progenitor cell population responsible for nephrogenesis was contained within the metanephric mesenchyme. It also highlighted the requirement for NPCs to receive an extrinsic signal to initiate differentiation, however, the specific molecule had yet to be

identified. About four decades later, co-culturing metanephric mesenchyme with WNT1 expressing cells also initiated nephrogenesis (Herzlinger et al., 1994). WNT4 knockout mice were also studied and mutant mice were unable to form pre-tubular aggregates (Stark et al., 1994). WNT4 is expressed in the metanephric mesenchyme from E11.5 and expression is maintained within the S-shaped body and the distal segment of the nephron which fuses to the collecting duct. These results highlighted the role WNT-signalling plays in differentiating NPCs, however the specific WNT-ligand in the developing kidney was not yet identified. WNT9b expression was identified in the developing kidney (Qian et al., 2003) and later shown to be specifically expressed in the nephric duct and ureteric bud (Carroll et al., 2005). Using a WNT9b knockout mouse model, mutant mice fail to initiate tubulogenesis and only form kidney rudiments at birth (Carroll et al., 2005). However, WNT9b can activate both canonical and non-canonical WNT pathways. To determine the specific pathway involved in initiating nephrogenesis, a group knocked out β-catenin in *Six2*(+) cells in the cap mesenchyme. The resulting kidney phenotype resembled the phenotype of *Wnt9b* knockout mouse, indicating WNT9B is signalling through the canonical WNT pathway to initiate nephrogenesis (Park et al., 2007). To further corroborate this observation, β-catenin was found to be both necessary and sufficient to induce expression of WNT9B target genes in an ex vivo organ culture model (Karner et al., 2011). Additionally, high levels of canonical WNT-signalling activity were observed in both the metanephric mesenchyme and ureteric bud tip using a β-catenin reporter mouse (Iglesias et al., 2014). Canonical WNT agonists RSPO1 and RSPO3 were shown to be expressed in the cap mesenchyme at E10.5 (Vidal et al., 2019). RSPO receptors LGR4 and LGR5 were also detectable at early stages in the developing kidney, however knockout of both receptors had no effect on kidney morphogenesis (Vidal et al., 2019). The importance of the canonical WNT-pathway has been well established in the developing kidney, however, a complete picture of the specific molecular components involved in transducing the WNT9B-signal has yet to be established.

1.3 Kidney Injury overview

Acute kidney injury (AKI) is a disorder characterized by a sudden loss of renal function, primarily a decrease in glomerular filtration rate (GFR), and subsequent increase in serum creatinine levels and a decrease in urine output (Levey and James. 2017). AKI occurs in approximately 13 million

people and accounts for 1.7 million deaths worldwide every year (Mehta et al., 2015). The mortality rate of patients receiving the current therapies, including renal replacement therapy (dialysis) is approximately 50% (Levey and James. 2017). Individuals that survive an episode of AKI are often left with permanent renal damage that develops into chronic kidney disease (CKD) and end-stage renal disease (ESRD) (Parr and Siew. 2016; Takaori et al., 2016). This highlights the need for advances in therapeutic options in this field of study.

Analysis of zebrafish kidneys in response to injury, identified the capacity for new nephron formation in adult life from a pool of NPCs (Diep et al., 2011). Adult zebrafish develop a limited number of nephrons at birth, making this mechanism essential for maintenance of renal function throughout their lifetime. Mammals on the other hand, generate thousands to millions of nephrons at birth but do not have the capacity to form new nephrons, however, nephrons do show the ability to repair themselves to a certain extent after injury. The mechanism describing how repair is achieved is a highly debated topic in nephrology.

1.3.1 Repair of kidney injury in mammalian kidneys

The proximal tubule section of the nephron is responsible for reabsorbing a majority of molecules within the glomerular filtrate. Due to the increased metabolic activity within this compartment, proximal tubule cells are often highly damaged in response to injury (Chang-Panesso et al., 2019). Interestingly, very little apoptosis is observed in proximal tubule cells in response to induced kidney injury in mice, instead, the cells appear to undergo a burst of proliferation (Guo et al., 2012). Additionally, cells in the proximal tubule have been shown to re-express proteins typically expressed during nephrogenesis including PAX2 (Villanueva et al., 2006). As previous mentioned, the source of these nephrogenic cells is still debated. One of the two prevailing theories of how mammalian kidneys repair themselves in response to injury argues that epithelial cells within the mature nephron, de-differentiate into mesenchymal progenitor cells that go on to repopulate the nephron (Bonventre. 2003; Kusaba et al., 2014). The second theory argues the existence of a pool of progenitor cells residing in the adult kidney with the ability to differentiate and integrate into a damaged nephron (Angelotti et al., 2012; Sagrinati et al., 2006). Nonetheless, episodes of induced AKI in mice often resolve within 7 days as demonstrated by a decrease in serum

creatinine, a biomarker for kidney function (Korrapati et al., 2012). Regardless of the source of cells, mammalian kidneys are able to initiate repair of injured tubular epithelia and in many cases, individuals can regain full kidney function after injury.

On the other hand, the repair process is not always completed normally. Incomplete/maladaptive repair of AKI can also occur which results in the formation of renal scarring/fibrosis and over time can progress into CKD (Ferenbach and Bonventre. 2015; Ronco et al., 2019). One mechanism AKI develops into CKD is though repeated episodes of acute injury. Once weekly induction of AKI in a mouse model led to elevated levels of inflammation in addition to increased expression of pro-inflammatory cytokines and profibrotic markers (Grgic et al., 2012). Another group demonstrated that expression of kidney injury molecule 1 (KIM-1) enhances repair of AKI in the short term, however, prolonged expression after injury increases kidney fibrosis and results in CKD (Humphreys et al., 2013; Yang et al., 2015). Increased fibrosis after AKI has also been linked to the appearance of myofibroblasts in the injured region which deposits collagen and other components of extracellular matrix (Humphreys et al., 2010; Bonventre and Yang. 2011). Therefore, it is evident that AKI needs to be resolved quickly and efficiently to prevent progression into CKD.

1.3.2 Cell therapy of AKI

In an attempt to aid the kidneys natural ability to repair itself, several groups have started looking into infusing cells capable of integrating into damaged renal tubules. Studies of embryonic mouse kidney identified CD24 as a cell surface marker that identifies the NPCs of the metanephric mesenchyme (Challen et al., 2004).

In humans, a population of quiescent cells have been identified as adult renal progenitor cells (ARPCs) (Sagrinati et al., 2006). As found in mice, ARPCs also express CD24, however they also express another cell surface marker, CD133. Additionally, ARPCs express transcription factors generally upregulated in stem cells, OCT4 and BMI1 (Sagrinati et al., 2006). Expression of specific cell surface markers makes this population of progenitor cells easy to isolate by flow cytometry. In vitro analysis demonstrated the capacity of ARPCs to self-renew and also differentiate into

several cell types, including proximal and distal tubule epithelial cells, adipocytes and osteogenic cells. In mice, AKI is modeled using intramuscular injections of glycerol which induces rhabdomyolysis. Muscle breakdown products enter the bloodstream where they are subsequently filtered by the kidney and specifically induce proximal tubule injury (Westenfelder et al., 1980). Using the glycerol-injury model, CD24(+)/CD133(+) cells isolated from human kidney demonstrated the ability to integrate into damaged nephrons and improve histological and functional readouts of AKI when injected into severe combined immunodeficient (SCID) mice (Lazzeri et al., 2007; Sagrinati et al., 2006). In addition to providing evidence of the multipotent nature of CD24(+)/CD133(+) cells, these studies also highlighted the potential use of a cell-based therapy to treat AKI. However, the source of the CD24(+)/CD133(+) cells highlights a major limitation of these studies, as they were isolated from aborted embryos or nephrectomised adult kidneys.

1.3.3 Minimally invasive sources of NPCs

Recent studies have investigated minimally invasive methods to isolate progenitor cells. In the 1960s/70s, it was shown that cells could be isolated and cultured from amniotic fluid and urine of newborn babies (Van Leeuwen et al., 1965; Sutherland and Bain. 1972). Amniotic fluid derived cells are multipotent and maintain characteristics of both embryonic and adult stem cells (De Coppi et al., 2007). Injection of amniotic fluid cells into an AKI mouse model resulted in improved recovery, however, the cells did not integrate into the damaged nephron (Rota et al., 2012). Instead, the cells were predominately located in peritubular regions, similar to results using hematopoietic stem cells (Morigi et al., 2008), suggesting they aid recovery through secretion of survival/growth factors and/or cytokines.

The lack of nephron integration observed by these studies led the field to shift focus to identify an easily isolated population of cells that resemble an NPC of the developing kidney. This was accomplished when characterizing cells isolated from neonatal urine, collected from newborns with a gestational age of 31-36 weeks. Gene expression and flow cytometry analysis showed that they express key markers of NPCs of the cap mesenchyme, including SIX2, CITED1 and cell surface receptor CD24 (Arcolino et al., 2016). Additionally, tubular epithelial cells treated with an

apoptosis-inducing agent were protected when co-cultured with urine-derived NPCs. Interestingly, NPCs could also be isolated from adult urine, although they had a slightly different expression profile. Adult urine-derived NPCs expressed CITED1, CD24 and CD133 but not SIX2 (Arcolino et al., 2016). Mice with AKI injected with adult urine-derived NPCs showed improved renal function and histology following injury compared to control mice (Tian et al., 2017; Sun et al., 2019; Zhang et al., 2020; Xiong et al., 2020). However, cells used in these studies were only characterized based on cell surface markers and not CITED1 and SIX2. Taking a closer look at immunofluorescence and histology images published by these groups, integration of NPCs into renal tubules can be observed, however, the number of integrated cells is somewhat limited. It is possible that the effectiveness of the injected NPCs could be increased if they express the correct NPC markers.

A potential method to augment cell integration is to take advantage of the canonical WNT-signalling pathway. Our laboratory previously demonstrated that the canonical WNT-signalling pathway is essential for cell integration into a mouse with AKI (Zhang et al., 2015). CD24+ cells from embryonic mouse kidneys showed high levels of integration into a glycerol injured mouse. However, pre-treating cells with canonical WNT-signalling inhibitor, IWR-1, significantly reduced the amount of integration. The status of the canonical WNT-signalling pathway has yet to be described in urine-derived NPCs. We hypothesize that priming NPCs with key molecules involved in enhancing the canonical WNT-signalling pathway will increase integration into renal tubules after AKI.

1.4 Wilms Tumour Overview

The importance of the WNT-signalling pathway in NPCs to allow nephrogenesis to proceed normally has been described, however, dysregulation of this pathway can lead to renal abnormalities, in particular, formation of precursor lesions that often result in Wilms Tumour (WT) development. WTs account for 90% of all pediatric renal tumours with an incidence of 8 cases per million children in the United States (Treger et al., 2019; Davidoff. 2009). WTs usually develop before the age of 5 years old and can present as unilateral (one kidney), bilateral (both kidneys) or multifocal (multiple tumours in one kidney) tumours (Rivera and Haber. 2005).

Unilateral cases of WT often arise sporadically and account for approximately 90% of WTs, while the bilateral and multifocal cases often contain inherited mutations or de novo germline mutations (Rivera and Haber. 2005; Treger et al., 2019). One of the first descriptions of WT was published in 1880 by William Osler. Although, at that time it was not named WT, he described two children with renal tumours containing both muscle fibres and epithelial cells (Osler. 1880). In 1899, Max Wilms published a monograph describing "mixed" tumours of the kidney (Coppes-Zantingal and Coppes. 1999; Wilms. 1899). Using histological analysis, he provided the first highly detailed microscopic description of what became to be known as WT. In this article, he describes tumours with a "triphasic" histology containing a mix of blastemal, epithelial and stromal cells. A recent microarray analysis of 300 WT samples identified three "archetypes" in which the tumour was classified as being predominantly blastemal, epithelial or stromal. Tumours that fell within the blastemal archetype over-expressed genes of the cap mesenchyme (including Cited1 and Six2), stromal WTs over-expressed genes of the surrounding uninduced mesenchyme and epithelial WTs over-expressed nephric epithelial markers (Trink et al., 2018). This demonstrates WTs develop from developmentally arrested NPCs.

The initial observation of the WT histology was crucial in determining the mechanism in which WTs develop, as it became evident that WTs developed as a result of abnormal nephrogenesis. WTs contain characteristic lesions referred to as "nephrogenic rests" which are detected in 100% of bilateral/multifocal WTs and 30-40% of sporadic WTs (Rivera and Haber. 2005). Nephrogenic rests are rarely found in normal kidneys and usually disappear in the first year of life (Park et al., 1993). Characterization of the lesions demonstrated that they are clusters of undifferentiated NPCs from the metanephric mesenchyme that failed to differentiate in response to the WNT9B signal from the UB (Bove and McAdams. 1978; Machin. 1980). Genetic analysis of two WT cases helped identify the role nephrogenic rests play in WT formation. Both subjects did not have germline WT1 mutations, however, identical somatic mutations in *WT1* were detected in both the tumour tissue and nearby nephrogenic rests, suggesting the WT is a clonal derivative of the nephrogenic rest (Park et al., 1993).

Knudson's two-hit hypothesis, first established when studying inherited versus sporadic cases of retinoblastoma (Rb), highlighted a feature that certain genes linked to tumourigenesis likely require mutations in both alleles and that these genes function as tumour suppressors. This theory is based on the observation that inherited cases of Rb developed at an earlier age than sporadic cases (Knudson. 1971). He theorized that inherited cases only require one additional mutation compared to sporadic cases which require two separate mutational events. Knudson also applied this hypothesis to WTs where he observed the same trend. Inherited cases of WTs developed at an earlier age and were often bilateral instead of unilateral when compared to sporadic cases, implying the gene responsible for Wilms tumourigenesis likely functions as a tumour suppressor (Knudson and Strong. 1972).

1.4.1 WT1 and canonical WNT-signalling in Wilms Tumour

The first gene associated with Wilms tumourigenesis was *WT1* and was discovered by studying subjects with WAGR syndrome, a disorder in which WTs develop in approximately 50% of subjects (Muto et al., 2002). WAGR syndrome was linked to deletions within chromosome band 11p13 (Francke et al., 1979; Riccardi et al., 1978). A decade later, *WT1* was identified as a zinc-finger transcription factor located within that region (Call et al., 1990; Gessler et al., 1990). As described in section 1.1.1, WT1 was subsequently shown to be essential for normal kidney development and is also mutated in approximately 20% of WTs.

Interestingly, activating mutations in *CTNNB1* are detected in approximately 15% of WTs (Koesters et al., 1999; Maiti et al., 2000). In one report, 95% of the analyzed WTs containing *CTNNB1* mutations also contained a *WT1* mutation (Maiti et al., 2000). *CTNNB1* mutations in WTs alter the serine residue important for phosphorylation by the destruction complex (Kusafuka et al., 2002; Amit et al., 2002). This prevents *CTNNB1* from being marked for ubiquitination and proteasomal degradation, allowing for constitutive cytoplasmic and nuclear accumulation which drives rapid cell growth (Koesters et al., 2003). Microdissection analysis detected a *CTNNB1* mutation in the tumour tissue but not nephrogenic rests, however, Wt1 mutations were detected in both suggesting the *CTNNB1* mutation is a late event in Wilms tumourigenesis (Fukuzawa et al., 2007). Loss of *WT1* and subsequent downregulation of *CTNNB1* appears to put a selective

pressure to drive proliferation which may explain the increased frequency of *CTNNB1* mutations in WT1-negative WTs.

Recently, our laboratory discovered that WT1 directly suppresses transcription of PRC2 complex member, Enhancer of zeste homolog 2 (*EZH2*). EZH2 is a methyltransferase that methylates histone H3 lysine 27 (H3K27) which generally results in downregulation of transcription. Specifically, the *CTNNB1* promoter is targeted by EZH2 and in vitro overexpression of WT1 or knockdown of *EZH2* decreased levels of repressive H3K27 marks and increased *CTNNB1* mRNA transcript levels (Akpa et al., 2015). Epigenetic analysis of active and repressive histone H3 marks revealed WTs resemble embryonic stem cells more than adult kidneys, with number of key differentiation genes epigenetically silenced in the tumour (Aiden et al., 2010). Together, these results suggest that loss of *WT1* during kidney development increases formation of nephrogenic rests due to increased EZH2 expression resulting in epigenetic silencing of differentiation genes. This once again highlights that WTs form as a result of arrested development.

1.4.1.1 Other Wilms Tumour mutations

Recently, a number of WT mutations have been identified in microRNA processing genes including loss of function mutations in *DROSHA*, *DICER1*, *DGCR8*, *XPO5* and *TARBP2* (Wegert et al., 2015; Wu et al., 2013; Walz et al., 2015; Rakheja et al., 2014; Torrezan et al., 2014). Interestingly, with regard to the DROSHA mutation, in the subset of WTs studied, 9% of the samples contained WT1 mutations, however, 40% WTs with DROSHA mutations also contained a WT1 mutation (Torrezan et al., 2014). Our laboratory also showed WT1 significantly alters miRNA profiles within a cell and interacts with members of the RNA-induced silencing complex (RISC), DICER1 and AGO2, to mediate silencing of some genes, specifically EZH2 (Akpa et al., 2016). Although DROSHA functions upstream of the RISC complex and is involved in generating mature miRNAs, loss of WT1 may put a selective pressure on all miRNA processing machinery in order to push WT1-null cells to proliferate.

Additional mutations in a number of genes have been identified in WTs including: genes commonly mutated in various cancers TP53 (Maschietto et al., 2014; Ooms et al., 2016), MYCN

(Williams et al., 2015) and IGF2 (Scott et al., 2012; Satoh et al., 2006)]; kidney development markers SIX1, SIX2 (Walz et al., 2015; Wegert et al., 2015) and AMER1 (formerly WTX) (Wegert et al., 2009; Rivera et al., 2007).

1.4.1.2 WT1 as a master regulator

Together, it appears WT1 acts as a master regulator during kidney development and also plays a key role in understanding Wilms tumourigenesis. Given the frequent occurrence of secondary mutations within WT1-negative tumours, it leads one to question the genomic stability of these cells. This is an area that, to this point, has not been well studied. During kidney development, high levels of proliferation can be detected in the cap mesenchyme in E14.5 kidneys and continues until nephrogenesis completes around P1/P2 (O'Hara et al., 2019; Saifudeen et al., 2002). Studies of both human and mouse embryonic stem cells (ESCs) demonstrate enhanced DNA repair mechanisms when compared to differentiated cells (Maynard et al., 2008; Saretzki et al., 2004). Proliferating cells activate a cell cycle checkpoint in response to DNA damage to protect the genomic integrity of the daughter cells (Bartek and Lukas. 2007). CHEK1, a protein involved in the DNA-damage induced cell cycle checkpoint, is expressed in proliferating cells but is downregulated in terminally differentiated cells (Lukas et al., 2001). Comparison of naïve and differentiated mouse ESCs showed downregulation of a number of DNA repair genes after differentiation (Saretzki et al., 2008). It is evident that DNA-repair genes are differentially expressed in proliferating and differentiating cells. NPCs of the developing kidney would likely require a mechanism to upregulate DNA repair genes during this period of robust proliferation. However, investigation of DNA-repair genes in NPCs, in particular, WT1-dependent repair genes has yet to be completed. This study would not only provide insight into the normal developmental process but may also explain a potential mechanism of genomic instability in WT1(-) nephrogenic rests.

1.4.2 DNA repair

Maintenance of genomic integrity is a critical cellular function. DNA polymerases are enzymes that catalyze the addition of nucleotides to newly synthesized DNA during replication. Although eukaryotic DNA polymerases are considered to be high fidelity, meaning they make few errors,

it is estimated that an error is made every 10^4 to 10^5 nucleotides, however mutations are only detected every 10^9 to 10^{10} base pairs per cell division (McCulloch and Kunkel. 2008; Kunkel. 2009; Hsieh and Yamane. 2008). This discrepancy is due to the DNA repair pathways allowing mutations to be corrected. During replication, errors are corrected by two separate mechanisms: intrinsic 3'→5' exonucleolytic proofreading activity within the DNA polymerase and also through activation of mismatch repair (MMR). The 3'→5' exonucleolytic activity of DNA polymerases switches the enzyme into editing mode to excise incorrectly added nucleotides (Shevelev and Hübscher. 2002). MMR identifies mismatched bases that escaped proofreading by the DNA polymerase and also small insertions/deletions that may have been incorporated (Jiricny. 2006). When an error is detected, it degrades the damaged region and allows the DNA polymerase to continue DNA synthesis. In addition to mutations introduced by DNA replication machinery, cells are constantly exposed to a number of endogenous and exogenous genotoxic stresses which damages DNA. Endogenous stresses include oxidative damage caused by reactive oxygen species, DNA methylation, spontaneous base deamination, and abasic sites. Exogenous stresses include both ionizing and ultraviolet radiation in addition to a number of chemical agents that can either be inhaled or ingested (Chatterjee and Walker. 2017). Exposure to either endogenous or exogenous DNA-damage causing agents often results in the addition of specific adducts to base pairs. The damaged base pairs must be repaired before DNA replication begins, otherwise, mutations will be incorporated into newly synthesized strands of DNA. Cells have a number of DNA-damage repair pathways, capable of responding to very specific DNA lesions to protect genomic integrity. Unsurprisingly, mutations in DNA repair genes results in genomic instability which is a mechanism for initiation of tumourigenesis (Preston et al., 2010). The major DNA-repair pathways and associated cancers will be described in more detail below.

1.4.2.1 Mismatch Repair (MMR)

As previously mentioned, MMR is involved in repairing mutations to improve the fidelity of DNA replication. The proteins in the MMR pathway include MSH2, MSH6, MLH1, MSH3 and PMS2 which form various combinations of heterodimeric complexes to perform detect different forms of DNA damage (Jiricny. 2006). The MMR machinery assembles to detect and excise the damaged region to allow for synthesis of error-free DNA (Genschel et al., 1998; Baretti and Le. 2018). A

common site for insertions and deletions are microsatellites, which contain stretches of repeated DNA motifs no longer than 10 nucleotides (Garrido-Ramos. 2017). DNA replication machinery often slips off of microsatellite regions which often cause small insertions or deletions. A consequence of either insertion or deletion is the formation of an insertion-deletion loop (IDL) caused by mismatched base pairing. MMR complexes specifically detect IDLs to mediate excisions of the wrongly incorporated nucleotides (Baretti and Le. 2018).

Mutations in MMR genes have been shown to cause a mutator phenotype known as microsatellite instability (MSI) (Li and Martin. 2016). MSI occurs when IDLs formed during replication cannot be repaired which permanently changes the length of microsatellites. This increases the number of missense and frameshift mutations introduced into coding regions, ultimately, increasing the risk of developing cancer. Germline mutations in all MMR genes cause Lynch Syndrome (LS), a disorder characterized by an increased risk in developing a number of cancers, most commonly colorectal and endometrial cancer (Lin et al., 1998). Fifteen to twenty percent of sporadic colorectal cancers also arise due to loss of function in MMR genes, however, instead of a loss of function mutation within the gene, it is primarily caused by hypermethylation of the MLH1 promoter, suppressing MLH1 transcription (Herman et al., 1998; Poulogiannis et al., 2010). Mutations in MMR genes have been reported in gastric, brain and hematologic tumours, highlighting the importance of a functional MMR pathway (Keller et al., 1996; Wimmer and Kratz. 2010).

1.4.2.2 Nucleotide Excision Repair (NER)

NER is a DNA-repair pathway responsible for correcting bulky/double helix distorting DNA lesions (Kusakabe et al., 2019). Commonly repaired lesions include 6-4 pyrimidine-pyrimidone photoproducts (6-4PPs) and cyclobutane-pyrimidine dimers (CPDs) which are generated after UV exposure (Liakos et al., 2017). NER can be separated into two pathways: global genome NER (GG-NER) and transcription coupled NER (TC-NER). GG-NER is constantly scanning the genome for helix-distorting lesions, while TC-NER is recruited to lesions when RNA polymerase II stalls while transcribing a gene (Torgovnick and Schumacher. 2015). Both NER pathways involve recognition of the DNA lesion, unwinding of DNA surrounding the lesion followed by excision of the damaged

DNA and synthesis of a new strand (Spivak. 2015). Given the multitude of functions required to complete repair using NER, it is evident that a number of proteins are involved and loss of function at different steps in the pathway can lead to various phenotypes.

One of the most well-studied disorders caused by NER defects is Xeroderma Pigmentosum (XP). XP is an autosomal recessive disorder characterized by increased sensitivity to sunlight and a 2000-fold increased risk of developing melanoma (Bradford et al., 2011). XP develops as a result of mutation in one of 7 proteins involved in NER genes, Xeroderma pigmentosum, complementation group A (XPA) to XPG (Lehmann et al., 2011). Loss of function in any of these genes reduces repair efficiency or completely abolishes NER function, allowing for mutations to accumulate. Another disorder associated with deficient NER is Cockayne syndrome (CS), an autosomal recessive disorder caused by mutations in TC-NER specific proteins, CSA (ERCC8) and CSB (ERCC6) (Wilson et al., 2016). Individuals with CS, present with microcephaly, growth failure and photosensitivity (Liakos et al., 2017). Although TC-NER function is altered in CS, there is no increased risk in developing skin cancers as seen in XP patients (Wilson et al., 2016). Single nucleotide polymorphisms (SNPs) in NER genes have shown weak association with the development of head and neck cancers (Carles et al., 2006), however, very few cancers aside from skin cancer have been linked to mutations in NER genes.

1.4.2.3 Base Excision Repair (BER)

BER functions to repair small DNA lesions that do not distort the DNA double helix, often caused by endogenous agents. Lesions most commonly repaired by BER include oxidized bases (i.e. 8-oxoguanine formed by reactive oxygen species), alkylated bases (ie. 3-methyladenine), deaminated bases and incorrect incorporation of uracil (Kim and Wilson. 2012). It is estimated that more than 20,000 of these lesions are generated in a cell every day, highlighting the importance of maintaining normal function of this repair pathway (Errol et al., 2006). The BER pathway involves damaged base recognition and removal (DNA glycosylase), DNA backbone incision (apurine/apyrimidine endonuclease), addition of appropriate terminal ends (phosphodiesterase or lyase) followed by filling the gap by DNA polymerase β and re-ligation of the DNA backbone (DNA ligase) (Kim and Wilson. 2012). Two subdivisions exist in the BER

pathway: short patch BER (removal of a single nucleotide) and long patch BER (removal of 2-10 nucleotides) (Krokan and Bjørås. 2013).

Several mouse models harbouring BER gene knockouts are embryonic lethal, again highlighting the essential nature of this pathway (Wallace et al., 2012). In humans, several groups identify an increased risk in developing gastric, colorectal, prostate and lung cancers linked to SNPs in various BER genes (Kuasne et al., 2011; Canbay et al., 2010; Rusin et al., 1999; Yamada et al., 2002). One of the most well-established associations is between mutations in BER DNA glycosylase, mutY DNA glycosylase (MUTYH), and increased risk of developing colorectal cancer and familial adenomatous polyposis (Farrington et al., 2005; Al-Tassan et al., 2002). Interestingly, promoter methylation resulting in downregulation of DNA glycosylases methyl-CpG-binding domain protein 4 (MBD4) and Nei Like DNA Glycosylase 1 (NEIL1) has been observed in colorectal, gastric and head/neck cancers (Howard et al., 2009; Shinmura et al., 2004; Chaisaingmongkol et al., 2012).

1.4.2.4 Homologous Recombination (HR) and Non-homologous End Joining (NHEJ)

Double strand breaks (DSBs) primarily form as a result of stalled/collapsed replication forks during DNA replication or by exposure to ionizing radiation (IR) (Jeggo et al., 2011). NHEJ has been shown to repair DSBs throughout the entire cell cycle while HR primarily functions in the synthesis (S) phase and gap 2 (G_2) phase of the cell cycle (Rothkamm et al., 2003). NHEJ begins when heterodimeric complex of Ku70 and Ku80 bind to both ends of the DSB and recruit the rest of the NHEJ machinery (Yang et al., 2016). This complex contains endo- and exo-nucleases which resects 4 or fewer nucleotides from a single strand on each end to generate microhomology to direct the repair process (Pannunzio et al., 2018). Specific DNA polymerases are recruited to the NHEJ complex to fill in the gaps created by nucleases; however, their activity is linked to incorporation of small insertions (Sishc and Davis. 2017). The final step in NHEJ involves the activity of a DNA ligase IV (LIG4) to seal the two ends together (Chang et al., 2017). NHEJ has the ability to repair DSBs with 100% accuracy, however, insertion/deletions are commonly introduced making it the more error-prone pathway of DSB repair (Ranjha et al., 2018).

Unlike NHEJ, HR requires a template sequence to complete repair, increasing the accuracy of the repair process. HR begins by recruitment of endo- and exo-nucleases which resect large stretches of DNA on either side of the break, leaving a portion of single stranded DNA (ssDNA). The ssDNA is subsequently coated with RAD51 which is a protein that helps search for a homologous sequence in the sister chromatid. This is followed by the removal of RAD51 and synthesis of new DNA by a DNA polymerase. An intermediate structure called a double Holliday junction forms. When DNA synthesis is completed, the two Holliday junctions are cut, and the backbone is re-ligated (Ranjha et al., 2018).

Maintaining normal NHEJ and HR pathway function is critical to prevent disease, highlighted by the number of published mutations in several genes of both pathways. In addition to repairing DSBs, NHEJ is essential for V(D)J recombination which is a critical process to allow for adaptive immunity (Smith et al., 2019). Therefore, loss of function mutations within genes of the NHEJ pathway often alters an individual's immune response. Unsurprisingly, mutations in both LIG4 and NHEJ1, which form a complex at DSBs, result in severe combined immunodeficiency (SCID) (Buck et al., 2006a; Buck et al., 2006b). SNPs and changes in expression levels of NHEJ genes are also found in a number of tumour tissues [reviewed Sishc and Davis 2017]. BRCA1 and BRCA2 are two genes identified as breast and ovarian cancer susceptibility genes (Miki et al., 1994; Wooster et al., 1995). Both BRCA1/2 bind to RAD51 to help facilitate HR and interestingly, recent studies detected variants in RAD51 that increase risk of developing breast and ovarian cancer (Golmard et al., 2013; Song et al., 2015). Bloom's syndrome, Werner's syndrome and Rothmund-Thomson syndrome are all caused by mutations in ReqQ helicase genes (primarily used in HR), involved in unwinding dsDNA to allow for DNA repair of DSBs (Ellis et al., 1995; Yu et al., 1996; Kitao et al., 1999). All three syndromes are associated with an increased risk of developing a number of cancers. Mutations in other HR genes have been associated with an increased risk in developing Hodgkin's lymphoma, colon cancer and uterine leiomyoma (Hiramoto et al., 1999; Schoenmakers et al., 1999).

1.4.2.5 DNA repair and Wilms tumour

In addition to the WT mutations described above, studies have also identified a small subset of tumours containing mutations or altered expression of DNA repair genes. Mutations in MMR gene MLH1 (a missense and frameshift deletion) were detected in an individual with WT (Poley et al., 2007). Immunohistochemistry confirmed loss of MLH1 expression in the tumour tissue and also MSI was confirmed, demonstrating this individual had a loss of normal MMR function. Additional cases of WTs containing MSI have been reported in the literature, however, no mutations in MMR genes were detected (Mason et al., 2000; Tsunematsu et al., 2000; Diniz et al., 2013). Analysis of MLH1, PMS2, MSH2, and MSH6 by immunohistochemistry in a subset of tumours revealed decreased expression within tumour tissue which did not always correlate to MSI status (Diniz et al., 2013). Two truncating mutations in BRCA2 were detected in a pair of siblings with WT suggesting these individuals may have impaired HR repair (Reid et al., 2005). Interestingly, in vitro studies of cells transfected with WT1, show significant increases in mRNA of several HR genes in WT1(+) cells (Oji et al., 2015). BRAC2 levels did not reach significance, however, mRNA expression was increased. Two SNPs in NER gene, XPD, were associated with an increased risk of developing WT (Zhu et al., 2018). Additionally, proteins involved in regulating cell cycle and initiating DNA damage response pathways, including TP53 and TRIM28 have been mutated in WT (Diets et al., 2019; Bardeesy et al., 1994; Maschietto et al., 2014). Taken together, mutations in DNA repair genes appear to be rare in WT, however, these pathways may be altered at the epigenetic/transcriptional level to downregulate expression. We hypothesize that in NPCs, WT1 upregulates specific DNA-repair genes to prepare cells for the burst of proliferation induced by the WNT9B signal. Additionally, *Wt1*(-) nephrogenic rests fail to upregulate these repair genes, leaving these cells vulnerable to acquire additional mutations as observed in WT.

1.5 Research aims

The central hypothesis of my thesis is two-fold: firstly, we hypothesize that early NPCs are unprimed and do not express the required molecular components to respond to the inductive WNT9B signal from the UB. Additionally, identification of these components will help in generating the ideal NPC to use in a cell-based therapy of AKI. Secondly, we hypothesize that

NPCs within the MM express a specific profile of DNA-repair genes to prepare for the burst of proliferation induced by WNT9B.

Objective 1

My first objective is to characterize the cell surface receptor components required to render an early NPC responsive to the inductive WNT9B signal. M15 cells are representative of an early NPC and respond poorly to WNT9B, making them a perfect model for experimentation. We systematically add members of the FZD, LRP and RSPO family to determine the co-receptor combination which provides robust activation of the canonical WNT-signalling pathway in response to WNT9B.

Objective 2

Once the molecular components involved in WNT9B signaling have been identified, my second objective is to generate an optimal NPC to use in a cell-based therapy for AKI. Given the requirement for canonical WNT-signalling activity to allow cell integration, infused cells must have a robust WNT-signaling capacity to increase effectiveness of the treatment. We hypothesize that pre-treatment of NPCs with recombinant RSPO1 protein will increase the number of cells integrated into glycerol-injured mouse kidneys. These results are described in Appendix I.

Objective 3

WT1 has been established as a master regulator during nephrogenesis and plays an important role in rendering NPCs responsive to the canonical WNT9B signal from the UB. WT1 also regulates gene expression within NPCs to promote transition from a progenitor-like state to a proliferating/differentiating cell. NPCs with biallelic *WT1* mutations form a population of developmentally arrested cells that often acquire mutations in *CTNNB1* which drive tumorigenesis. We hypothesize that WT1 upregulates specific DNA-repair genes in NPCs to prepare cells for the burst of proliferation induced by WNT9B. We speculate that downregulation of WT1-dependent DNA-repair genes could be a plausible mechanism explaining the common occurrence of secondary *CTNNB1* mutations in WT1(-) NPCs.

Chapter 2: Determinants of WNT9B Responsiveness Signalling in NPCs

2.1 Overview

The WNT-signalling pathway plays a critical role in kidney development as nephron progenitor cells (NPCs) in the metanephric mesenchyme become fully primed by E11.5. By this stage, NPCs appear to express all the machinery required to respond to the inductive WNT9B signal secreted by the UB (Carroll et al., 2005). WNT9B is essential for NPC differentiation and survival, demonstrated by the anephric phenotype observed in *Wnt9b*-null mice (Carroll et al., 2005). Specifically, activation of the canonical/β-catenin WNT-signalling pathway by WNT9B drives nephrogenesis demonstrated by the intense β-catenin activity observed in the cap mesenchyme (Iglesias et al., 2014). Additionally, knockout of the β-catenin gene or *Wnt9b* specifically in mouse NPCs both result in an anephric phenotype (Park et al., 2007). Although the WNT-signalling pathway has been shown to be significant for normal differentiation and maturation of the nephron, the specific molecular components involved in transducing this signal in NPCs has yet to be described.

Canonical WNT-ligands require a co-receptor complex consisting of a member of the FZD family (10 different FZD receptors) and a LRP5/6 receptor. The combination of FZD and LRP determines the specificity for the WNT ligand. Some WNT-ligands are promiscuous, in that they have the ability to bind to several co-receptors. However, binding of some WNT ligands is very specific. Additionally, WNT-responsiveness can be augmented in the presence of agonists, in particular, a family of R-spondins which have recently been shown to be essential for development and maintenance of various cell types in a number of organ systems.

In section 2.2 , we describe the components involved in transducing the WNT9B signal in a cell line representative of an uninduced NPC. We took a systematic approach to determine the essential cell surface FZD and LRP receptors required. Here, we show that a co-receptor complex of FZD5 and LRP6 are required for WNT9B signalling. Interestingly, this response was only detectable in the presence of WNT agonists, RSPO1. Finally, we confirm expression of *Fzd5*, *Lrp6* and *Rspo1* in a bona fide *Cited1*(+) NPC from embryonic mouse kidney. We conclude that the

NPCs of the cap mesenchyme need to acquire FZD5, LRP6 and RSPO1 to become fully primed and able to respond to the inductive WNT9B signal to drive nephrogenesis.

2.2 Molecular determinants of WNT9b responsiveness in nephron progenitor cells

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Running Title: WNT9b responsiveness in NPCs

Citation: PLoS One (2019) 14(4): e0215139.

2.2.1 Abstract

Primed nephron progenitor cells (NPCs) appear in metanephric mesenchyme by E11.5 and differentiate in response to the inductive WNT9b signal from the ureteric bud. However, the NPC WNT-receptor complex is unknown. We obtained M15 cells from E10.5 mesonephric mesenchyme and systematically analyzed components required for canonical WNT9b-responsiveness. When M15 cells were transfected with a β-catenin luciferase reporter plasmid, exposure to recombinant WNT9b resulted in minimal luciferase activity. We then analyzed mRNA-expression of WNT-pathway components and identified *Fzd1-6* and *Lrp6* transcripts but not *Rspo1*. When M15 cells were treated with recombinant RSPO1 the response to transfected WNT9b was augmented 4.8-fold. Co-transfection of M15 cells with *Fzd5* (but no other *Fzd* family member) further increased the WNT9b signal to 16.8-fold and siRNA knockdown of *Fzd5* reduced the signal by 52%. Knockdown of *Lrp6* resulted in 60% WNT9b signal reduction. We confirmed *Fzd5*, *Lrp6* and *Rspo1* mRNA expression in CITED1(+) NPCs from E15.5 embryonic mouse kidney. Thus, while many WNT signaling-pathway components are present by E10.5, optimum responsiveness of E11.5 cap mesenchyme requires that NPCs acquire RSPO1, FZD5 and LRP6.

2.2.2 Introduction

The mammalian kidneys are derived from progenitor cells in the embryonic intermediate mesoderm, expressing the transcription factor, OSR1. Fate mapping studies of the embryonic kidney reveal that cells labeled by the *Osr1* promoter at embryonic day E7.5 give rise to all elements of the maturing kidney [1] and *Osr1* knockout mice are anephric [2, 3]. Around E8.5-E9, a subset of OSR1-positive kidney progenitor cells are transformed into polarized epithelia, forming the paired nephric duct structures that elongate down the embryo [4]. Concurrently, another subset of cells upregulate Wilms' tumor 1 (WT1) while retaining a mesenchymal phenotype. [5, 6]. The columns of WT1(+) cells flanking each nephric duct are committed to the nephron progenitor cell (NPC) fate; interestingly, *Wt1* knockout mice fail to develop functional kidneys [7]. Development of the metanephric kidney begins in earnest when ureteric buds emerge from each nephric duct (E10.5), begins to arborize as it grows into the adjacent column of metanephric mesenchyme and induces local NPCs to begin nephrogenesis.

In the 1950s, Grobstein demonstrated that the metanephric mesenchyme can generate renal tubular structures when co-cultured with inductive tissues that mimic the ureteric bud signal [8]. This fundamental observation showed that the proper signal from the ureteric bud could trigger differentiation in the committed NPCs from the metanephric mesenchyme. Key observations by Herzlinger [9] and Carroll [10, 11] established the canonical WNT9b/β-catenin signaling pathway as the central mechanism by which the ureteric bud initiates nephrogenesis. Secretion of WNT9b by the ureteric bud is required for the early inductive events in the developing kidney. Transgenic mice with a beta-catenin reporter display intense canonical WNT-signaling activity in the cap mesenchyme [12, 13].

It is uncertain when NPCs become competent to respond to the inductive WNT signal, however, WT1 expression is a crucial element in this process. Biallelic mutations of *WT1* in humans result in the formation of nephrogenic rests, clonal developmentally arrested cells which lack canonical WNT-signalling activity and are unresponsive to inductive signals from the ureteric bud [14]. We discovered that this is accomplished by WT1 suppression of EZH2, de-repressing epigenetically silenced genes of the differentiation cascade [15]. Prior to arrival of the ureteric bud (E10.5-E11), maturing WT1(+) NPCs express a panel of genes, including retinoic acid receptor-alpha (*Rara*), cadherin 11 (*Cdh11*) and CD24 [13, 16]. However, the stage at which they are fully competent to respond to the WNT9b signal is unknown. Furthermore, the molecular basis for WNT9b responsiveness in NPCs is unknown.

The canonical WNT signaling pathway is full of redundancies. Here we take a systematic approach to identifying the crucial components of the WNT9b signaling pathway in embryonic mouse kidney.

2.2.3 Materials and methods

2.2.3.1 Cell culture

M15 cells are WT1-expressing cells isolated from E10.5 mouse mesonephric mesenchyme expressing the large T protein of polyoma virus under control of the early viral enhancer. The M15 cell line was establish following the protocol described by Larsson et al (1995) and donated

by the Hastie lab (Edinburgh, Scotland) [17]. Cells growing in monolayer attached to plastic culture vessels in the presence of DMEM culture medium with 10% Fetal Bovine Serum and 1% Penicillin/ Streptomycin.

2.2.3.2 Luciferase reporter transfections and dual luciferase assay

Transient transfections were performed using a canonical WNT-signalling reporter plasmid, Super 8X TOPFlash (TOPFlash). M50 Super 8x TOPFlash was a gift from Randall Moon (Addgene plasmid # 12456 ; http://n2t.net/addgene:12456 ; RRID:Addgene_12456) [18]. The Renilla luciferase expression vector pRL-SV40 (Promega, Madison, WI, USA) was used to normalize for transfection efficiency. Transfections for each condition were performed in triplicate and repeated three times on different days. The following frizzled plasmids were gifts from Chris Garcia & Jeremy Nathans: pRK5-mFzd1-1D4, pRK5-mFzd2-1D4, pRK5-mFzd3-1D4, pRK5-mFzd4-1D4, pRK5-mFzd5-1D4, pRK5-mFzd6-1D4, pRK5-mFzd7-1D4, pRK5-mFzd8-1D4, pRK5-mFzd9-1D4, pRK5-mFzd10-1D4 and pRK5-Wnt9b [19] (Addgene, Cambridge, MA, USA). *Lrp5* (Clone ID: 3154246) and *Lrp6* (Clone ID: 6409058) plasmids were purchased from Dharmachon (Lafayette, CO, USA).

One day prior to transfection, 20,000 M15 cells were seeded in 24-well plates and transfected at 80% confluency using Lipofectamine 2000 Transfection Reagent according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). Plasmids were transfected in the following amounts: *Fzd* (50 ng), TOPFlash (44 ng), *Lrp* (5 ng), *Wnt* (50 ng), Renilla (1 ng). Recombinant WNT9b (3669-WN/CF, R&D Systems, Minneapolis, MN, USA) was added at a concentration of 50 ng/mL to transfection media at the time of transfection in corresponding conditions. In R-spondin conditions, either 200 ng/mL of recombinant mouse RSPO1 (3474-RS – R&D Systems, Minneapolis, MN, USA) or 200 ng/mL of recombinant mouse RSPO3 (4120-RS/CF – R&D Systems, Minneapolis, MN, USA) was added to each well 24 hours post transfection. Firefly and renilla luciferase reporter activities were measured after 48h using the Dual Luciferase Assay System reagents and quantified in a GLOMAX 96 microplate luminometer (Promega, Madison, WI, USA). The reporter activity was expressed as a Firefly luciferase/ Renilla luciferase ratio.

The same procedure as described above was followed to monitor luciferase activity. For siRNA experiments, cells were transfected with Silencer pre-designed siRNA targeting mouse *Fzd1* (siRNA ID: 75730), *Fzd2* (siRNA ID: 57265), *Fzd5* (siRNA ID: 14367) and *Lrp6* (siRNA ID: 62715) (Ambion, Carlsbad, CA, USA) using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer instructions.

2.2.3.3 RNA isolation and real-time PCR analysis

RNA was isolated using the QIAGEN RNeasy kit according to the manufacturer's instructions (QIAGEN, Toronto, ON, Canada). RT-PCR was performed using the iScript cDNA synthesis kit (Bio-Rad, Mississauga, ON, Canada). Quantitative real-time PCR was performed using the SsoFast EvaGreen Supermix with Low ROX (Bio-Rad, Mississauga, ON, Canada) and specific primer sets in a LightCycler 480 II (Roche Applied Science, Laval, QC, Canada).

2.2.3.4 Immunoblotting

Protein content was quantified in cellular extracts using the BCA assay (Pierce, Rockford, IL, USA). Twenty-five micrograms of protein extract were loaded onto SDS-PAGE gel and subjected to electrophoresis following standard immunoblotting techniques. The following primary antibodies and titres were used: anti-WT1 (antibody C19: sc-192, 1/200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Actin (A5441, 1/10000, Sigma-Aldrich, Oakville, ON, Canada). Immunoreactive bands were detected using species-specific horseradish peroxidase-conjugated secondary antibodies (1/2000, Cell Signaling, Danvers, MA, USA) and visualized and analyzed using the GE Healthcare ECL Plus Western Blotting Detection Reagents and the BioRad Imager Scanner and software (GE Healthcare, Mississauga, ON, Canada).

2.2.3.5 In situ hybridization

In situ hybridization of E11.5 embryos was performed according to the protocol listed on the GUDMAP website: https://www.gudmap.org/chaise/recordset/#2/Protocol:Protocol@sort(RID). cDNAs were purchased from ThermoFischer/Open Biosystems. For each gene, we include the clone ID, the restriction enzyme used to linearize the plasmid and the polymerase used to synthesize the antisense probe. *Fzd1* (Clone ID: 5697795) SalI/T3, *Fzd2* (Clone ID: 6411627)

SalI/T3, *Fzd3* (Clone ID: 30084926)EcoRI/T3, *Fzd4* (Clone ID: 4238940) SalI/T7, *Fzd5* (Clone ID: UI-M-CGOP-BRL-B-03-0-UI) EcoRI/T3, *Fzd6* (Clone ID: 3983985) SalI/T7, *Fzd7* (Clone ID: 6844727) SalI/T3, *Fzd8* (Clone ID: 3992722) SalI/T7, *Fzd9* (Clone ID: UI-M-CGOP-BGI-E-03-0-UI), *Fzd10* (Clone ID: 556296) Pst1/T7.

2.2.3.6 Mice

All animal experiments followed the guidelines provided by the Canadian Council of Animal Care and were approved by the McGill University Facility Animal Care Committee (FACC), including an analysis of the 3Rs of animal use in research. Cited1-Cre mice were donated from Dr. Mark de Caestecker [20]. B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J ($Tom^{flox/flox}$)mice were bought from Jackson Laboratories. All animals were housed at the Research Institute of the McGill University Health Centre animal facility and monitored daily by animal care staff. Support staff followed McGill University Standard Operating Procedure #508 for rodent husbandry guidelines (https://mcgill.ca/research/files/research/508_-_rodent_husbandry_-_march_2016_1.pdf).

Cited1-Cre males were crossed with homozygous $Tom^{flox/flox}$ females to generate double transgenic embryos. All genotypes generated from this cross were viable and healthy. For immunofluorescence experiments, at 17 dpc, 0.1 mg/g body weight of Tamoxifen (Sigma) was administered to pregnant females via intraperitoneal injection in their home cage [21]. No adverse events were observed in the pregnant female or embryos at this dose of tamoxifen administration. Females were sacrificed 24 hours later, and embryos were harvested. For ddPCR experiments on Cited1/Tom cells, pairs of embryonic kidneys were plated in a single well of a 6-well plate after digestion in a collagenase B digestion solution at 37˚C for 1 hour. These cells were subsequently treated with 2.5 µg/mL of 4-hydroxytamoxifen added to culture media. Digested embryonic kidneys from one pregnancy were pooled and cells were grown at 37˚C in tissue culture flasks in NPC growth media [22].

2.2.3.7 Tissue preparation and confocal microscopy

Embryonic mouse kidneys (E18) from Cited1/Tom mice were fixed overnight in 4% PFA at 4°C. Kidneys were then transferred into 15% Sucrose in PBS and rocked at room temperature for 30 mins followed by rocking overnight at 4˚C in 30% sucrose. Next, kidneys were placed into a 1:1

mixture of 30% sucrose/PBS and OCT and rocked at 4˚C for 2 hours and then were embedded in OCT and stored at -80˚C until sectioned. Cryosections (7uM) were obtained using a Leica Cryostat. Nuclei were counterstained with VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Images were obtained with a laser scanning confocal microscope (LSM780) and the ZEN2010 software (Carl Zeiss Canada Ltd., Toronto, ON, Canada) at room temperature and processed by Adobe Photoshop and Illustrator software.

2.2.3.8 Fluorescence activated cell sorting (FACS)

Whole embryonic kidneys were isolated and activated with tamoxifen as previously described. Cells were then washed in PBS and re-suspended into 500 µL of 2% FBS in PBS solution and kept at 4˚C until they were sorted. Cell sorting was performed by immunophenotyping core facility staff using a BD FACSAria Fusion. Isolated Cited1/Tom cells isolated were immediately pelleted and frozen at -80˚C.

2.2.3.9 Droplet digital PCR (ddPCR)

RNA was extracted from Cited1/Tom cells followed by cDNA synthesis as previously described (n=4). Droplets were formed in a QX200 Droplet Generator and PCR was performed using the QX200 ddPCR EvaGreen Supermix (Bio-Rad, Mississauga, ON, Canada) and specific primer sets in a C1000 Touch Thermal Cycler (Bio-Rad, Mississauga, ON, Canada). Droplets were read using the QX200 Droplet Reader machine and results were displayed in QuantaSoft software.

2.2.3.10 Statistical analysis

Graphs are presented as mean ± SEM of three or more independent results. Statistical significance was assessed by a one-way ANOVA followed by a Dunnett correction for multiple comparisons. ddPCR results were analyzed by unpaired t-tests.

2.2.4 Results

2.2.4.1 M15 cells

A committed lineage of NPCs emerge from the OSR1(+)/WT1(+) intermediate mesoderm as early as embryonic day E7.5 [1]. To model early events that render NPCs responsive to the inductive WNT9b signal from ureteric bud, we analyzed the M15 cell line. M15 cells are derived from E10.5

mesonephric mesenchyme of mice bearing the large T protein polyoma virus under the control of an early viral enhancer [17]. These cells are thought to represent the NPC phenotype one day prior to arrival of the ureteric bud at E11.5. To validate the lineage specification of M15 cells, we confirmed the expression WT1 (Figs 2.1A and 2.1B) and the pattern of additional transcripts characteristic of the early NPC lineage (Table 2.1). We detected transcripts of key early NPC markers including *Osr1* and *Cited1* but not markers of NPCs after exposure to the ureteric bud, such as *Wnt4* and *Rara* (Table 2.1). We then screened M15 cells for mRNA expression (RT-PCR) of candidate genes in the canonical WNT/β-catenin signaling pathway (Table 2.1). We identified expression of β-Catenin, *Lrp*6, *Lgr4/6* and *Fzd1-6*. Notably absent were *Rspo1* and 3, *Fzd7-10*, *Lrp5* and *Lgr5.*

Table 2.1. mRNA expression of WNT/β-catenin pathway components in M15 cells.

Wnt-signalling genes				NPC markers	
Gene	Expression	Gene	Expression	Gene	Expression
Fzd1	+	*Lrp5*	-	*WT1*	+
Fzd2	+	*Lrp6*	+	*Osr1*	+
Fzd3	+	*Rspo1*	-	*Cited1*	+
Fzd4	+	*Rspo3*	-	*Six2*	-
Fzd5	+	*CTNNB1*	+	*Wnt4*	-
Fzd6	+	*Wnt9b*	-	*Rara*	-
Fzd7	-	*Lgr4*	+		
Fzd8	-	*Lgr5*	-		
Fzd9	-	*Lgr6*	+		
Fzd10	-				

2.2.4.2 M15 cells are unresponsive to external WNT9b

To ascertain whether M15 cells are primed to respond to a WNT9b signal, we transiently transfected the cells with TOPFlash, a β-Catenin/luciferase reporter, and exposed them to

recombinant WNT9B protein at concentrations ranging from 50-400 ng/ml but detected only minimal response (1.05-fold) (Fig 2.1C).

2.2.4.3 RSPO1 enhances responsiveness of M15 cells to WNT9b

Considering M15 cells lack both R-spondins known to be expressed in NPCs of embryonic mouse kidney cap mesenchyme (GUDMAP), we reasoned that M15 cell WNT-responsiveness might be limited by the stability of the WNT-receptor complex at the cell surface [23-25]. To test this hypothesis, we first transfected M15 cells with TOPFlash and assessed the response to a co-transfected WNT9b expression plasmid. As seen in Fig 2.1D we detected a significant (5-fold) increase in luciferase activity. We then added recombinant RSPO1 (200 ng/ml) or RSPO3 (200 ng/ml) which further increased the signal to 22- and 27-fold above baseline, respectively ($p<0.0001$) (Fig 2.1D). Preliminary dose-response studies showed that no further signal increase was obtained with higher concentrations of either R-spondin protein. To dissect the importance of other canonical WNT-pathway components, we added *Wnt9b* plasmid and recombinant RSPO1 (200 ng/ml) in all subsequent experiments.

2.2.4.4 Frizzled receptor expression in cap mesenchyme

To identify candidate Frizzled receptors responsible for transducing the WNT9b response in NPCs, we performed *in situ* hybridization for the Frizzled family members (*Fzd1-10)* in E11.5 mouse kidney, except for *Fzd9* which was unsuccessful. As seen in Fig 2.2A, embryos cross-sectioned across both nephric fields show several Frizzled family members (*Fzd2, Fzd3, Fzd5 and Fzd7*) with diffuse expression patterns but with concentrated expression in the cap mesenchyme; in contrast to *Fzds* with weak expression in the cap mesenchyme (*Fzd4* and *Fzd10*) or strong expression restricted to ureteric bud branch tips (*Fzd6* and *Fzd8*).

2.2.4.5 Transfection of M15 cells with Fzd5 enhances WNT9b responsiveness

To confirm whether one of the *Fzd* receptors is rate limiting in M15 cells, we transfected each member of the *Fzd* receptor family (*Fzds 1-10*) individually into M15 cells expressing TOPFlash. Cells were co-transfected with *Wnt9b* and exposed to recombinant RSPO1 (200 ng/ml) in each experiment. As seen in Fig 2.2B, the only *Fzd* which significantly augmented WNT9b-induced

TOPFlash signal was *Fzd5*. When M15 cells were co-transfected with *Fzd5*, activity of the canonical WNT/β-Catenin reporter was increased 3.5-fold (p=0.0002). We then performed similar experiments in M15 cells co-transfected with an siRNA targeting *Fzd5*, previously shown to knock down *Fzd5* expression level by 70%. As seen in Fig 2.2C, presence of the *Fzd5* siRNA reduced WNT9b-dependent TOPFlash activity by 52% (p=0.005), whereas knockdown of *Fzd1* and *Fzd2* resulted in non-significant changes.

2.2.4.6 *Lrp6* is required for optimal responsiveness of M15 cells to WNT9b

To examine the importance of *Lrp* expression to the canonical WNT9b-responsiveness, we transiently transfected M15 cells with *Wnt9b*, TOPFlash and a *Lrp6* siRNA. A scrambled siRNA was transfected in another condition as a control. As seen in Fig 2.3, addition of the *Lrp6* siRNA reduced WNT9b-dependent TOPFlash signal by 66% (p<0.0001) whereas the scrambled siRNA had no effect. Interestingly, additional co-transfection with *Lrp5* was unable to rescue WNT9b pathway activity in the presence of *Lrp6* siRNA. Co-transfection of M15 cells with *Lrp5* (in the absence of siRNA) had no effect on its own.

2.2.4.7 Responsiveness to extrinsic WNT9b is restored by addition of *Fzd5* and RSPO1

To ascertain whether M15 cell responsiveness to an external source of WNT9b could be restored by addition of suboptimal WNT-pathway components, we transfected the cells with TOPFlash and *Fzd5*. We then treated them with recombinant RSPO1 and measured luciferase activity. As seen in Fig 2.4, no response was detected in cells exposed to WNT9b, RSPO1 or *Fzd5* alone. However, the signal was increased 3.3-fold over baseline in M15 cells exposed to recombinant WNT9b and RSPO1. The signal was increased to 11.1-fold over baseline in M15 cells transfected with *Fzd5* and exposed to recombinant WNT9b and RSPO1 (p<0.0001) (Fig 2.4).

2.2.4.8 Cited1 cells isolated from embryonic mouse kidney express *Wt1*, *Fzd5*, *Lrp6* and *Rspo1*

To confirm expression of the key components of the WNT9b signaling pathway identified above in a primary NPC, we isolated Cited1-expressing cells from embryonic mouse kidneys. Six2 is a commonly used cap mesenchyme marker, however, Cited1 has been shown to have overlapping expression with Six2 and also is downregulated before NPCs begin differentiation into mature

tubules [26, 27]. To identify Cited1 cells in the cap mesenchyme, we crossed mice with a floxed tdTomato (TomatoRed) transgene to mice bearing a tamoxifen-inducible *Cited1*-driven Cre Recombinase [20]. The Cited1-Cre mouse also contains EGFP, however, we were not able to specifically isolate the Cited1 population of cells due to high green autofluorescence observed in the kidney. As seen in Fig 2.5A, tamoxifen administered to the pregnant mother at E17 activated TomatoRed in NPCs of the cap mesenchyme. Although activation of the Cre-recombinase was successful in vivo, this method required more time between tamoxifen injection and cell isolation which increased the likelihood of including differentiated cells into our analysis. To circumvent this issue and isolate NPCs rapidly after activation of the TomatoRed tag, we digested E15.5 embryonic kidneys from *Cited1*^{Cre}/TomatoRed mice with collagenase, dispersed the cells into monolayer culture and added 4-hydorxytamoxifen (2.5µg/ml) to induce Cre-recombinase expression *in vitro* (Fig 2.5B). After 12 hours, TomatoRed(+) cells were isolated by FACS for analysis. This method ensured that fewer red-labelled cells would differentiate before FACS isolation. We extracted RNA from Cited1/TomatoRed(+) cells of 17 embryonic kidneys pooled from 4 litters (two litters per sample; sample 1: n= 9 embryonic kidneys; sample 2: n=8 embryonic kidneys) and analyzed transcripts levels by droplet digital PCR (ddPCR) due to the limited number of cells isolated per kidney. As seen in Fig 2.5C, we confirmed mRNA expression of *Wt1, Fzd5, Rspo1* and *Lrp6* in the *Cited1*/TomatoRed(+) NPCs from E15.5 cap mesenchyme. Each condition was compared to the Cited1/TomatoRed(-) fraction of cells obtained from the same kidneys and normalized to beta-2-microglobulin (B2M) transcript levels. As the Cited1/TomatoRed(+) population of cells represents approximately 6% of the E15.5 kidneys after FACS, we expected the Cited1/TomatoRed(-) population of cells to also express some level of our markers of interest, therefore we used this condition as a positive control.

2.2.5 Discussion

Around embryonic day E9.0 of mouse development, a lineage of WT1-expressing progenitor cells emerge within the OSR1(+) intermediate mesoderm. To model this early NPC prior to the arrival of the ureteric bud, we studied the M15 cell line isolated from E10.5 mouse kidneys [17]. These cells express *Osr1*, WT1 and *Cited1*, placing them in the early NPC lineage. Previous studies from our lab showed the essential role of WT1 for responsiveness to the inductive WNT9b signal

through suppression of EZH2, a histone H3K27 methyltransferase. EZH2 suppression in turn opens up chromatin, permitting exit from the stem cell state [15, 28]. Thus, WT1 is essential for maturation of the nephron progenitor cell lineage. Nevertheless, we found that M15 cells were unresponsive to WNT9b *in vitro*. This suggests that WT1 expression alone is not sufficient to prime the NPC for WNT-responsiveness and that the early NPC must acquire additional molecular properties by the time the ureteric bud arrives at E10.5-E11.

Although M15 cells are unresponsive to WNT9b, they are derived from the Osr1/WT1(+) lineage in embryonic kidney and afford an informative *in vitro* model in which to explore the molecular basis for WNT9b responsiveness. M15 cells express many components of the canonical WNT-signaling pathway, including 4 frizzled receptors (*Fzd1, Fzd2, Fzd3* and *Fzd5*) which can be detected in the cap mesenchyme surrounding each ureteric bud tip. M15 cells also express the frizzled co-receptor *Lrp6* and complex-stabilizing proteins *Lgr4/6,* shown by the GUDMAP consortium to be present in cap mesenchyme [24, 25]. Strikingly, however, they do not express members of the R-spondin family. Several investigators have shown that canonical WNT-signal transduction is dramatically increased by stabilization of the FZD/LRP6/WNT complex at the cell surface as a result of the presence of R-spondins [23].

The R-spondin family binds to the WNT-receptor complex through its association with an LGR family member [29] and ZNRF3/RNF43. ZNRF3 is a negative regulator of canonical WNT-signalling and has a role of ubiquitinating FZD receptors, targeting them for destruction and also preventing phosphorylation of LRP receptors, keeping them in their inactive form [30, 31]. RSPO1 binds to ZNRF3 which in turn associates with an LGR receptor to remove ZNRF3 from the cell membrane and allows the WNT-receptor to remain active at the cell surface [31]. RSPO1 transcripts are strongly expressed in the cap mesenchyme of E11.5 mouse kidney [32] but were entirely absent in M15 cells. In our study, pre-treatment of M15 cells with RSPO1 enhanced WNT9b-induced canonical signaling activity 4-fold. When the cells were transfected with additional *Fzd5*, RSPO1 augmented WNT9b-responsiveness 11-fold. Thus, RSPO1 appears to be critical for a robust response to WNT9b and its absence in M15 cells precludes measurable signal transduction. We

postulate that RSPO1 is not expressed in the early developing kidney (E10.5) and the effects of WT1 on NPC chromatin alone are insufficient to induce RSPO1 expression. RSPO1 expression may be a late priming event in the maturation of the NPC.

The effects of RSPO1/LGR interactions are crucial for normal nephrogenesis. Three LGRs (*Lgr4*, *Lgr5*, *Lgr6*) interact with R-spondin proteins [29, 33-36]. LGR5 has been well-studied in intestinal epithelia where it was shown to have an important function to promote intestinal stem cell renewal [37-39]. We detected both Lgr4 and Lgr6 transcripts in M15 cells which is in concordance with the data found on GUDMAP, where expression was detected in the NPC lineage. Current commercially available siRNAs are non-specific and result in knockdown of both transcripts, therefore we cannot determine which protein is most important in NPCs. Another group studying *Lgr4*-knockout mice observed increased apoptosis in NPCs and disruption of the process by which NPCs condense around ureteric bud tips [40], suggesting Lgr4 may be the primary determinant of WNT9b signal transduction in cap mesenchyme. In contrast, murine knockout of the *Rspo1* gene has no renal phenotype [41], likely reflecting redundancy between RSPO1 and RSPO3, both of which are expressed in the cap mesenchyme (GUDMAP). This is in keeping with our *in vitro* observations indicating that both recombinant RSPO1 and RSPO3 enhance WNT-responsiveness in M15 cells.

Few studies have investigated frizzled expression in the developing kidney. Ureteric bud specific expression of FZD4 and FZD8 in E11.5 kidneys was previously examined using *Fzd4*-lacZ and *Fzd8*-lacZ mouse models [42]. Additionally, widespread renal expression of FZD2 and FZD7 was observed in 12, 13 and 18-week human fetal kidneys [43]. Our in situ hybridization data revealed distinct Frizzled expression patterns in E11.5 mouse kidneys. We detected *Fzd1*, *Fzd2*, *Fzd3*, *Fzd5* and *Fzd7* expression in the cap mesenchyme, whereas *Fzd4*, *Fzd6* and *Fzd8* expression was highly restricted to the ureteric bud. *Fzd10* expression was relatively non-specific and *Fzd9* in situ hybridization did not work for technical reasons. Interestingly, we found that in the presence of RSPO1, only *Fzd5* was limiting the WNT-response as the canonical signal was amplified by transfecting cells with *Fzd5* but none of the other Fzd family members. Furthermore, siRNA

knockdown of *Fzd5* (but not *Fzd1* or *Fzd2*) reduced WNT9b responsiveness. These observations suggest that FZD5 is the primary WNT co-receptor involved in transducing the inductive WNT9b signal in mammalian kidney. Moreover, it raises the possibility that the other FZDs expressed in cap mesenchyme might be involved in transduction of other canonical and non-canonical WNT ligands, such as WNT6 and WNT11 from ureteric bud tips [44, 45] or WNT2b and WNT4 from the metanephric mesenchyme of the developing kidney [46, 47].

Phylogenetic analysis of human frizzled proteins established five distinct frizzled subgroups [48], one of which consisted of FZD5 and FZD8. Our in situ hybridization studies of E11.5 embryonic mouse kidney demonstrate expression of *Fzd5* in the cap mesenchyme while *Fzd8* is exclusively expressed in the ureteric bud. Interestingly, WNT9b was demonstrated to bind and form a complex with FZD8 and LRP6 [49]. It is conceivable that *Fzd8* mediates the robust canonical WNT signaling activity in ureteric buds reported by Bridgewater and Iglesias [13, 50].

The renal stroma, marked by the *Foxd1* promoter, is another major compartment of the developing kidney which surrounds NPCs in the cap mesenchyme. *Foxd1* knockout mice develop smaller kidneys with disorganized tubular structures suggesting the Foxd1(+) stroma is required for nephrogenesis to proceed normally [51-53]. Das et al (2013) propose a model in which the renal stroma promotes NPC differentiation through secretion of Fat4. Ultimately, this process results in phosphorylation of YAP/TAZ which promotes transcription of Class I beta-catenin targets (differentiation) rather than Class II beta-catenin targets (self-renewal) [51]. In Foxd1 knockout mice, NPCs do not receive the Fat4 signal from the renal stroma and remain in a state of self-renewal. However, initiation of differentiation or self-renewal both require Wnt9b to bind to its cell surface receptor.

Based on our data and the observations above, we propose a model of renal development in which WT1(+) NPCs in E10.5 embryonic mouse kidney express some, but not all, components of the canonical WNT-signaling pathway. By E11.5, additional events (expression of RSPO1 and

increased expression of FZD5) have primed NPCs forming the cap mesenchyme, allowing responsiveness to the anticipated WNT9b signal from ureteric bud (Fig 2.6).

2.2.6 Acknowledgements

The authors would like to acknowledge background information drawn from the GUDMAP consortium public database (www.GUDMAP.org). We would like to acknowledge co-investigators of the CIHR/FRSQ/ERARE consortium who gave advice and critical analysis of the manuscript.

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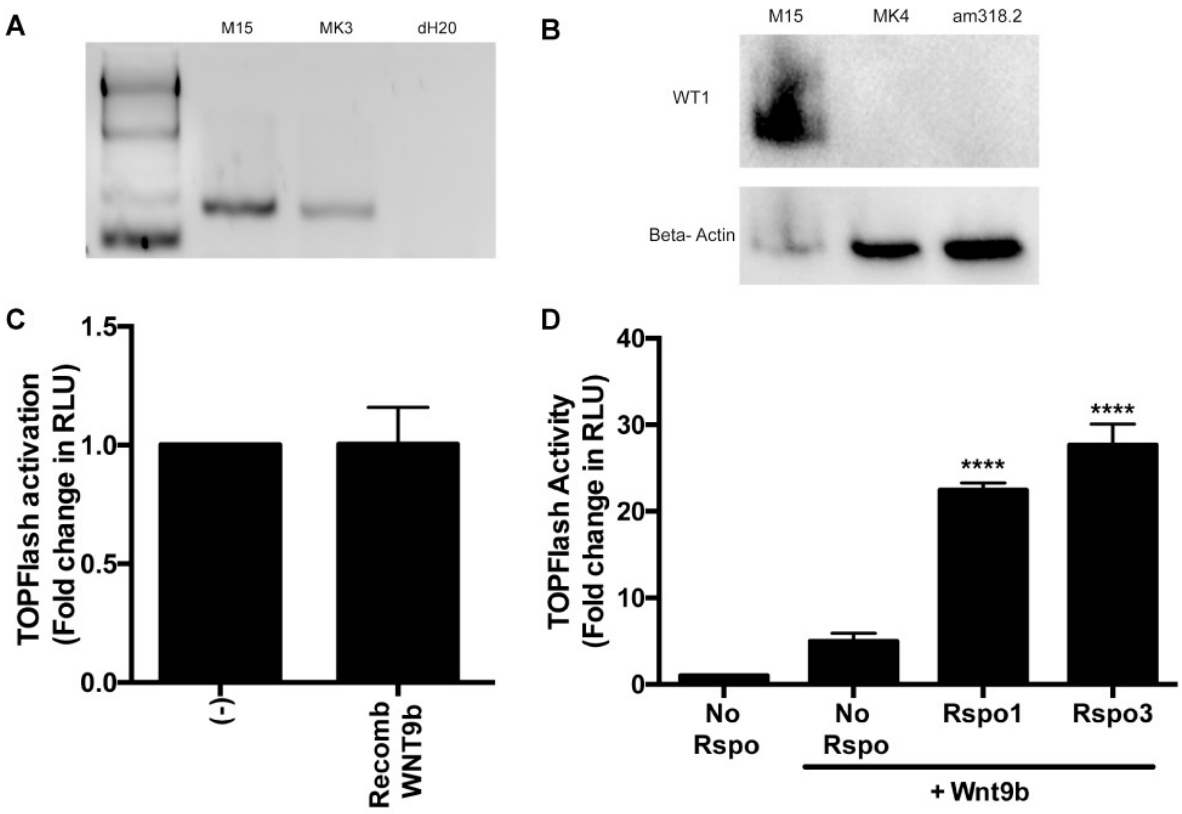


Figure 2.1. Effect of recombinant RSPO1 on responsiveness of M15 cells to WNT9b.
(A) mRNA from E10.5 mouse mesonephric mesenchyme (M15 cells) was analyzed by RT-PCR for *Wt1* mRNA expression in M15 cells and MK3 (positive control) cells vs water blank. (B) Lysates of M15 cells vs E14.5 MK4 (negative control) or am318.2 mesenchymal stem cells from 20-week gestation human amniotic fluid were analyzed by Western immunoblotting for WT1 protein (upper panel) and Beta actin (lower panel). (C) M15 cells were transiently transfected with β-catenin-luciferase reporter (TOPFlash) and Renilla-luciferase reporter. The cells were exposed to recombinant WNT9b (50 ng/ml). After 48 hours, TOPFlash to Renilla signal (RLU) was measured in a luminometer. An unpaired two-tailed Welch's t-test was performed. (ns) p=0.98. (D) M15 cells were transfected with TOPFlash, Renilla and *Wnt9b* plasmids and cultured for 24 hours; recombinant RSPO1 or RSPO3 (200 ng/ml) were added for an additional 24 hours and TOPFlash to Renilla signal was measured. A one-way ANOVA followed by a Dunnett correction for multiple comparisons was performed. (****) = p <0.0001.

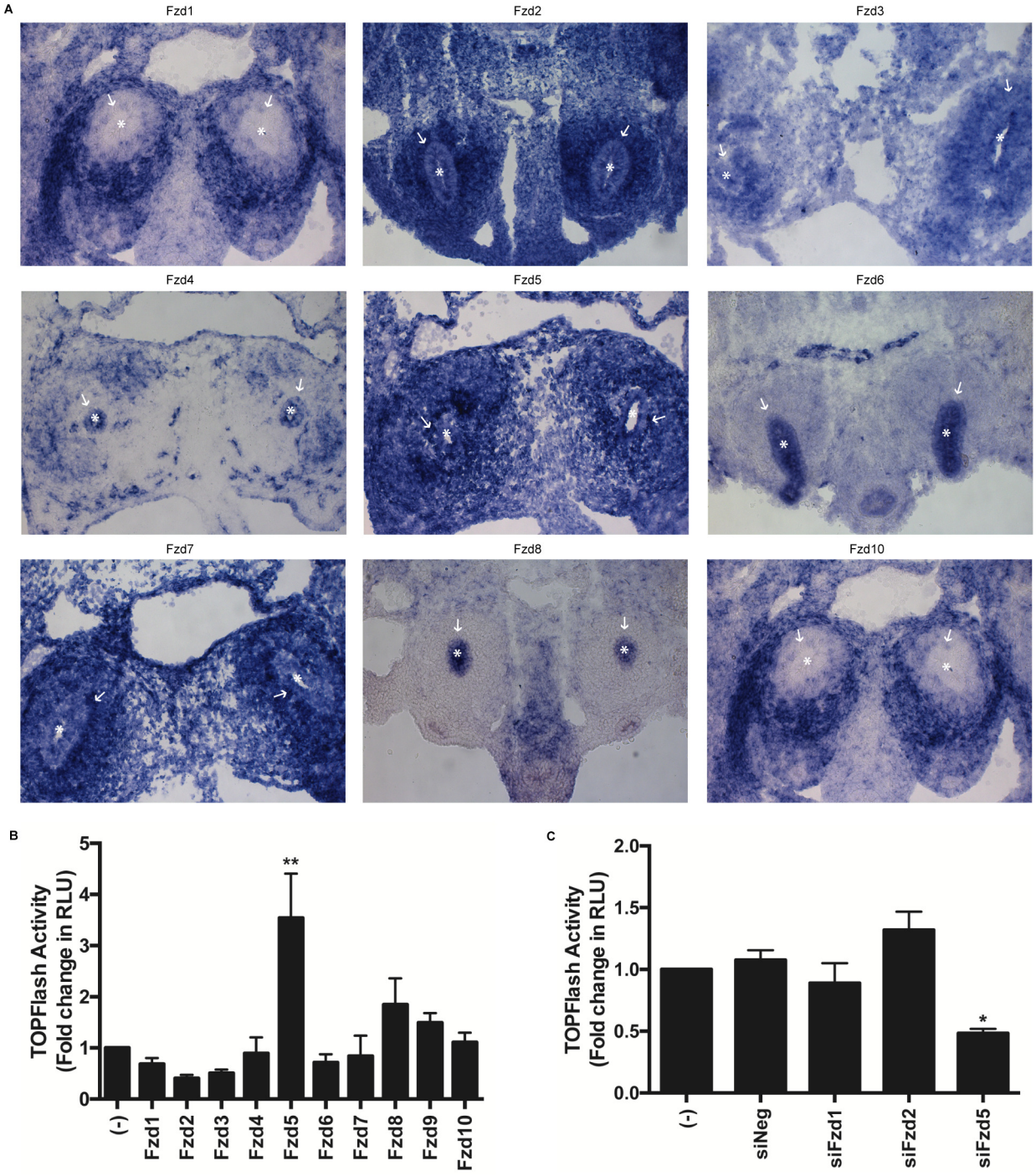
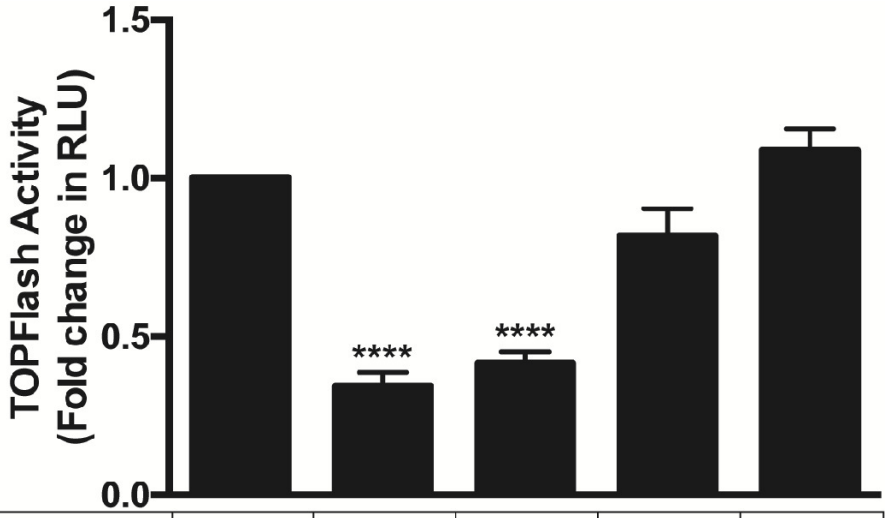


Figure 2.2. Effect of *Fzd* expression on WNT9b responsiveness in M15 cells.
(A) Cross sections of E11.5 embryos displaying both nephric fields were assessed by *in situ* hybridization using riboprobes for *Fzd1-10*, except *Fzd9* which was unsuccessful for technical reasons. Asterisk (*) marks ureteric buds. Arrow (→) marks cells of the cap mesenchyme. (B) M15

cells were transiently transfected with β-catenin-luciferase reporter (TOPFlash), Renilla-luciferase reporter, *Wnt9b*-expression vector and various *Fzd*1-10 expression plasmids in the presence of recombinant RSPO1 (200 ng/ml). TOPFlash to Renilla signal was measured after 48 hours. A one-way ANOVA followed by a Dunnett correction for multiple comparisons was performed. (**) p=0.0002 (C) M15 cells were transiently transfected with β-catenin-luciferase reporter (TOPFlash), Renilla-luciferase reporter, *Wnt9b*-expression vector and siRNAs targeting *Fzd*1, *Fzd*2 or *Fzd*5 vs a scrambled negative control siRNA in the presence of recombinant RSPO1 (200 ng/ml). TOPFlash to Renilla signal was measured. A one-way ANOVA followed by a Dunnett correction for multiple comparisons was performed. (*) p=0.005



siLrp6	-	+	+	-	-
Lrp5	-	-	+	+	+
scrambled siRNA	-	-	-	+	-

Figure 2.3. *Lrp6* is required for optimal responsiveness of M15 cells to WNT9b.
M15 cells were transiently transfected with β-catenin-luciferase reporter (TOPFlash), Renilla-luciferase reporter and a *Wnt9b* expression vector and treated with RSPO1 (200 ng/ml). The cells were co-transfected with an siRNA targeting *Lrp*6 or a scrambled negative control siRNA in the presence of recombinant RSPO1 (200 ng/ml). After 48 hours, TOPFlash to Renilla signal was measured. In another experiment, the cells were co-transfected with a *Lrp5* expression plasmid to assess its effect on WNT9b pathway activity. A one-way ANOVA followed by a Dunnett correction for multiple comparisons was performed. (****) $p<0.0001$

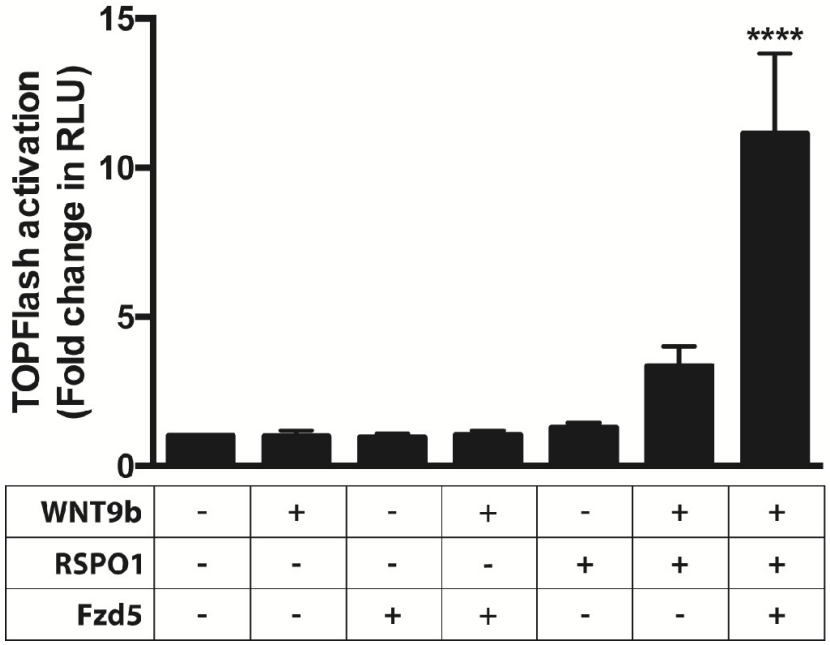


Figure 2.4. Responsiveness to extrinsic WNT9b is restored by addition of *Fzd* and RSPO1.
In all conditions, M15 cells were transiently transfected with β-catenin-luciferase reporter (TOPFlash) and Renilla-luciferase reporter; in some experiments the cells were co-transfected with *Fzd*5 expression plasmid. TOPFlash to Renilla signal was measured in the presence or absence of recombinant WNT9b (50 ng/mL) and/or recombinant RSPO1 (200 ng/ml). A one-way ANOVA followed by a Dunnett correction for multiple comparisons was performed. (****) = $p<0.0001$

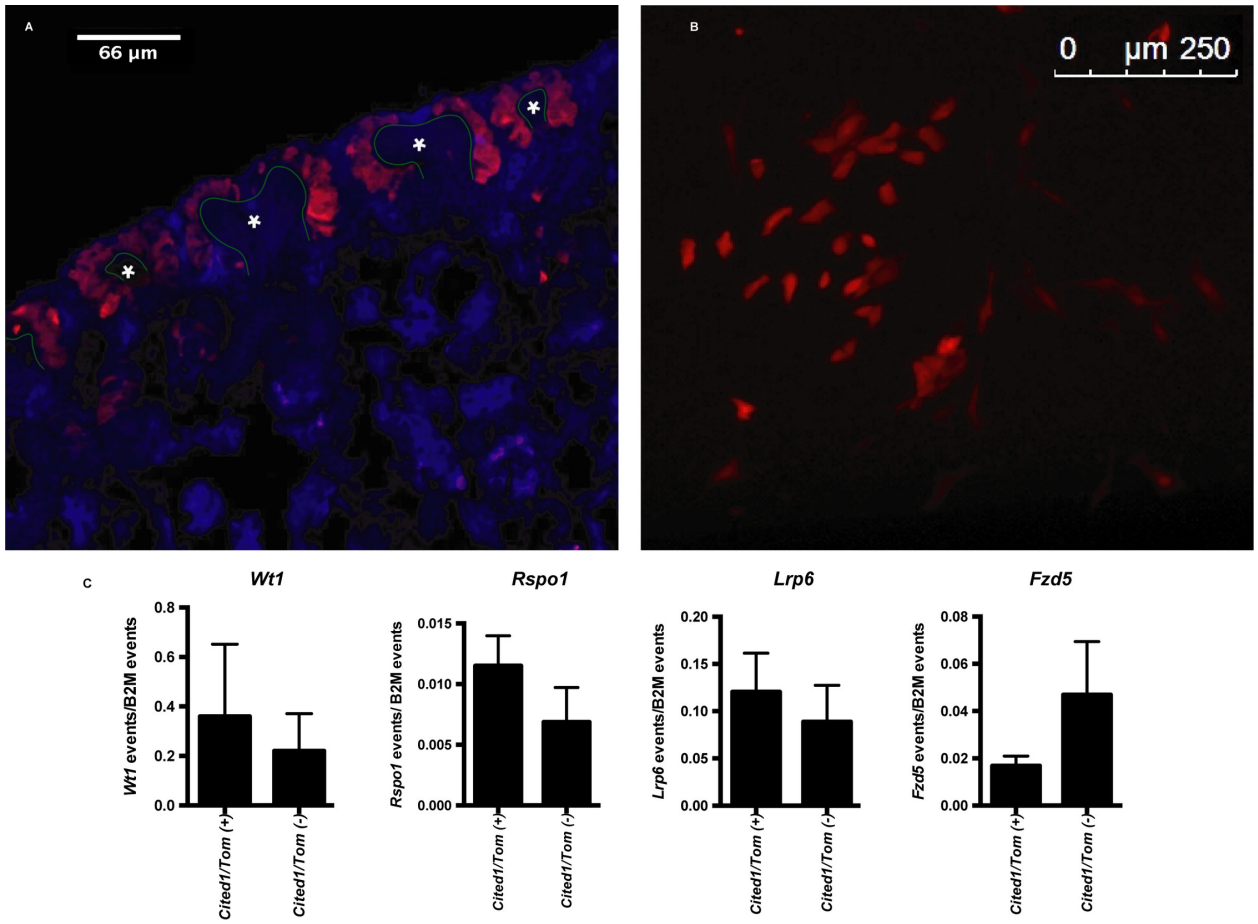


Figure 2.5. Identification and isolation of Cited1 expressing cells from embryonic kidneys.
(A) Cryosections of E18 embryonic kidneys isolated from Cited1^{Cre}/TomatoRed mice were assessed by immunofluorescent microscopy for the presence of TomatoRed in cap mesenchyme surrounding ureteric bud tips. (*) Ureteric Bud outlined in green. (B) Whole E15.5 embryonic kidneys were dispersed into monolayer culture in the presence of tamoxifen (2.5 µg/ml) for 16 hours and TomatoRed(+) cells were visualized by immunofluorescent microscopy. (C) Expression of WNT9b pathway component transcripts in Cited1^{Cre}/TomatoRed cells isolated by FACS from E15.5 embryonic mouse kidney quantified by ddPCR. Bars represent mean number of events of gene of interest normalized to B2M events (n=2, 17 total pooled embryonic kidneys). Error bars represent standard error of the mean.

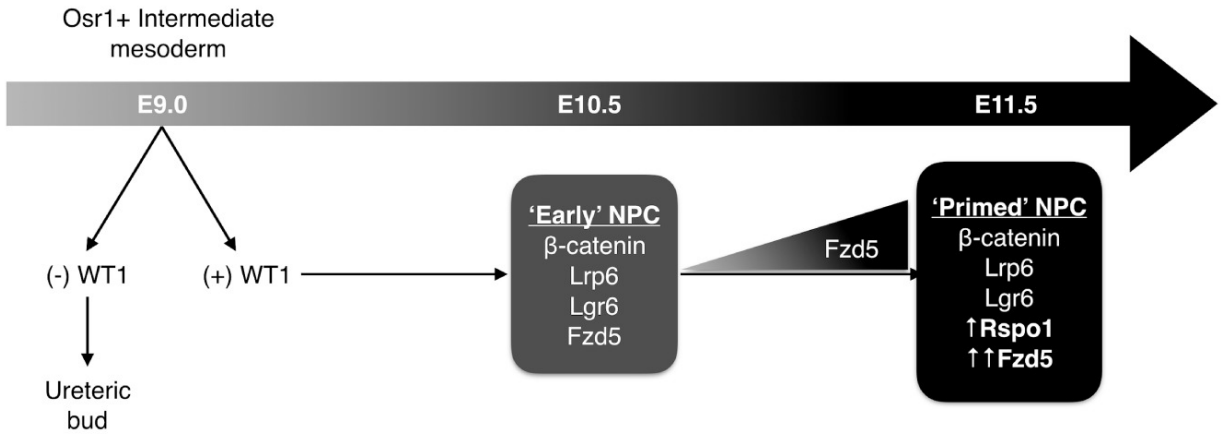


Figure 2.6. Proposed model of nephron progenitor cell development in embryonic mouse kidney.

Early WT1(+) NPCs express a number of important molecules in the canonical WNT-signalling pathway. By E11.5, increased expression of Fzd5 and addition of Rspo1 render NPCs fully competent to respond to the inductive WNT9b signal.

End of manuscript.

2.3 Supplementary Statistical Anaylsis Information

All luciferase experiments detailed in Chapter 2 (Fig 2.1C, 2.1D, 2.2B, 2.2C, 2.3 and 2.4) were performed in triplicate (technical replicates) on three different days (biological replicates; n=3). All subsequent figures in Chapter 3 and Appendix I include more detailed statistical information in the figure legend.

2.4 Connecting Chapter 2 and Chapter 3

In Chapter 2, we show that NPCs need to express FZD5, LRP6 and RPSO1 for optimal responsiveness to WNT9B. This observation is not only interesting in understanding the biology of kidney development, it also has implications in developing cell-based therapies for AKI and CKD. Our lab has shown that the canonical WNT-signalling pathway is essential for cell integration into a model of AKI. Therefore, knowing the specific molecular components required for enhancing WNT-signalling in an NPC can be translated to generate an optimal cell to use in treating kidney injury. We show that cells simply treated with RSPO1 prior to cell infusion increases cell integration into renal tubules of mice with AKI (Refer to Appendix I).

Another characteristic of NPC biology yet to be described is the role of DNA-repair genes during the early priming process. This is of particular importance because the precursor lesions that result in WT formation are comprised of developmentally arrested NPCs that failed to differentiate into renal tubules in response to the WNT9B signal. WT1 acts as a master regulator to allow for the differentiation cascade to proceed normally and is commonly mutated in WTs. Interestingly, secondary mutations in the β-catenin gene (*CTNNB1*) are often detected in *WT1*(-) tumours, suggesting the genomic stability of these developmentally arrested cells may be compromised. In the following chapter, we investigate DNA-repair expression profiles in NPCs of the cap mesenchyme and determine whether expression is dependent on WT1.

Chapter 3: DNA repair in Nephron Progenitor Cells

3.1 Overview

In early embryonic kidney, the inductive WNT9B signal from the UB initiates a burst of rapid proliferation in NPCs, permitting cells to begin differentiation (Carroll et al., 2005). Proliferation in the nephrogenic zone can be observed in the kidney from the initial induction event until the end of nephrogenesis which occurs postnatally in mice (O'Hara et al., 2019; Saifudeen et al., 2002). Prior to the arrival of the UB, tumour suppressor gene, WT1, is expressed in the intermediate mesoderm and primes the NPC population to respond to WNT9B. However, loss of *WT1* in NPCs results in the formation of clones of developmentally arrested cells called "nephrogenic rests" (Call et al., 1990). Nephrogenic rests are considered WT precursor lesions and contain cells expressing NPC marker CITED1 (Lovvorn et al., 2007). Epigenetic analysis of WTs revealed similar expression patterns when comparing tumour tissue to fetal kidney and embryonic stem cells, with key genes of the differentiation cascade remaining epigenetically silenced (Aiden et al., 2010). Additionally, all three cell lineages of the developing kidney – blastema, epithelia and stroma – are detected in WT tissue. This characteristic triphasic histology demonstrates that WTs arise from early progenitor cells retaining some level of multi-potency (ie. NPCs). Nephrogenic rests may persist within the normal kidney and resorb over time. However, secondary genetic lesions are commonly acquired – most often, mutations in the *CTNNB1* gene, resulting in constitutive activation of β-catenin which drives unregulated proliferation (Zirn et al., 2006; Fukuzawa et al., 2007; Uschkereit et al., 2007; Koesters et al., 1999). Approximately 15% of all WTs and 50% of WTs with mutations in *WT1*, acquire activating mutations in *CTNNB1*, indicating that loss of WT1 appears to have an effect on the genomic stability of nephrogenic rests (Li et al., 2004).

The role of WT1 in regulating DNA-repair pathways is an area that has been under investigated. Using an in vitro model, over-expression of WT1 isoform C was shown to upregulate genes involved in the homologous recombination DNA-repair pathway (*Xrcc2*, *Rad51D*, and *Rad54*) (Oji et al., 2015). Additionally, analysis of human WT samples identified an association between increased risk of developing WT with a polymorphism in a NER gene, *XPD* (*ERCC2*) (Zhu et al., 2018). While these studies show a potential link between WT1 and DNA-repair, they do not

examine the role of WT1 in NPCs of the cap mesenchyme. In this study, we investigate the expression of DNA-repair genes in *Cited1*(+) NPCs from embryonic mouse kidney and determine whether expression is dependent on WT1. We hypothesize that WT1 expression in NPCs of the cap mesenchyme is essential to activate specific DNA-repair genes in anticipation of the burst of proliferation induced by WNT9B.

In section 3.2, we show that NPCs express a specific DNA-repair gene profile which is regulated in part by WT1. Cells isolated from *Cited1*(+) NPCs have elevated expression levels of BER gene, *Neil3*, compared to adult kidney and total embryonic kidney. In contrast, we did not detect upregulation of *Neil3* in UB cells, however, other repair genes were specifically upregulated in that compartment (*Ung*, *Rad51b*, *Brca1*). Knockdown of *Wt1* in *Cited1*(+) NPCs resulted in a corresponding downregulation of *Neil3* expression. Finally, we show that WT1 directly binds the *Neil3* promoter to drive transcription. We speculate that this could be a potential mechanism responsible for the common occurrence of *CTNNB1* mutations in *WT1*(-) nephrogenic rests.

3.2 WT1 Regulates Expression of the DNA-Repair Gene *Neil3* During Nephrogenesis

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Keywords: DNA-repair gene, WT1, Wilms Tumor, Neil3, nephron progenitor cell, kidney development

3.2.1 Abstract

Background: Individuals who inherit a germline mutation in *WT1* often acquire a second inactivating mutation within nephron progenitor cells during kidney development. This renders the cells unresponsive to the inductive WNT9B signal. These developmentally arrested cells frequently acquire activating mutations in *CTNNB1* that drive Wilms tumorigenesis. However, the mechanism is unclear. We hypothesize that WT1 upregulates expression of DNA-repair genes within NPCs prior to induction by WNT9B.

Results: First, to identify DNA-repair systems that might be WT1-dependent, we analyzed DNA-repair gene transcript levels in embryonic versus adult kidney and selected those with >20-fold increase for further study. The repair gene, *Neil3,* was enriched in mouse *Cited1*(+) NPCs. Cre-recombinase driven knockout of *Wt1* in *Cited1*(+) NPCs (60% knockout efficiency) caused 58% reduction in *Neil3*-expression. ChIP-PCR analysis showed that WT1 binds to the *Neil3* promoter. Transfection of a *Neil3* promoter-reporter into WT1(-) and WT1(+) cells increased reporter activity 2-fold in WT1(+) cells, demonstrating WT1 activates *Neil3* transcription. Thus, *Neil3* expression in cap mesenchyme NPCs is WT1-dependent.

Hypothesis: We propose that loss of the WT1-dependent enzyme *Neil3* compromises genomic stability, permitting constitutively-active mutations of *CTNNB1* to arise in *WT1*(-) nephrogenic rests.

3.2.2 Introduction

Mammalian kidneys develop from the embryonic intermediate mesoderm. Reciprocal interactions between the nephric duct and the metanephric mesenchyme are required for normal kidney development to occur. As the nephric duct extends caudally in the embryo, it reaches a subset of *Wt1*-expressing mesenchyme, committed to form nephron progenitor cells (NPCs). WT1 is a transcription factor that sets the cell fate and oversees the departure from a primitive stem cell state to a specialized progenitor, equipped to respond to inductive signals (Narlis et al., 2007; Wilm and Munoz-Chapuli. 2016). The nephric duct/metanephric mesenchyme interaction induces an outgrowth of the nephric duct, called the ureteric bud (UB) at

approximately E10.5 in mouse kidney development (Dressler. 2009). At this stage, NPCs forms a cap of cells around the UB and begin to express *Cited1* which has been shown to be a marker of an early/unprimed NPC (Boyle et al., 2007; Lovvorn et al., 2007; Mugford et al., 2009; Park et al., 2012).

In E11.5 mouse kidney, the UB begins to arborize within the metanephric mesenchyme (Dressler. 2009). Observations by Herzlinger in 1994 showed that nephrogenic precursors were induced to differentiate by canonical WNT/β-Catenin signals (Herzlinger et al., 1994). Subsequently, Carroll showed WNT9B was the specific WNT ligand secreted by the UB (Carroll et al., 2005; Karner et al., 2011). Dickinson et al. recently found that WNT9B binds to a Frizzled5 (FZD5) receptor complex that includes LRP5 and R-spondin1 (RSPO1) (Dickinson et al., 2019). The inductive WNT response in NPCs releases the differentiation cascade involving a new series of transcription factors (eg. *Six2*/*Pax8*) in parallel with a burst of cell proliferation which supports growth and segmentation of the emerging nephron (Stark et al., 1994; Carroll et al., 2005; Self et al., 2006; Kobayashi et al., 2008). Proliferation also continues in the nephrogenic zone to sustain the NPC pool until the end of nephrogenesis in the perinatal period (Saifudeen et al., 2002; O'Hara et al., 2019).

WT1 seems to be critical to the competence of NPCs. In the absence of *Wt1*, kidney development is blocked, and NPCs are unable to respond to WNT9B signals. Mice with biallelic *Wt1* mutations fail to develop kidneys (Kreidberg et al., 1993). Humans who inherit a heterozygous germline WT1 mutation develop competent NPCs that undergo normal nephrogenesis unless they acquire a somatic mutation of the second WT1 allele (Rivera and Haber. 2005). This gives rise to clones of developmentally arrested NPCs which are unresponsive to the inductive WNT9B signal (Call et al., 1990). These “nephrogenic rests” may persist within the normal kidney for long periods before they involute (Park et al., 1993). Alternatively, NPCs within the nephrogenic rests may acquire an additional genetic lesion, most often a constitutively-activating mutation of the β-Catenin gene (*CTNNB1*) which drives rapid cell growth and malignant transformation (Koesters et al., 1999; Fukuzawa et al., 2007; Zirn et al., 2006; Uschkereit et al., 2007). Patients who inherit

a germline *WT1* mutation often display bilateral/multifocal WTs, each with a unique, sporadic β-Catenin mutation (Rivera and Haber. 2005). Activating mutations of the β-catenin gene are present in 15% of all WTs and 50% of WTs with WT1 mutations (Li et al., 2004). This suggests that constitutively active β-catenin bypasses the requirement for WNT9B in releasing the differentiation cascade and rapid cell division that support nephrogenesis.

In this study, we hypothesize that WT1 is required for activation of specific DNA-repair pathways in *Cited1*(+) cells in the cap mesenchyme, thus, protecting the genome during the proliferative burst which characterizes nephrogenesis. We show that fetal mouse kidney expresses multiple DNA-repair genes at a high level compared to adult kidney. We developed a mutant mouse with *Cited1*-targeted knockout of *Wt1* to identify WT1-dependent DNA-repair genes specific to the cap mesenchyme.

3.2.3 Methods

3.2.3.1 DNA-repair-focused RT2 Profiler PCR Array

Pregnant C57BL/6J wildtype dams (N=3) were sacrificed when the embryos had matured to embryonic day 17.5 (E17.5). A dissecting microscope (Leica MZ75) and dissecting forceps were used to remove embryonic kidneys. The kidney pairs from all embryos within a litter were pooled into a single tube prior to RNA extraction. Kidney tissue was also harvested from 3 adult C57BL/6J males (4 months of age).

Total RNA was extracted from each pool (N=3) of E17.5 kidneys and from adult C57BL/6J wildtype kidney tissue (N=3) using the EZ-10 DNAaway RNA Miniprep Kit (Bio Basic, cat. # BS88133). Following kit specifications, 30 milligrams of frozen kidney tissue was used in each reaction. RNA was eluted in 30 μL of nuclease-free water and the quality and concentration of the RNA in each sample was determined by spectrophotometry. RNA was reverse transcribed into cDNA, using the RT2 First Strand Kit (Qiagen, cat.# 330401). 500 ng of RNA was added into each reverse transcription reaction.

A DNA-repair-focused RT2 Profiler PCR Array (Qiagen, cat. # PAMM-042Z) was used to compare DNA-repair gene expression in embryonic versus adult mouse kidneys. qPCR was performed on a LightCycler 480 Instrument II (Roche, product # 05015278001), following the thermocycling conditions recommended by the array manufacturer. The threshold cycle (CT) was calculated for each well using the second derivative max setting of the LightCycler 480 software.

Web-based PCR array data analysis software (Qiagen web portal at GeneGlobe, http://www.qiagen.com/geneglobe) was used to interpret the controls and to quantify the fold change in expression of each DNA-repair gene in embryonic kidneys compared to adult kidneys, using the delta-delta CT method. The CT value of each repair gene was normalized to that of B2m.

3.2.3.2 Mice

All animal experiments were approved by the McGill Facility Animal Care Committee (FACC). *Cited1*$^{CreER(T2)}$ mice were donated by Dr. Mark de Caestecker. B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J (Tom$^{flox/flox}$) mice were purchased from Jackson Laboratories and turn on tdTomato reporter expression when acted on by Cre- recombinase. *Wt1*$^{flox/flox}$ mice were donated by Dr. Peter Hohenstein. *Wt1* alleles in *Wt1*$^{flox/flox}$ mice have loxP sites flanking exons 8 and 9 of the *Wt1* gene, knocking out gene function in the presence of Cre-recombinase. *Hoxb7*^{GFP} were donated by Dr. Indra Gupta and these mice constitutively express GFP in Hoxb7 expressing tissues, including the ureteric bud.

To generate *Cited1*$^{CreER(T2)}$;Tom$^{flox/+}$ mice, *Cited1*$^{CreER(T2)}$ mice were mated with homozygous Tom$^{flox/flox}$ mice. To generate *Cited1*$^{CreER(T2)}$;*Wt1*$^{flox/flox}$;Tom$^{flox/+}$, *Cited1*$^{CreER(T2)}$ mice were mated with *Wt1*$^{flox/flox}$ mice. The resulting *Cited1*$^{CreER(T2)}$;*Wt1*$^{flox/+}$ were backcrossed to the *Wt1*$^{flox/flox}$ strain until *Cited1*$^{CreER(T2)}$;*Wt1*$^{flox/flox}$ mice were obtained. In parallel, *Wt1*$^{flox/flox}$ mice were crossed with the Tom$^{flox/flox}$ mice. The pups were self-crossed to obtain *Wt1*$^{flox/flox}$;Tom$^{flox/flox}$ mice. Finally, *Wt1*$^{flox/flox}$;Tom$^{flox/flox}$ mice were crossed with *Cited1*$^{CreER(T2)}$;*Wt1*$^{flox/flox}$ mice to generate *Cited1*$^{CreER(T2)}$;*Wt1*$^{flox/flox}$;Tom$^{flox/+}$ embryos which were harvested for downstream applications.

3.2.3.3 Isolation of Tomato red cells by FACS

Embryos were harvested at E17.5 A dissecting microscope (Leica MZ75) and dissecting forceps were used to remove embryonic kidneys. Kidneys were placed in a digesting solution at 37˚C for 45 minutes. Digestion solution contained 1 mg/mL Collagenase B (Roche, cat. # 11088807001), 2.5 mg/mL Dispase II (Roche, cat. # 04942078001), 2% FBS (Wisent, product # 080-450) and 1 Unit/mL DNase I (Roche Diagnostics, product # 118286650) in PBS (Corning). Embryonic kidney cells were placed in NPC growth media (Arcolino et al., 2016) within sterile cell culture flasks, and incubated for 24 hours at 37˚C. After 24 hours, the cells were treated with 2.5 µg/mL of (Z)-4-Hydroxy Tamoxifen (Toronto Research Chemicals, cat. # H954725), to activate Cre-recombinase activity. Cells were incubated at 37˚C with the (Z)-4-Hydroxy Tamoxifen for a further 24 hours, and then examined under a fluorescent microscope (Zeiss Axiovert 40 CFL). Flasks with visible red fluorescence were trypsinized for 5 minutes using 0.25% trypsin/EDTA (Corning, cat. # MT25053CI) and pooled into a single sample for FACS analysis. Flasks with no visible fluorescent cells were trypsinized for 5 minutes and combined into a single total kidney cell pool, which was pelleted and stored at -80°C until total RNA extraction.

The FACS sample was resuspended in 0.5-1 mL of FACS media, containing 2% FBS (Wisent) in PBS (Corning), filtered using a 70 μm cell strainer, and kept at 4˚C until sorting. Cell sorting was performed by MUHC immunophenotyping core facility staff using a BD FACSAria Fusion. Isolated *Cited1*$^{CreER(T2)}$;Tom$^{flox/+}$ or *Cited1*$^{CreER(T2)}$;*Wt1*$^{flox/flox}$;Tom$^{flox/+}$ cells were immediately pelleted and frozen at -80˚C.

The same method was followed to isolate fluorescent green *Hoxb7*^{GFP} cells from embryonic kidneys of *Hoxb7*^{GFP} mice, however cultured cells were not treated with (Z)-4-Hydroxy Tamoxifen, as these cells do not require Cre-recombinase activation in order to fluoresce.

3.2.3.4 Total RNA extraction from fluorescence-sorted cells and total kidney cell pools of littermates

Cited1(+) cells from 4 litters of *Wt1*$^{+/+}$ mice were pooled into a single sample to provide a sufficient number of cells for total RNA extraction (approximately 490,000 cells in total). Similarly,

Cited1(+) cells from 6 litters of *Wt1*$^{flox/flox}$ mice were pooled into a single sample of approximately 400,000 cells for total RNA extraction. *Hoxb7*(+) cells from 4 litters were pooled into a single sample of approximately 767,000 cells for RNA extraction.

RNA samples were prepared from 4 total kidney cell pools of *Wt1*$^{+/+}$ mice, 5 total kidney cell pools of *Wt1*$^{flox/flox}$ mice and 3 total kidney cell pools of *Hoxb7*^{GFP} littermates. Total RNA was extracted from each sample using the Zymo Research Quick-RNA Microprep Kit (Zymo Research, cat. # R1050). In all cases, RNA was eluted in 15 μL of nuclease-free water and the quality and concentration of the RNA in each sample was determined by spectrophotometry.

3.2.3.5 RT-qPCR Analysis of DNA Repair Gene Expression

10 ng of cDNA was loaded into each qPCR reaction, along with 5 µL of SsoFast EvaGreen Supermix (Bio-Rad, cat. # 1725211), Forward and Reverse primers each at a final concentration of 0.3 µM, and 2.4 µL of nuclease-free water. The total volume of each reaction was 10 µL and each reaction took place in a single well of a 96-well -qPCR plate. Quantitative measurement of mRNA was performed in technical triplicate for each cDNA sample, and the qPCR plate was read by a LightCycler 480 Instrument II (Roche, product # 05015278001).

The CT value of each repair gene was normalized to that of *B2m* for every sample. The normalized expression of each repair gene in *Cited1*(+) cells was computed as a fold change relative to average expression of the gene in 6 total kidney cell pools of *Wt1*$^{+/+}$ mice (N=6 for Standard Error of the Mean calculations). Similarly, the normalized expression of each repair gene in *Cited1(+)* cells of *Wt1*$^{flox/flox}$ mice was computed as a fold change relative to average expression of the gene in 5 total kidney cell pools of *Wt1*$^{flox/flox}$ mice (following treatment with 4-hydroxytamoxifen to activate Cre-recombinase activity). The normalized expression of each repair gene in *Hoxb7*(+) cells was computed as a fold change relative to average expression of the gene in 3 total kidney cell pools. In all instances, the expression of each repair gene was averaged across multiple pools of total kidney cells and used as a baseline against which all DNA repair gene expression measurements in the *Cited1*- or *Hoxb7*-compartments can be compared.

3.2.3.6 Chromatin Immunoprecipitation

M15 cells were grown, fixed, quenched and harvested following standard techniques. Sonication was performed on a Sonolab 7.1 (Covaris, Toronto, ON, Canada) to obtain 500 bp fragments. The immunoprecipitation was performed using Protein A/G agarose beads. The following antibodies and titers were used: Normal Rabbit IgG (2729, 5 µg Cell Signaling, Danvers, MA, USA), RNA polymerase II (17-620, 5ug, Sigma-Aldrich,), WT1 C19 (sc-192, 5µg Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Online database, MatInspector (Genomatix, Munich, Germany), was utilized to perform in silica analysis of the *Neil3* promoter to detect putative transcription factor binding sites. PCR primers surrounding 2 putative WT1 binding sites in the Neil3 promoter were designed using NCBI PrimerBlast (*Neil3*promoter-F: AGGCCTGACTGGAAAACAAC and *Neil3*promoter-R: GGCTCAGGAAATACCGATGACA).

3.2.3.7 *Neil3* promoter reporter assay

MK4 cells and MK4 cells stably-transfected with WT1 were seeded in 6-well plates (100,000 cells/well) and transiently transfected with a mCherry Neil3 promoter reporter plasmid (250ng/well, MPRM48810-PM02, GeneCopoeia, Rockville, MD, USA). Cells were prepared for FACS as described above and fluorescence between WT1(+) and WT1(-) cells were compared.

3.2.3.8 Statistical Analysis

P-value for the RT2 Profiler PCR Array was calculated using a Student's t-test of the normalized expression for each gene in the embryonic versus the adult kidney samples. *Neil3* promoter-reporter results were analyzed with an ordinary one-way ANOVA with multiple comparisons.

3.2.4 Results

During murine nephrogenesis, the ureteric bud begins to penetrate and arborize within the metanephric mesenchyme at around E11.5. WNT signals from the tip of each ureteric bud induce the adjacent cap mesenchyme to activate the differentiation cascade and undergo a phase of rapid cell division to form nephrons. To test the hypothesis that these cells require activation of a specific set of DNA-repair enzymes to protect the genome, we examined the transcript levels of 84 DNA-repair genes (normalized to *B2m*), using a RT2 Profiler PCR Array. As seen in Figure

3.1, 48 of the 84 genes were expressed in E17.5 kidney at a transcript level at least 2-fold above that in adult kidney ($P < 0.05$). Only one gene (*Parp3*) was suppressed > 2-fold compared to adult kidney, however this result was not statistically significant. The fold change value and corresponding P-value for each DNA-repair gene included in the RT2 Profiler PCR Array (listed alphabetically) are presented in Supplementary Table S3.1.

We reasoned that, if renal progenitor cells require a specific set of DNA-repair enzymes to protect the genome during nephrogenesis, then these genes would be highly enriched in cells of the cap mesenchyme. To test this idea, we selected genes upregulated > 20-fold in the embryonic kidney compared to the adult. These seven genes are listed in Table 3.1.

Table 3.1. DNA repair genes upregulated > 20-fold in embryonic versus adult mouse kidney.

Symbol	Embryonic Kidney Fold Change (comparing to adult kidney)	P-value
Exo1	34.78	0.020
Lig1	28.57	0.001
Neil3	28.05	0.003
Ung	27.16	0.002
Rad18	25.87	< 0.001
Rad51b	24.88	0.002
Brca1	24.59	< 0.001

Table Legend: mRNA expression for each gene was determined by qRT-PCR performed three times (n=3). This fold change value represents the normalized expression for the gene of interest in the embryonic kidney divided by its normalized expression in the adult kidney. Each P-value was calculated using a Student's t-test of the normalized expression for each gene in the embryonic versus the adult kidney samples.

To ascertain which of these DNA-repair enzymes were enriched specifically within the cap mesenchyme, we isolated E17.5 kidneys from embryonic mice bearing the NPC marker *Cited1/tdTomato*. *Cited1(*+) cells were isolated by FACS and assayed for individual transcript

levels (normalized to *B2m*). Figure 3.2 shows relative expression of each DNA-repair enzyme in *Cited1*(+) cells compared to average levels in the entire embryonic kidney. Several DNA-repair enzymes appeared to be suppressed in the cap mesenchyme (*Exo1, Rad51b* and *Ung*), but *Neil3,* a DNA glycosylase in the base excision repair pathway, was uniquely enriched (about 15-fold).

To ascertain whether any of the genes which were not enriched in cap mesenchyme were elevated in another rapidly-dividing embryonic kidney compartment, we also isolated cells expressing the ureteric bud marker *Hoxb7-GFP* and analyzed transcript level of each DNA-repair enzyme, as above. As seen in Figure 3.3, at least two of the DNA-repair enzymes downregulated in the *Cited1* (+) cells (*Rad51b* and *Ung*), were found to be enriched in the *Hoxb7*-compartment. Unlike the *Cited1*(+) cells, the *Hoxb7*(+) cells did not show enrichment in *Neil3* expression.

In order to estimate the efficiency of recombinase-inactivation of *Wt1*, we isolated and cultured primary cells from E17.5 mouse embryonic kidneys and treated them with (Z)-4-Hydroxy Tamoxifen (4OHT) in vitro. We then isolated *Cited1*(+) cells (expressing a fluorescent tdTomato marker) and compared *Wt1* expression level to that in the total kidney cell pools (before FACS separation). As seen in Figure 3.4A, *Wt1* expression in *Cited1*(+) cells of $Wt1^{+/+}$ mice was 3.86-fold higher than *Wt1* transcript level in the total kidney cell pool. In contrast, *Cited1(+)* cell expression of *Wt1* was only 1.38-fold higher than the total kidney cell pool in $Wt1^{flox/flox}$ mice. Thus, efficiency of recombinase-inactivation of *Wt1* was estimated to be approximately 64.2%.

Using the same samples, we then measured the effect of *Wt1* knockdown on *Neil3* transcript levels (Figure 3.4B). In $Wt1^{+/+}$ cells from wildtype E17.5 mice, *Neil3 w*as enriched in the *Cited1*-compartment by 14.9-fold. However, *Neil3* was enriched only 6.2-fold in the *Cited1*(+) cells from $Wt1^{flox/flox}$ mice. This represents a 58.4% reduction in *Cited1(+)* cell *Neil3* mRNA, paralleling the percent reduction in *Wt1*. There was no significant effect of *Wt1* knockdown on expression of the other 6 DNA-repair genes in *Cited1*(+) cells.

To determine whether WT1 regulates *Neil3* expression at the transcriptional level, we performed a ChIP-PCR to detect WT1 bound to the *Neil3* promoter. To accomplish this, we used WT1-expressing M15 cells, a cell line representative of an early NPC. We designed primers surrounding two predicted WT1-binding sites within the *Neil3* promoter (Figure 3.5A). We were able to detect regions of the *Neil3* promoter in our ChIP samples probed with a WT1 antibody (Figure 3.5B). Next, we performed a functional assay to confirm whether WT1-expression results in upregulation of *Neil3* mRNA transcription. For this experiment we used a WT1(-) cell line, MK4 cells, derived from partially differentiated metanephric mesenchyme from postnatal mouse kidney [Valerius et al 2002]. MK4 cells stably-transfected with WT1D were transiently transfected with a *Neil3* promoter-reporter (mCherry) vector and assessed for median fluorescence intensity (MFI) versus WT1(-) controls. We observed a 2.1-fold increase in MFI in WT1(+) cells compared to WT1(-) cells (Figure 3.5C). Since NPCs receive an inductive WNT9B signal to induce proliferation and differentiation, we tested the effect of exogenous canonical WNT9B (with or without WNT agonist, RSPO1) on *Neil3* promoter-reporter activity. However, we found no significant effect of WNT9B, RSPO1 or WNT9B/RSPO1 on *Neil3* promoter activity (Figure 3.5C).

3.2.5 Discussion

Studies into the developing kidney have highlighted WT1 as a critical transcription factor involved in priming early NPCs. Mice harbouring loss of function mutations in WT1 exhibit apoptosis of the metanephric mesenchyme and failure to develop the metanephric kidney (Kreidberg et al., 1993). Expression of WT1 in NPCs prior to the arrival of the UB unleashes a cascade of events in order to render cells responsive to the inductive WNT9B signal. Our lab has previously shown that loss of WT1 results in upregulation of histone H3 trimethylase, enhancer of zeste homolog 2 (EZH2), which then goes on to methylate the β-Catenin promoter and downregulate transcription (Akpa et al., 2015). Loss of β-Catenin expression reduces the ability of cells to activate the canonical WNT-signalling, a pathway critical to induce differentiation and proliferation in NPCs. Interestingly, individuals that inherit a germline mutation in *WT1*, including individuals with WAGR and Denys-Drash syndrome, often develop WTs at an earlier age than in sporadic cases. This is due to the requirement of only one additional *WT1* mutation which results in the formation of bilateral and multifocal nephrogenic rests within their kidneys.

Characterization of nephrogenic rests detected high levels of CITED1 expression, showing they are derived from early NPCs that have failed to differentiate into tubular epithelia (Lovvorn et al., 2007).

Wilms tumours arise in association with newly-acquired sporadic activating mutations of the β-catenin gene in WT1(-) progenitor cells. Analysis of WTs and nephrogenic rest cells immediately surrounding the tumour tissue showed loss of WT1 in both tissues, however, β-Catenin was only mutated in the tumour (Fukuzawa et al., 2007). β-catenin is predominantly localized to the cytoplasm in nephrogenic rests suggesting little to no canonical WNT-pathway activation. In contrast, nuclear localization, thought to drive unregulated proliferation, was observed in tumour tissue (Maiti et al., 2000). Studies of bilateral/multifocal WTs often identify different secondary mutations within each tumour of the same individual. We hypothesize that loss of WT1 undermines genomic stability by compromising DNA-repair mechanisms normally activated during nephrogenesis.

If so, we reasoned that specific DNA-repair enzymes could be activated in NPCs forming the cap mesenchyme in anticipation of the proliferative burst that initiates nephrogenesis. We first examined the DNA-repair gene expression profiles in embryonic kidneys compared to adult kidney. 48 out of 84 DNA-repair genes in our panel were upregulated in the embryonic kidney >2-fold compared to the adult kidney. A majority of these genes clustered in the base excision repair (BER) pathway (17), homologous recombination pathway (16) and nucleotide excision repair (NER) pathway (14), with some genes functioning in multiple pathways. Of these 48 genes, we focused on 7 with the highest transcript levels (>20-fold above adult level). Only one of these genes (*Neil3*) was enriched in the *Cited1*(+) NPCs of the cap mesenchyme. This suggests that *Neil3* may be a DNA-repair enzyme specifically activated in the primed NPC that protects the genome during nephrogenesis. NEIL3 is a DNA glycosylase involved in repairing DNA-damage through the BER pathway. This pathway specifically repairs minor lesions that do not cause distortion in the DNA double helix (primarily, oxidized, alkylated and deaminated bases or single strand breaks) (Kanamitsu and Ikeda. 2010).

One of the most common types of mutation observed in the β-catenin gene in WTs are missense mutations. These mutations allow constitutive activity, but it is unclear why these mutations should arise after failure to upregulate *Neil3*. Maiti et al (2000) described 17 *WT1*-mutant Wilms tumours where 10 tumours carried C>T missense mutations in an exon 3 codon containing a serine residue critical for regulating β-Catenin (Maiti et al., 2000). Mutation in this residue allows for constitutive nuclear accumulation of β-Catenin and constitutive activation of canonical WNT-signalling. Interestingly, C>T transition mutations in β-Catenin are common following oxidative base damage in *Escherichia coli* (Kreutzer and Essigmann. 1998). C>T transitions can also be caused by deamination of either 5-methylcytosine or cytosine (Krokan et al., 2002). Liu et al. showed that NEIL3 preferentially repairs oxidative lesions, specifically the further oxidation products of 8-oxoguanine (a common lesion resulting from free radical attack) (Liu et al., 2010). Therefore, it appears that the form of DNA damage preferentially repaired by NEIL3 (oxidative lesions), commonly results in base alterations (C>T transition) like those observed in the β-Catenin gene of *WT1*-mutant Wilms tumors.

WTs have also been associated with mutations in several genes involved in processing microRNAs (miRNAs). Approximately 40% of sporadic WTs are linked to WT1 mutations and loss of function mutations in *DROSHA* (Torrezan et al., 2014). Most commonly, mutations in the *DROSHA* RNase III domains involved a G>A transition. This class of mutation often occurs as a result of methylation of cytosine bases and is considered mutagenic due to increased spontaneous deamination to thymine (Parry. 2006; Cooper and Youssoufian. 1988). Lesions caused by deamination of cytosine are typically repaired by the BER pathway (potentially by NEIL3).

Whereas *Neil3* was enriched in NPCs of the cap mesenchyme, we wondered whether other DNA-repair enzymes were activated in other lineages of the developing kidney. Interestingly, of our top 7 genes, several were enriched in cells from the UB (*Ung*, *Rad51b* and *Brca1*). *Neil3* was not enriched in this compartment. UNG is also a DNA glycosylase that functions through the BER pathway. RAD51B and BRCA1 are members of the homologous recombination repair pathway.

In embryonic kidney, the UB undergoes branching morphogenesis and requires rapid cell division at the UB tip. This suggests that there are lineage specific DNA-repair systems brought into play during kidney development.

When we knocked down *Wt1* with Cre-recombinase to 64% of baseline, this was accompanied by a 58% reduction in *Neil3* transcript levels. Thus, *Neil3* expression could be entirely dependent on the presence of WT1. However, while our data clearly show that WT1 binds to the *Neil3* promoter and induces transcription in MK4 cells, the effect on expression was only 2-fold. This suggests that WT1 may need additional partners (not present in MK4 cells) for full activation of *Neil3*. We tested several candidates, including CITED1 (transcription factor unique to the cap mesenchyme) and WNT9B ± RSPO1 (that activate the differentiation cascade in the cap mesenchyme). However, none of these augmented the effect of WT1 on *Neil3* transcription. Thus, the additional factors accounting for the striking enrichment of *Neil3* in the cap mesenchyme (15-fold) remain a matter of conjecture.

It is intriguing that WTs arise from β-catenin mutations in nephrogenic rests, a clonal population of slowly-proliferating, developmentally arrested cells. However, Beerman et al demonstrated that quiescent hematopoietic stem cells (HSCs) from aged mice accumulate more DNA strand breaks than more rapidly-proliferating HSCs from young mice (Beerman et al., 2014). HSCs (aged or young) were able to repair the accumulated lesions when induced to proliferate due to an upregulation in DNA-repair genes. Slowly growing HSCs appear to exhibit lower levels of non-homologous end joining (NHEJ) repair (involved in double strand breaks repair) compared to their differentiated progeny (Biechonski et al., 2018). Thus, it is plausible that nephrogenic rest cells, like slow-growing HSCs, have reduced levels of specific DNA-repair pathways and increased risk of mutation.

Our analysis focused on 7 candidate genes with the highest levels of upregulation (>20-fold increased transcript level) in embryonic kidney versus adult. However, the 41 genes with an intermediate level of upregulation (between 2 and 20-fold increased transcript level) may also

be important in cap mesenchyme NPCs. It is entirely possible that there are additional WT1-dependent genes in this group which also contribute to genomic instability in *WT1*-null nephrogenic rests.

3.2.6 Summary

Our data show that DNA-repair genes are expressed in a lineage-specific manner during kidney development. Among the most highly expressed DNA-repair genes in fetal kidney, we identified *Neil3* as being specifically enriched in NPCs of cap mesenchyme. In contrast, *Ung*, *Rad51b* and *Brca1* were specifically enriched in UB cells.

We found that *Neil3*-expression in NPCs of cap mesenchyme is regulated by the Wilms tumour suppressor gene, WT1. WT1 binds directly to the *Neil3* promoter to drive transcription. This effect is likely to be part of NPC priming since WNT9B ± RSPO1 had no additional effect on *Neil3*-transcript levels.

3.2.7 Speculation

In embryonic kidney, targeted expression of *NEIL3* primes the NPC and protects the genome during nephrogenesis. Loss of *WT1* in nephrogenic rests may compromise expression of *NEIL3*, contributing to the risk of *CTNNB1* mutations associated with malignant transformation.

3.2.8 Acknowledgements

We would like to thank Ekaterina Iourtchenko and Marie-Hélène Lacombe of the RI-MUHC Immunophenotyping Platform.

3.2.9 Funding

This work was supported by operating grants to Dr. Goodyer from the Kidney Foundation of Canada, the Canadian Institutes of Health/ Fonds de recherche du Québec - Santé/ERA-Net for Research Programs on Rare Diseases and an infrastructure support grant to the McGill University Health Center Research Institute from the Fonds de la Recherche en Santé du Québec (FRSQ). Kyle Dickinson was the recipient of a graduate studentship award from the Research Institute of

McGill University Health Centre and Desjardins Group. Paul Goodyer is the recipient of a CIHR James McGill Research Chair. Leah Hammond was the recipient of a Desjardins Studentship in Child Health Research award.

3.2.10 Competing Interests

No competing interests declared.

3.2.11 References

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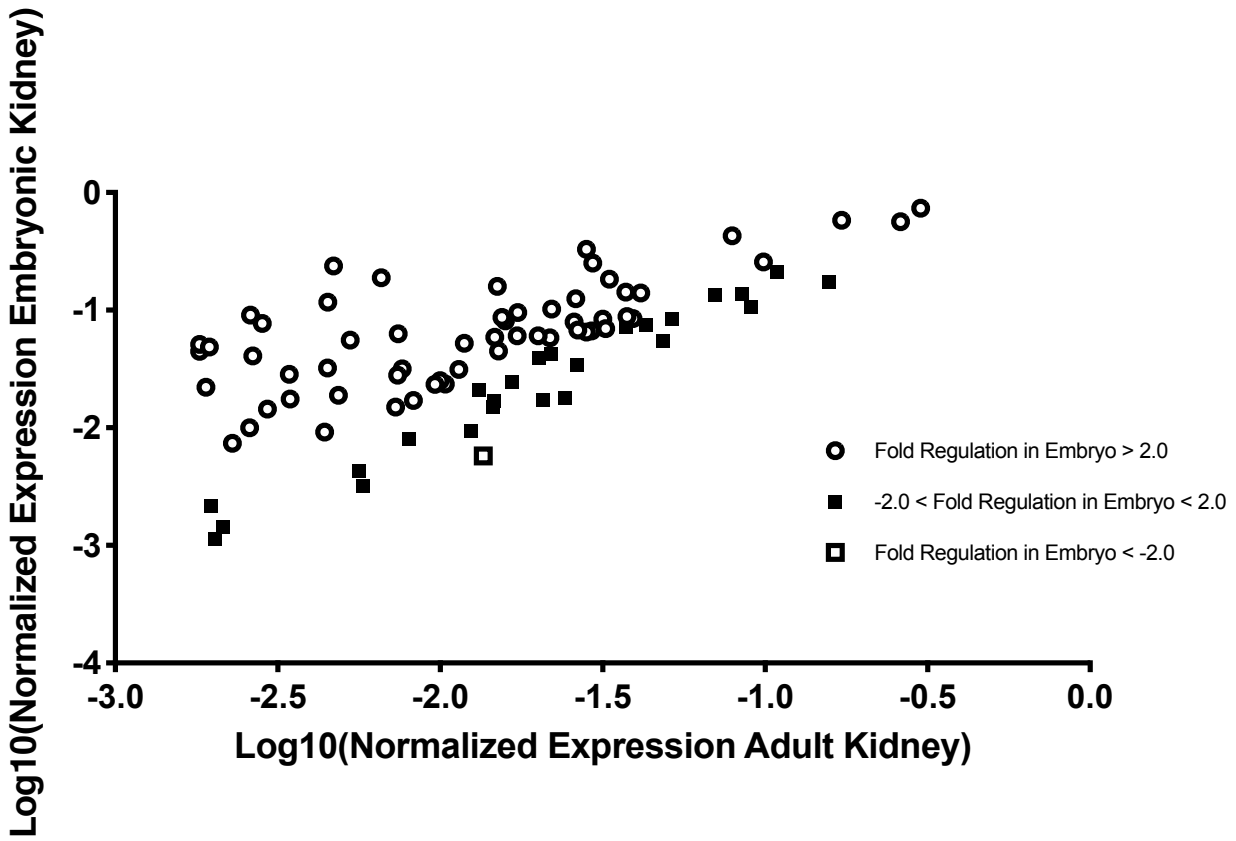

Figure 3.1. Normalized expression of DNA repair genes in embryonic versus adult mouse kidney.

This scatterplot shows the log-transformed normalized expression of 84 DNA repair genes. Expression in the embryonic kidney is plotted on the y-axis and expression in the adult kidney is plotted on the x-axis. Genes upregulated greater than 2-fold in the embryo are shown as white circles. Genes with a fold-regulation between -2 and 2 in the embryo, representing genes relatively unchanged in expression level between embryo and adult, are shown as black squares. Genes with a fold-regulation value less than -2 are shown as white squares. These genes are under-expressed in the embryo compared to the adult.

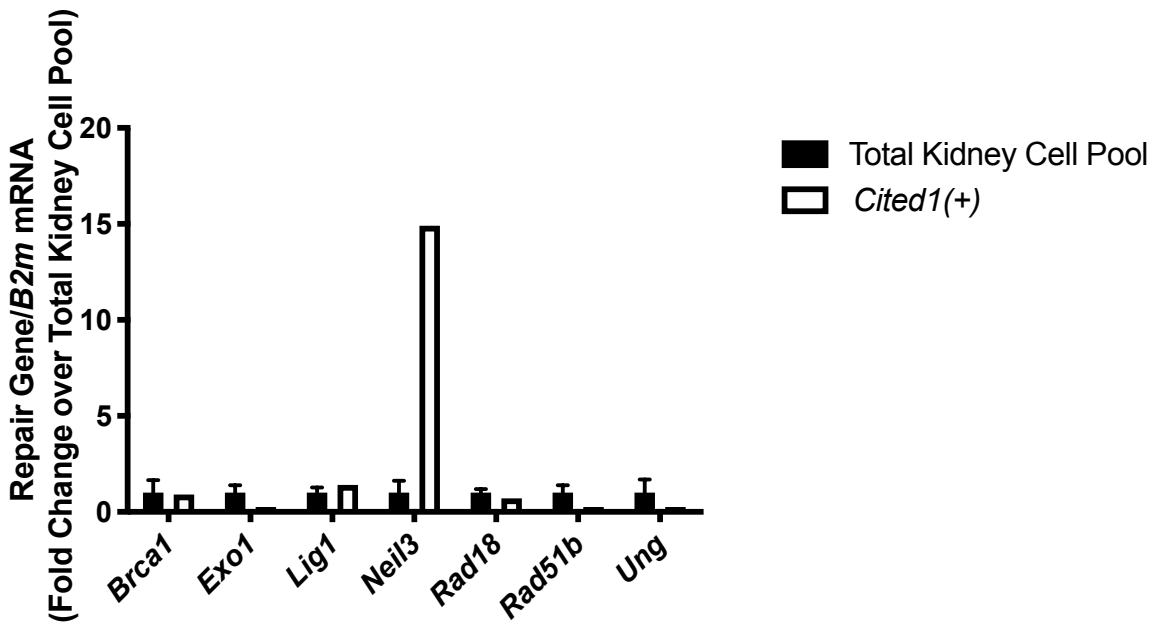

Figure 3.2. Fold change in DNA repair gene mRNA in *Cited1*(+) cells versus total kidney of E17.5 mice.

The expression of each repair gene was averaged across 6 pools of total kidney cells and used as a baseline against which all DNA repair gene expression measurements in the *Cited1*-compartment can be compared. The average repair gene expression in total kidney cell pools plus/minus standard error of the mean is plotted in black with the fold change in expression for the *Cited1*(+) cells shown in grey. The expression of each repair gene was normalized to *B2m* for every sample. Total kidney cell pool samples were analyzed 4 times (biological replicates) in triplicate (technical replicates). Due to the limited number of cells isolated per litter, Cited1(+) samples were analyzed once (biological replicate), however, contained cells pooled from 4 litters.

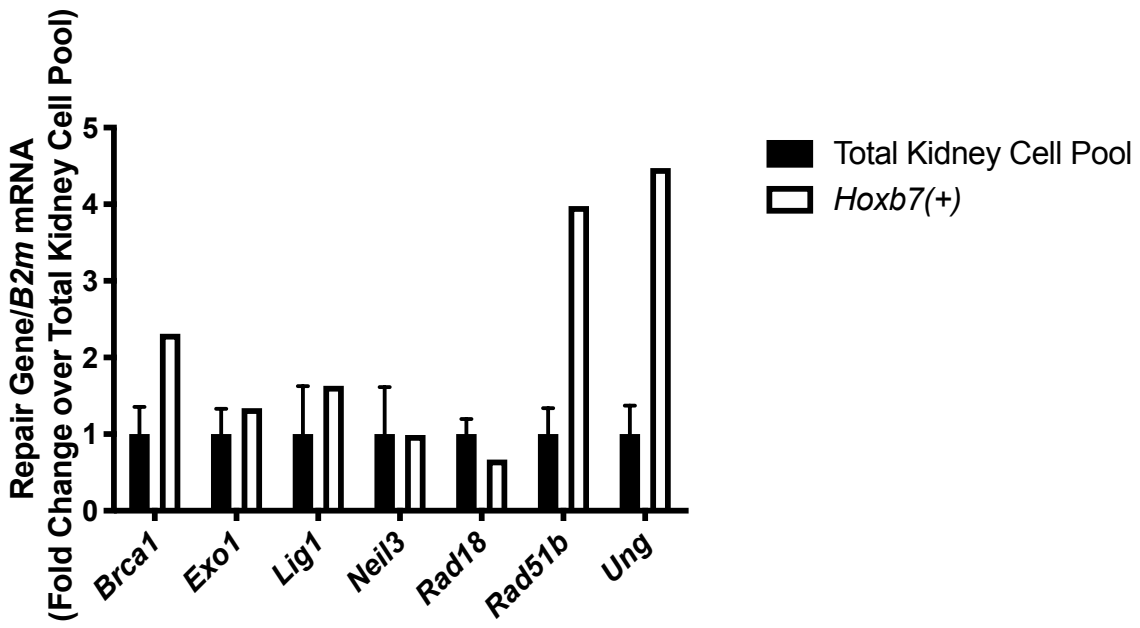

Figure 3.3. Fold change in DNA repair gene mRNA in *Hoxb7*(+) cells versus total kidney of E17.5 mice.

The expression of each repair gene was averaged across 3 pools of total kidney cells and used as a baseline against which all DNA repair gene expression measurements in the *Hoxb7*-compartment can be compared. The average repair gene expression in total kidney cell pools plus/minus standard error of the mean is plotted in black with the fold change in expression for the *Hoxb7*(+) cells shown in grey. The expression of each repair gene was normalized to *B2m* for every sample. Total kidney cell pool samples were analyzed 3 times (biological replicates) in triplicate (technical replicates). Hoxb7(+) samples were analyzed once (biological replicate), however, contained cells pooled from 3 litters.

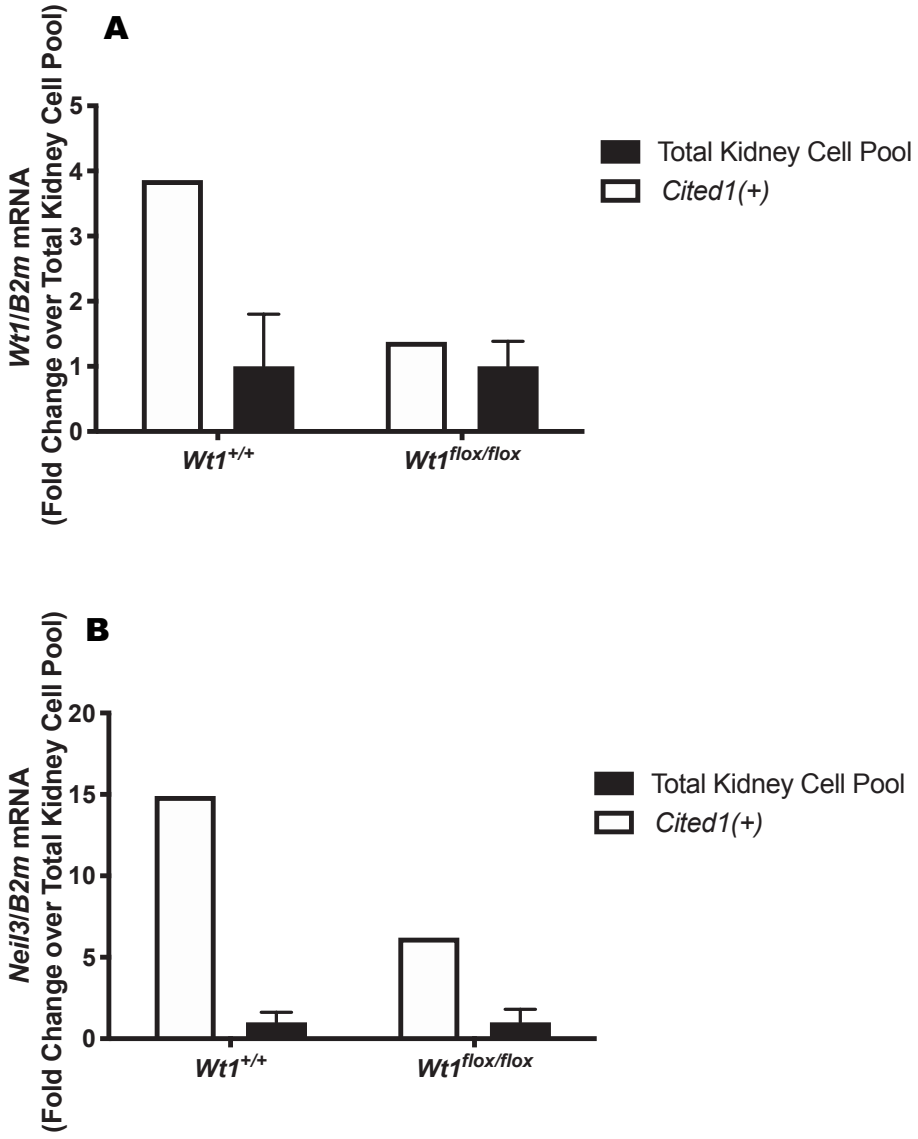

Figure 3.4. Expression of *Neil3* in the *Cited1*-compartment of E17.5 mice is reduced following knockdown of *Wt1.*

(A) Fold change in *Wt1* mRNA in *Cited1*(+) cells versus total kidney for $Wt1^{+/+}$ mice and $Wt1^{flox/flox}$ mice. The expression of wildtype Wt1 was averaged across multiple pools of total kidney cells and used as a baseline against which all Wt1 expression measurements in the *Cited1*-compartment can be compared. The average Wt1 expression in total kidney cell pools plus/minus standard error of the mean is plotted in black with the fold change in expression for the *Cited1*(+) cells shown on the left for $Wt1^{+/+}$ mice and on the right for $Wt1^{flox/flox}$ mice (white bars). The expression of Wt1 was normalized to B2m for every sample. (B) Fold change in *Neil3* mRNA in

Cited1(+) cells versus total kidney for *Wt1*$^{+/+}$ mice (on the left) and *Wt1*$^{flox/flox}$ mice (on the right). The expression of *Neil3* was averaged across multiple pools of total kidney cells and used as a baseline against which *Neil3* expression measurements in the *Cited1*-compartment can be compared. The average repair gene expression in total kidney cell pools plus/minus standard error of the mean is plotted in black with the fold change in expression for the *Cited1*(+) cells shown in grey. The expression of each repair gene was normalized to B2m for every sample. Total kidney cell pool samples for *Wt1*$^{+/+}$ mice were analyzed four times (biological replicates) in triplicate (technical replicates). Total kidney cell pool samples for *Wt1*$^{flox/flox}$ mice were analyzed six times (biological replicates) in triplicate (technical replicates). Cited1(+) samples were analyzed a single time (n=1), however, contained pooled samples from 4 litters for *Wt1*$^{+/+}$ mice and 6 litters for *Wt1*$^{flox/flox}$ mice.

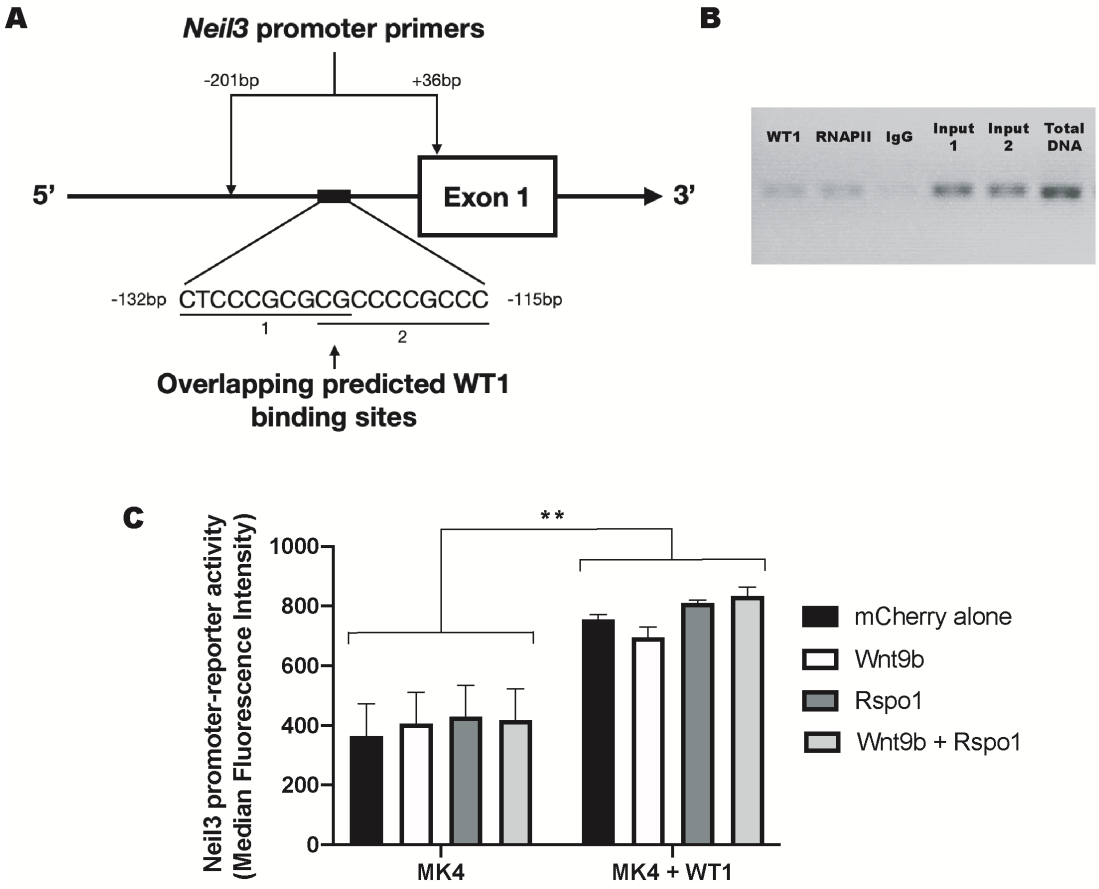

Figure 3.5. WT1 binds to the Neil3 promoter and activates transcription.
(A) Schematic of the WT1 binding sites within the *Neil3* promoter. Base pair positions are measured as distance from the transcription start site. (B) Detection of *Neil3* promoter in DNA bound to WT1. ChIP samples using a WT1 antibody pull down (Lane 1), RNAPII antibody (Lane 2) and IgG antibody (Lane 3). Input samples (1% - Lane 4 & 5) and total sample DNA (Lane 6) were also included in analysis. (C) Measurement of *Neil3* promoter activity in WT1(-) and WT1(+) MK4 cells. N=3 (biological replicates) for each condition. Promoter activity is represented as median fluorescence intensity (MFI). Cells conditions are as follows: promoter reporter (black bars), WNT9B treated (white bars), RSPO1 treated (dark grey bars) or WNT9B and RSPO1 treated (light grey bars). Values of the untreated parental cell line was subtracted from each condition. Error bars represent SEM. (** = $p<0.01$)

Supplementary Table S3.1. Fold change values and associated P-values for each DNA repair gene included in the RT2 Profiler PCR array.

Symbol	Embryonic Kidney Fold Change (comparing to adult kidney)	P-value (comparing to adult kidney)
Apex1	**10.6295**	**0.000839**
Apex2	**4.1602**	**0.004169**
Atm	**2.061**	0.054225
Atr	**2.2815**	**0.009319**
Atxn3	1.1251	0.966323
Brca1	**24.59**	**0.000162**
Brca2	**7.1768**	**0.002343**
Brip1	**8.3013**	**0.002834**
Ccnh	**2.6027**	**0.012589**
Ccno	0.6643	0.648675
Cdk7	**5.0982**	**0.002982**
Ddb1	**2.4509**	**0.008761**
Ddb2	**3.4983**	**0.000034**
Dmc1	**3.8548**	0.111203
Ercc1	**4.6268**	**0.000447**
Ercc2	1.9141	0.128101
Ercc3	**5.5022**	**0.001004**
Ercc4	1.4641	0.300542
Ercc5	**2.3134**	**0.038097**
Ercc6	**2.7447**	**0.00856**
Ercc8	**2.271**	0.057208
Exo1	**34.7755**	**0.019745**
Fen1	**10.5074**	**0.01858**
Lig1	**28.5747**	**0.001224**
Lig3	**5.5919**	**0.003499**
Lig4	**2.6697**	**0.008114**
Mgmt	1.9141	**0.009647**
Mlh1	**3.8282**	**0.039664**
Mlh3	0.8312	0.604044
Mms19	**2.0849**	0.062483

Mpg	**3.0175**	**0.001569**
Mre11a	**3.0667**	**0.003511**
Msh2	**11.6587**	**0.014532**
Msh3	1.7451	0.07426
Msh4	0.5599	0.560657
Msh5	1.0943	0.859842
Msh6	**8.515**	**0.013548**
Mutyh	**11.6318**	**0.016968**
Neil1	0.7596	0.544057
Neil2	0.5561	0.341552
Neil3	**28.0514**	**0.003121**
Nthl1	**2.9759**	**0.013747**
Ogg1	1.2894	0.103845
Parp1	**4.7789**	**0.003536**
Parp2	**3.793**	**0.003625**
Parp3	0.4253	0.239346
Pms1	**2.0562**	0.05736
Pms2	**8.4561**	**0.000006**
Pnkp	0.7492	0.390417
Polb	1.1096	0.822934
Pold3	**4.4076**	**0.008083**
Poll	1.9498	**0.031352**
Prkdc	1.6021	0.168439
Rad18	**25.8722**	**0.000154**
Rad21	**3.3714**	**0.000592**
Rad23a	1.6208	0.094769
Rad23b	**2.1585**	**0.002455**
Rad50	**3.9908**	**0.007216**
Rad51	**50.5626**	0.071733
Rad51c	**4.9132**	**0.000395**
Rad51b	**24.8757**	**0.002358**
Rad51d	0.7667	0.668549
Rad52	**2.6512**	0.080292
Rad54l	**15.3837**	**0.003551**
Rfc1	**5.5277**	**0.00057**
Rpa1	**2.1634**	0.064496

Rpa3	**5.4264**	0.084011
Slk	1.9453	**0.021347**
Smug1	1.1514	0.795024
Tdg	**3.2266**	**0.008855**
Top3a	**2.5198**	**0.000601**
Top3b	1.6396	0.063164
Trex1	**3.8816**	**0.016992**
Ung	**27.1585**	**0.001891**
Xab2	**2.1585**	**0.048162**
Xpa	1.1701	0.321335
Xpc	1.0401	0.869525
Xrcc1	**3.3792**	**0.000737**
Xrcc2	**5.0747**	**0.001289**
Xrcc3	**2.4284**	0.056838
Xrcc4	**2.3457**	**0.009416**
Xrcc5	1.9274	**0.003558**
Xrcc6	**2.5491**	**0.049805**
Xrcc6bp1	1	0.887347

Table Legend: mRNA expression for each gene was determined by qRT-PCR performed three times (n=3). This fold change value represents the normalized expression for the gene of interest in the embryonic kidney divided by its normalized expression in the adult kidney. Fold change values greater than 2.0 are indicated in bold. Each P-value was calculated using a Student's t-test of the normalized expression for each gene in the embryonic versus the adult kidney samples. P-values less than 0.05 are indicated in bold.

End of manuscript.

Chapter 4: Discussion and Future Directions

4.1 Background

Critical events early in mammalian kidney development determine the final kidney size and nephron number. Reduction in total nephron number increases the chances of developing disease during childhood or early adult life (Hoy et al., 2005). Associations have been made between decreased nephron number and development of hypertension later in life. One group showed that hypertensive subjects had 46% fewer nephrons compared to healthy controls (Keller et al., 2003). Children with oligomeganephronia develop proteinuria, hypertension and renal failure as a result of a reduction in nephron number and kidney size (Kandasamy et al., 2013). Unsurprisingly, kidney development is a tightly regulated, highly coordinated sequence of events involving multiple pathways to complete nephrogenesis. However, a majority of the mature nephron structure is derived from NPCs of the cap mesenchyme, thus, making it critical to understand the biology which permits these cells to allow nephrogenesis to proceed normally.

Investigations into NPC biology have identified two major contributors to cell survival and proliferation: canonical WNT-signalling and WT1. NPCs receive a WNT9B signal from the adjacent UB which induces cell proliferation and differentiation, marking the beginning of nephrogenesis. Mice harbouring mutations in *Wnt9b* develop vestigial kidneys with segments of UB-derived epithelia but no nephrons (Carroll et al., 2005). Nephrons also failed to form in mice harbouring a NPC-specific knockout in β-catenin (Park et al., 2007). Interestingly, overexpression of β-catenin in NPCs was sufficient to induce tubulogenesis in vitro, highlighting the importance of the canonical WNT-signalling pathway in this process. However, prior to arrival of the UB around E10.5, the NPC population develops normally in both mouse models. These early NPC priming events are dependent on WT1 expression. Expression of WT1 in a portion of the IM sets the cell fate for the population of cells which become NPCs. Mice with biallelic knockout of *Wt1* fail to generate the primed population of NPCs and are anephric (Schedl and Hastie. 1998). Therefore, WT1 appears to act as a master regulator of the early events of nephrogenesis. Additionally, loss of WT1-expression in NPCs has been identified as a mechanism of Wilms tumorigenesis. *WT1*(-) NPCs fail to be induced by the WNT9B signal and remain in a progenitor-like state in the postnatal kidney. These quiescent cells may resorb into the kidney; however, they often acquire secondary

mutations in the β-catenin gene which promote tumour formation. Approximately 95% of WTs with β-catenin mutations also have mutations in *WT1* (Maiti et al., 2000). β-catenin mutations in WTs were shown to occur secondary to the *WT1* mutation, suggesting the genomic integrity of *WT1*(-) cells is compromised, however, a plausible mechanism to explain this observation has yet to be described.

4.2 NPCs need to acquire *Fzd5*, *Rspo1* and *Lrp6* for optimal responsiveness to WNT9B

Our first objective was to determine the specific molecular components which render NPCs responsive to the inductive WNT9B signal. To accomplish this, we obtained the M15 cell line isolated from E10.5 kidney, representative of an early NPC prior to induction by WNT9B (Larsson et al., 1995). M15 cells express key markers of early NPCs including *Cited1* and *Osr1*. Crucially, M15 cells also express high levels of WT1 which our lab has previously shown to be an important transcription factor required to render cells responsive to a canonical WNT signal (Akpa et al., 2015). WT1 indirectly controls β-catenin transcription through transcriptional regulation of EZH2, a histone H3K27 methyltransferase responsible for placing suppressive histone modifications on chromatin. WT1 suppresses EZH2 which allows upregulation of β-catenin transcription. Therefore, we expected to observe activation of the canonical WNT-pathway in our WT1(+) M15 cell line, however, they were unresponsive when treated with WNT9B. Interestingly, when we transfected a *Wnt9b* vector, we were able to observe low levels of WNT-activation. We reasoned that early NPCs must acquire additional cell surface molecules to become fully primed to respond to the WNT9B signal.

It became evident that M15 cells were missing essential components involved in binding and transducing the WNT-signal. Therefore, our next step was to analyze expression of WNT-signalling molecules in M15 cells. First, we referenced microarray data from the GUDMAP consortium to determine canonical WNT-molecules expressed in cap mesenchyme NPCs and compared it to M15 cells. Of the 12 molecules involved in dimerizing to form WNT co-receptors, we detected expression of *Fzd1*, *Fzd2*, *Fzd3*, *Fzd5* and *Lrp6* in M15 cells which were also expressed in cap mesenchyme cells according to GUDMAP. However, M15 cells did not express WNT-

agonist *Rspo1* and its receptor *Lgr4*/*Lgr6* which have been shown to prolong expression of the WNT-receptor in the cell membrane.

R-spondin proteins associate with WNT-receptors to modulate membrane stability through binding of LGR and ZNRF3 receptors. ZNRF3 functions to negatively regulate canonical WNT-signalling by targeting FZD receptors for degradation and also preventing activation of LRP receptors (Koo et al., 2012; Hao et al., 2012). RSPO1 simultaneously binds to ZNRF3 and an LGR receptor to initiate membrane clearance of ZNRF3, preventing degradation of the WNT-receptor complex (Hao et al., 2012). Expression of two members of the R-spondin family, *Rspo1* and *Rspo3*, were detected in cap mesenchyme NPCs of the developing kidney (Motamedi et al., 2014). Interestingly, we were unable to stimulate robust WNT9B responsiveness in M15 cells without treating them with recombinant RSPO1 protein. A study, published subsequent to our Chapter 2 manuscript, generated *Rspo1* and *Rspo3* knockout mouse models to describe the resulting kidney phenotype. Knockout of either *Rspo1* or *Rspo3* had minimal adverse effect on nephrogenesis and kidney development suggesting they have redundant functions in NPCs (Vidal et al., 2019). However, *Rspo1* and *Rspo3* double knockout mice fail to generate nephrons due to significant reduction in proliferation and survival of the *Six2*(+) NPC population. This in vivo study further validates our in vitro findings of the importance of R-spondins in augmenting canonical WNT-signalling to allow for optimal WNT9B responsiveness in NPCs.

The RSPO/LGR axis has been studied in other organ systems. Specifically, LGR5 was shown to mark stem cells within intestinal crypts and be essential for self-renewal and maintenance of this population (Barker et al., 2010; Barker et al., 2007). In the intestine, RSPO3 is produced by stromal cells neighbouring the LGR5(+) stem cells and is necessary for self-renewal (Greicius et al., 2018). Knockout of *Rspo3* in stromal cells co-cultured with stem cells resulted in a significant reduction in the number of stem cells. However, cell survival could be rescued by addition of recombinant RSPO3 protein. Given the importance of the RSPO/LGR axis in intestinal stem/progenitor cells, we also wanted to investigate the LGR status in our NPC model. M15 cells express both *Lgr4* and *Lgr6*, but not *Lgr5*. We were able to amplify WNT-responsiveness in M15 cells without addition

of *Lgr5* so we reasoned that the receptor involved in binding RSPO1 must have already been expressed in our cell line. Using siRNA, we knocked out both *Lgr4* and *Lgr6* and observed a reduction in β-catenin reporter activity when M15 cells were treated with WNT9B and RSPO1. However, analysis of *Lgr4* and *Lgr6* mRNA transcript levels to assess the specificity of the siRNAs used in our study revealed that they were non-specific, and each siRNA resulted in downregulation of both genes (data not shown). Although LGR receptors appear to be essential in NPCs, we could not identify the specific receptor involved in augmenting canonical WNT-activity in response to WNT9B and RSPO1. Interestingly, *Lgr4*-null mice develop severe renal hypoplasia and contain approximately 90% fewer nephrons compared to wildtype controls (Kato et al., 2006). Further analysis of the renal phenotype of *Lgr4*-null mice discovered increased NPC apoptosis in addition to a failure of NPCs to condense around the UB (Mohri et al., 2012). The renal phenotype of *Lgr4*-null mice mirrors the phenotype of both *Wnt9b* and *CTNNB1* knockout mice, suggesting LGR4 is likely the receptor involved in binding RSPO1/3 in NPCs. Nonetheless, these observations further validate the vital role of RSPO/LGR agonist activity in permitting NPC responsiveness to WNT9B.

The remaining component involved in transducing a WNT-signal is the FZD/LRP co-receptor complex. Two members of the LRP family (LRP5 and LRP6) are involved in canonical WNT-signalling. To determine the receptor involved in binding WNT9B, we transiently transfected M15 with an siRNA against *Lrp6* and observed a significant reduction in WNT-activity. This effect could not be rescued by overexpression of *Lrp5*, suggesting *Lrp6* is sufficient to activate WNT9B-signalling. Expression of FZD receptors, in particular, the specific receptor involved in transducing a WNT9B signal in the NPC population has yet to be described. To accomplish this, we first performed in situ hybridization in E11.5 kidneys and detected expression of *Fzd1, Fzd2, Fzd3, Fzd5* and *Fzd7* in the cap mesenchyme. Although we had identified a few candidates, we systematically transfected *Fzd1 – Fzd10* into M15 cells in addition to *Wnt9b* and measured canonical WNT-signalling activity. Only *Fzd5* resulted in significant upregulation of WNT-activity, however, this was only observed in the presence of RSPO1. Phylogenetic analysis of human FZD receptors place FZD5 and FZD8 as a pair of receptors sharing significant homology (MacDonald

and He. 2012). Additionally, WNT9B was shown to bind to a co-receptor complex consisting of FZD8 and LRP6 (Bourhis et al., 2010). Our results, combined with the high homology shared with FZD8, a proven WNT9B receptor, help to establish FZD5 as being responsible for transducing the WNT9B signal in NPCs.

We propose a model of NPC priming in which they must turn on expression of RSPO1, FZD5 and LRP6 prior to the arrival of the UB and the inductive WNT9B signal to allow for optimal responsiveness to induce nephrogenesis.

4.3 Treating NPCs with RSPO1 prior to injection improves cell integration

In addition to improving the overall knowledge of kidney development and NPC biology, our results from Chapter 2 also have implications in the field of cell based therapy of AKI. Mammalian kidneys have a significant capacity to repair themselves in response to injury, however, repair can often be incomplete, resulting in fibrosis/scarring which progresses into CKD over time. Therefore, cell-based therapy for AKI may augment the kidneys natural repair mechanisms to allow for full repair of the damaged kidney. Injection of either HSCs or amMSCs into mice with AKI improve renal function compared to controls (Morigi et al., 2008; Rota et al., 2012). However, both cell types localize in peritubular blood vessels and exert their protective effects primarily through secretion of cytokines. Peritubular localization of the infused cells raises concerns about the long-term benefit of this treatment. Due to the stem-like nature of HSCs and amMSCs, it is plausible that over time these cells could transform/mutate and increase the likelihood of developing a renal tumour. Interestingly, cells isolated from either embryonic mouse kidney or nephrectomized adult kidney show integration into damaged nephrons of acutely injured mice (Angelotti et al., 2012; Sagrinati et al., 2006). However, these cells are isolated from highly invasive procedures, thus, reducing the potential clinical usefulness of this therapy. Additionally, our lab previously showed that canonical WNT-signalling activity is essential for NPC integration to occur (Zhang et al., 2015). Therefore, it is important to obtain a renal/nephron progenitor-like cell from a minimally invasive source, with a robust canonical WNT-signalling capacity which should increase the ability to integrate into damaged nephrons.

In Appendix I, to model an optimal NPC, we tested M15 cells and UD-NPCs. Our investigations of M15 cells have identified them as early NPCs which are not fully primed to respond to a WNT9B signal. However, we identified the required WNT-receptor molecules and specifically highlighted the importance RSPO1 plays in this process. The UD-NPCs used in our study were isolated from urine of neonatal urine and confirmed to express key markers of early NPCs, however, do not express *RSPO1*. Due to the necessity of the canonical WNT-signalling pathway for cell integration, we reasoned that pre-treating cells with RSPO1 prior to infusion will increase the percentage of cells integrated into tubular epithelia.

First, we assessed the WNT-signalling capacity of our cell lines and observed increased responsiveness to WNT9B in the presence of RSPO1 in both M15 cells and UD-NPCs. Next, we infused RSPO1-treated NPCs into mice with glycerol-induced AKI. Although these are preliminary results, we detected increased integration of RSPO1-treated M15 cells versus controls. However, RSPO1 appeared to have less of an effect on UD-NPC integration. We postulated that this was due to lack of expression of other components of the WNT-signalling pathway. Our UD-NPCs do not express *FZD5*, the receptor we identified as essential for transducing a WNT9B signal. Although *FZD5* can be transfected/transduced into UD-NPCs, our aim was to establish a simple method for cell isolation and treatment prior to infusion into subjects with AKI. Adding additional steps to the protocol would diminish the usefulness when translated to the clinic.

Despite the poor integration observed in our RSPO1- treated UD-NPC conditions, this area of investigation remains promising due to the number of different nephron progenitor-like cell types isolated from neonatal and adult urine. Our study only assessed a single, clonally-derived cell line isolated from neonatal urine; however, additional cell lines were generated from the same individual and have differential expression of key NPC and WNT-signalling markers. UD-NPCs can also be isolated from adult urine and again show different expression profiles compared to the neonatal derived cells (Arcolino et al., 2016). Therefore, the possibility exists that an optimal UD-NPC exists and can be identified with further characterization and testing in models of AKI. These findings can also be extended and applied for use in CKD. Similar to early studies of

cell based therapy of AKI, MSCs have been tested in mouse models of CKD and have comparable results. Infusion of MSCs improve the renal phenotype and reduce the renal fibrosis/scarring in treated mice, however, these cells remain in peritubular regions and fail to differentiate and integrate into the kidney (Sedrakyan et al., 2012; Franquesa et al., 2012). Once again mirroring AKI studies, integration was observed when NPCs from aborted human fetal kidneys were injected in a mouse with CKD (Harari-Steinberg et al., 2013). Once again, this highlights the requirement of cells to be partially differentiated towards the renal lineage to allow for integration into damaged tubules. However, the availability of aborted human fetal kidneys remains a limiting factor in the usefulness of this treatment. Therefore, use of UD-NPCs, isolated from a non-invasive procedure, could also have potential implications for the treatment of CKD.

Therefore, we are proposing a method to modify WNT-signalling in NPCs prior to injection to improve cell integration following AKI. The minimally-invasive UD-NPC collection method and the simplicity of adding recombinant RSPO1 protein the growth media prior to injection, vastly improves the clinical utility of our proposed treatment method.

4.4 WT1 directly binds to the *Neil3* promoter to upregulate transcription in NPCs

We have described the essential role of the canonical WNT-signalling pathway in early NPCs to allow for nephrogenesis to proceed normally, highlighted by the anephric phenotype observed in both WNT9B and β-catenin knockout mice. However, WT1 expression in the IM precedes the arrival of the UB and establishes the genetic landscape in cells destined to become NPCs. Our lab previously showed that WT1 indirectly plays a role in activating transcription of β-catenin. Cells lacking WT1 have reduced canonical WNT-signalling activity due to increased levels of repressive histone H3K27 trimethylation marks in the β-catenin gene promoter (Akpa et al., 2015). Therefore, WT1 is another critical element in rending NPCs responsive to WNT9B. However, kidneys in individuals who inherit a single germline mutation in *WT1* develop normally until they acquire a somatic mutation in the second *WT1* allele (Rivera and Haber. 2005). Biallelic *WT1* loss of function mutations in NPCs results in generation of characteristic precursor lesions called a nephrogenic rests. These lesions are clusters of developmentally arrested NPCs which are unable to respond to WNT9B and remain undifferentiated in the postnatal kidney (Call et al., 1990).

Interestingly, β-catenin localizes in the cytoplasm in nephrogenic rest cells, suggesting the canonical WNT-signalling pathway is inactive (Fukuzawa et al., 2007). Nephrogenic rest cells often acquire mutations in *CTNNB1* which result in constitutive activation of canonical WNT-signalling which drives Wilms tumourigenesis (Uschkereit et al., 2007; Zirn et al., 2006; Koesters et al., 1999). *CTNNB1* mutations were confirmed to occur secondary to *WT1* mutations by analyzing the mutational status of tumour tissue and the surrounding nephrongenic rest cells. Biallelic *WT1* mutations were detected in both nephrogenic rests and neighbouring WT tissue, however, *CTNNB1* was only mutated in the tumour (Fukuzawa et al., 2007). We hypothesized that the genomic stability of *WT1*-null nephrogenic rests may be compromised due to loss of expression of WT1-dependent DNA-repair genes.

Mammalian cells are exposed to endogenous genotoxic stresses which induce DNA-damage. It is estimated that approximately 70,000 DNA lesions are generated in mammalian cells a day, highlighting the importance of maintaining normal function of DNA-repair pathways (Tubbs and Nussenzweig. 2017). We aimed to identify the DNA-repair pathways/genes expressed in cap mesenchyme NPCs. Firstly, we analyzed differential expression levels of a panel of DNA-repair genes in embryonic mouse kidney versus adult mouse kidney. We detected 48 DNA repair genes upregulated (>2 fold) in the embryo versus the adult. However, we reasoned that genes with the highest upregulation compared to the adult kidney may potentially have increased importance in the developing kidney. Therefore, we focused our subsequent experiments on 7 genes upregulated >20-fold. In *Cited1*(+) cap mesenchyme NPCs, we only detected enrichment of a single gene, *Neil3* (BER pathway), suggesting it may be expressed in order to protect the NPC genome during nephrogenesis.

To determine whether the observed enrichment in *Neil3* expression was specific to NPCs, we also tested our top 7 candidate genes in UB cells. We detected upregulation of *Ung*, *Rad51b* and *Brca1* but not *Neil3* in *Hoxb7*(+) UB cells compared to total embryonic kidney. The UB, much like cap mesenchyme NPCs, undergo a period of rapid proliferation to complete branching morphogenesis during kidney development. However, differentiation in UB cells is stimulated by

a different set of growth factors (GDNF, TGF-β and BMP4) compared to cap mesenchyme NPCs (WNT9B) (Moore et al., 1996; Sainio et al., 1997; Davies et al., 1999; Milbrandt et al., 1998; Ritvos et al., 1995; Piscione et al., 1997). We postulate this could contribute to the lineage-specific expression of DNA-repair enzymes during kidney development.

After confirming *Neil3* is highly expressed in cap mesenchyme NPCs, we wanted to determine whether expression was dependent on the status of WT1. In NPCs with a *Wt1*-knockdown, *Neil3* expression was significantly reduced. Subsequently, we detected WT1 directly binds to the *Neil3* promoter which results in a corresponding activation of *Neil3* transcription. However, using our reporter assay, the observed increase in activation was only 2-fold, therefore, we postulated whether additional partners are required for more robust activation of *Neil3* transcription. We tested the effect of several candidates involved in nephrogenesis (*Cited1*, WNT9B ± RSPO1) but did not detect any additional activation in *Neil3* expression. Thus, *Neil3* enrichment in NPCs may need occur prior to the arrival of the UB and the inductive WNT9B signal to prepare cells for the burst of rapid proliferation.

We showed *Neil3* levels are reduced in WT1(-) NPCs, however, it is uncertain how this could result in acquisition of characteristic mutations in *CTNNB1* in WT1-null nephrogenic rests. *Neil3* is a member of the BER pathway which is involved in repairing DNA lesions that do not cause distortion in the DNA double helix (oxidized bases, alkylated bases, deaminated bases and single strand breaks) (Kanamitsu and Ikeda. 2010). WT1(-) WTs often acquire missense mutations (C>T transition) in *CTNNB1* which alters an important regulatory serine residue in β-catenin, resulting in constitutive activation of canonical WNT-signalling (Maiti et al., 2000). C>T transition mutations occur as a result of failure to repair oxidative DNA damage and deamination of 5-methylcytosine (Kreutzer and Essigmann. 1998; Krokan et al., 2002). Specifically, oxidative damage commonly results in formation of 8-oxoguanine lesions which NEIL3 preferentially repairs (Liu et al., 2010). Thus, it is entirely possible that loss of NEIL3 in WT1(-) nephrogenic rests could result in the common C>T transition mutations detected in WTs with *CTNNB1* mutations.

WT1(-) WTs also commonly acquire inactivating mutations in *DROSHA*, a key enzyme involved in miRNA processing. One study detected approximately 40% of sporadic WTs with both *WT1* and *DROSHA* mutations (Torrezan et al., 2014). *DROSHA* mutations in WTs are commonly G>A transitions within an important catalytic RNase III domain. G>A transitions often occur as a result of methylation of cytosine which subsequently deaminates to thymine (Parry. 2006; Cooper and Youssoufian. 1988). BER also functions to repair this type of lesion, thus, opening the possibility that NEIL3 may be involved in the repair process.

Although nephrogenic rests are a population of non-proliferating, developmentally arrested progenitor cells, they appear to acquire mutations at a higher rate than the surrounding terminally differentiated cells. The rate of mutation in non-proliferating HSCs was compared to the rate in proliferating HSCs. It was demonstrated that non-proliferating/quiescent HSCs from aged mice accumulated more DNA strand breaks than HSCs from younger mice (Beerman et al., 2014). Interestingly, inducing aged HSCs to proliferate resulted in upregulation of DNA-repair genes which reduced the number of observed DNA strand breaks. It was subsequently shown that NHEJ activity was reduced in non-proliferating HSCs compared to their differentiated progeny (committed hematopoietic progenitor cells) (Biechonski et al., 2018). Thus, it is plausible that similar to HSCs, nephrogenic rest cells also exhibit reduced levels of specific repair pathways and loss of WT1 may compound the inherent genomic instability of quiescent cells.

Our data demonstrates lineage-specific expression of DNA-repair genes in the developing kidney. We show that *Neil3* in cap mesenchyme NPCs is upregulated as a result of direct binding of WT1 to the *Neil3* promoter. Therefore, it is conceivable that *WT1*-null nephrogenic rests have a corresponding reduction in *Neil3* which could explain the increased number of secondary mutations in *CTNNB1* commonly observed in *WT1*(-) WTs.

4.5 Conclusion

In conclusion, my investigations have provided further insight into the biology of early NPCs of the cap mesenchyme. I have shown that early NPCs are unable to respond to the inductive WNT9B signal from the UB until they acquire specific components of the WNT-signalling pathway,

including *Rspo1*, *Fzd5* and *Lrp6*. These findings were subsequently applied to develop a method for easy isolation and preparation of cells to inject in models of AKI. Simple pre-treatment of NPCs with RSPO1 prior to infusion appeared to improve cell integration into damaged tubules.

Lastly, I showed that DNA-repair genes are expressed in a lineage specific manner in the developing kidney. In particular, *Neil3* was enriched in NPCs compared to the adjacent UB and total embryonic kidney. *Neil3* enrichment in NPCs resulted from direct binding of WT1 to the *Neil3* promoter which activated transcription. Taken together, these results describe a potential mechanism to explain the frequent occurrence of secondary *CTNNB1* mutations in individuals with biallelic *WT1* mutations.

4.6 Future directions

Altogether, these findings provide insight into the molecular events occurring in early NPCs of the developing kidney. However, these results have also opened up additional avenues of investigation as described below:

4.6.1 Method optimization for using RSPO1-treated UD-NPCs as a cell-based therapy of AKI

As seen in our studies, we investigated a potential treatment option for AKI involving a simple method for cell isolation and preparation. However, we believe we can optimize this method to increase the number of UD-NPCs that integrate into damaged tubules. Primarily, we must perform additional genetic screening of UD-NPCs to help identify an optimal cell line to test in this model. Due to the variation in cells obtained from a single urine sample, propagating single cell clones will generate a large number of candidates to screen. Additionally, we can also begin screening adult UD-NPCs which will further improve the clinical utility of this treatment option. In practice, UD-NPCs can be obtained from individuals suffering from AKI or CKD which would reduce the need to screen for histocompatibility. Finally, we must also assess renal function using biomarkers such as serum creatinine and blood urea nitrogen in our mouse models. These functional readouts will provide a more complete picture of the effect UD-NPCs have on repairing AKI.

4.6.2 Investigating *Neil3* status in bona fide nephrogenic rest cells and WT tissue

We have proposed a potential mechanism of Wilms tumourigenesis, however, additional studies must be performed to prove our hypothesis. We can measure rates of mutation in *Neil3* knockout/knockdown cells to determine whether genomic stability is altered. Specifically, we can analyze the *CTNNB1* gene to assess whether the characteristic activating mutation commonly found in WTs also occurs in *Neil3*-null cells. Additionally, we can study the role of *Neil3* in NPCs in a mouse model. Biallelic knockout of WT1 in mouse *Cited1*(+) NPCs results in WT formation (Huang et al., 2016). Using a similar model, we can knockout *Neil3* in early NPCs and determine whether WTs develop. Lastly, DNA-repair pathways could be analyzed in human WT tissue samples and cell lines (obtained from the Children's Oncology Group).

4.6.3 Analyze DNA-repair genes moderately upregulated in embryonic versus adult kidney.

Our analysis of DNA-repair genes expressed in the cap mesenchyme focused on genes upregulated >20-fold in embryonic kidney versus adult kidney. However, an additional 41 genes were upregulated between 2 and 20-fold, therefore, the possibility exists that repair genes other than *Neil3* are enriched the NPCs. If additional candidates are identified, studies can be performed to determine whether expression is regulated by WT1.

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Appendix I: Cell-based therapy of Acute Kidney Injury

Introduction

AKI is a disease that affects 1 in 5 adults and 1 in 3 children worldwide and is characterized by a sudden drop in renal function. Mortality rates of individuals with AKI are high with approximately 1 in 5 of adults and 1 in 7 children dying of complications related to injury (Susantitaphong et al., 2013). Current treatments for AKI involve replacing the normal function of a kidney in maintaining homeostasis. This therapy often results in subjects going on dialysis to remove toxins from the blood and receive intravenous fluids to maintain fluid balance (Levey and James. 2017). These measures in addition to the capacity of the kidney to naturally repair itself may help individuals with an episode of AKI, however, these treatments do not always prevent the formation of renal scarring/fibrosis (Ronco et al., 2019). Over time, a decrease in renal function as a result of renal fibrosis can lead to the development of chronic kidney disease and long-term complications (Parr and Siew. 2016).

Current studies are focused on developing a cell-based therapy to treat AKI. Using mouse models, infusion of a population of ARPCs improved renal function after induction of AKI (Angelotti et al., 2012; Sagrinati et al., 2006). However, ARPCs must be obtained by an invasive nephrectomy, thus decreasing the potential to translate this treatment into a practical therapy, therefore, focus shifted to find a minimally invasive source of cells to treat AKI. Hematopoietic stem cells, although obtained through a fairly invasive procedure, were also tested in an AKI mouse model and were shown to improve renal function (Morigi et al., 2008). Additionally, stem cells isolated from amniotic fluid were also shown to improve renal function (Rota et al., 2012). However, hematopoietic and amniotic fluid stem cells both failed to show integration into damaged renal tubules and instead were detected in peritubular blood vessels. Recently, progenitor cells expressing key markers of the developing kidney have been isolated from fetal and adult urine, making them a promising cell type to use to treat AKI (Arcolino et al., 2016). Infusion of these cells into a mouse model again showed improvement in functional readouts of AKI, however, these cells showed a small amount of integration into renal tubules.

Our lab previously showed that cell integration into an AKI mouse model is dependent on canonical WNT/β-catenin signalling. Treating cells with a β-catenin inhibitor significantly reduced the number of integrated cells (Zhang et al., 2015). Therefore, it is possible that cell integration and the effectiveness of cell-therapy could be enhanced by augmenting the WNT-signalling pathway in these cells. We hypothesize that increasing canonical WNT-signalling activity in NPCs prior to injection will improve integration into renal tubules after AKI injury. Here we show an early mouse NPC and human urine-derived progenitor cells treated with WNT-agonist, RSPO1, prior to injection show increased integration compared to baseline.

Methods

Cell culture (M15 and hNPCs)

M15 cells were donated by Dr. Nicholas Hastie and were grown in DMEM + 10% FBS at 37˚C. Human urine derived NPCs were donated by Dr. Elena Levtchenko and grown in conditions established by her laboratory (Arcolino et al., 2016). Recombinant RSPO1 protein (3474-RS-050, R&D Systems, Minneapolis, MN, USA) was added to culture medium for 48 hours before injection. Immediately prior to cell injection, cells were stained with PKH26 (PKH26GL, Sigma-Aldrich, Oakville, ON, Canada) according to the manufacturers protocol.

Luciferase assay

Transient transfections of human urine derived NPCs were performed using a canonical WNT-signalling reporter plasmid, Super8XTOPFlash. Renilla luciferase expression vector pRL-SV40 (Promega, Madison, WI, USA) was co-transfected and used to normalize for transfection efficiency. Transfections were performed in triplicate and repeated three times on different days. One day prior to transfection, 20,000 human urine derived NPCs were seeded in 24-well plates and transfected at 80% confluency using Lipofectamine 2000 Transfection Reagent according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). Plasmids were transfected in the following amounts: Super8XTOPFlash (44 ng), *Wnt9b* (50 ng), Renilla (1 ng). Recombinant human WNT9B (ab159815, Abcam, Toronto, ON, Canada) was added at a concentration of 50 ng/mL. In R-spondin conditions, 200 ng/mL of recombinant human RSPO1 (4645-RS-025– R&D Systems, Minneapolis, MN, USA) was added to each well 24 hours post

transfection. Cells were lysed 48 hours after transfection and luciferase activity was measured using the Dual Luciferase Assay System reagents and quantified in a GLOMAX 96 microplate luminometer (Promega, Madison, WI, USA).

Glycerol injury + Cell injection

To induce AKI, adult C57/BL6 or NOD-SCID mice were injected (intramuscular) with 8ul/g of a 1:1 Glycerol/PBS solution or PBS alone. Each condition was repeated in three mice. On the third day after glycerol injury, 500,000 cells were injected intravenously via the tail vein per mouse on two consecutive days. On the seventh day after glycerol injury, mice were sacrificed and kidneys were harvested washed in PBS and placed in 4% PFA in PBS solution overnight at 4˚C. Kidneys were transferred into a 15% sucrose/PBS solution at room temperature until kidneys sunk and then transferred into a 30% sucrose/PBS solution and placed at 4˚C overnight. The following morning, kidneys were transferred into a 1:1 mixture of OCT and 30% sucrose/PBS and rocked at 4˚C for 2 hours. They were subsequently placed into Cryomolds filled with OCT, snap frozen on dry ice and stored at -80˚C. Cryosections (7uM) were obtained using a Leica Cryostat.

Confocal microscopy

Prior to imaging, nuclei were counterstained with VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Proximal tubules were labelled using fluorescein-labelled lotus tetragonolobus lectin (Vector Laboratories, Burlingame, CA, USA). Images were obtained with a laser scanning confocal microscope (LSM780) and the ZEN2010 software (Carl Zeiss Canada Ltd., Toronto, ON, Canada) at room temperature and processed by Adobe Photoshop and Illustrator software.

Results

M15 and Urine Derived NPCs (UD-NPCs)

We used M15 cells isolated from E10.5 embryonic mesonephric mesoderm and human UD-NPCs isolated from neonatal urine to test in an AKI model. As described by Dickinson et al, M15 cells express key markers of the developing kidney and represent an unprimed NPC that poorly responds to the inductive WNT9B signal (Dickinson et al., 2019). To confirm UD-NPCs accurately

represent the NPC lineage, we confirmed mRNA expression of early NPC markers, CD24, *WT1*, *CITED1*, *SIX2* and *OSR1*. However, UD-NPCs did not express key molecules of the canonical WNT-signalling pathway *FZD5* and *RSPO1*. To test whether UD-NPCs are responsive to an exogenous WNT9B signal, we transiently transfected them with TOPFlash (a β-Catenin luciferase reporter) and exposed them to recombinant WNT9B. As seen in Figure A1.1A, exposing UD-NPCs to WNT9B does not activate the TOPFlash reporter, however, exposing cells to both WNT9B and RSPO1 resulted in a 3.6-fold increase. Similarly, as seen in Figure 3.1B, exposing WNT9B was unable to activate the TOPFlash reporter in M15 cells, however, treatment with both WNT9B and RSPO1 resulted in a 3.3-fold increase in activation. Due to technical and time limitations, *FZD5* could not be added to UD-NPCs, however, addition of WNT9B, RSPO1 and *Fzd5* to M15 cells resulted in a 11.1-fold increase in TOPFlash activation (Fig A1.1B).

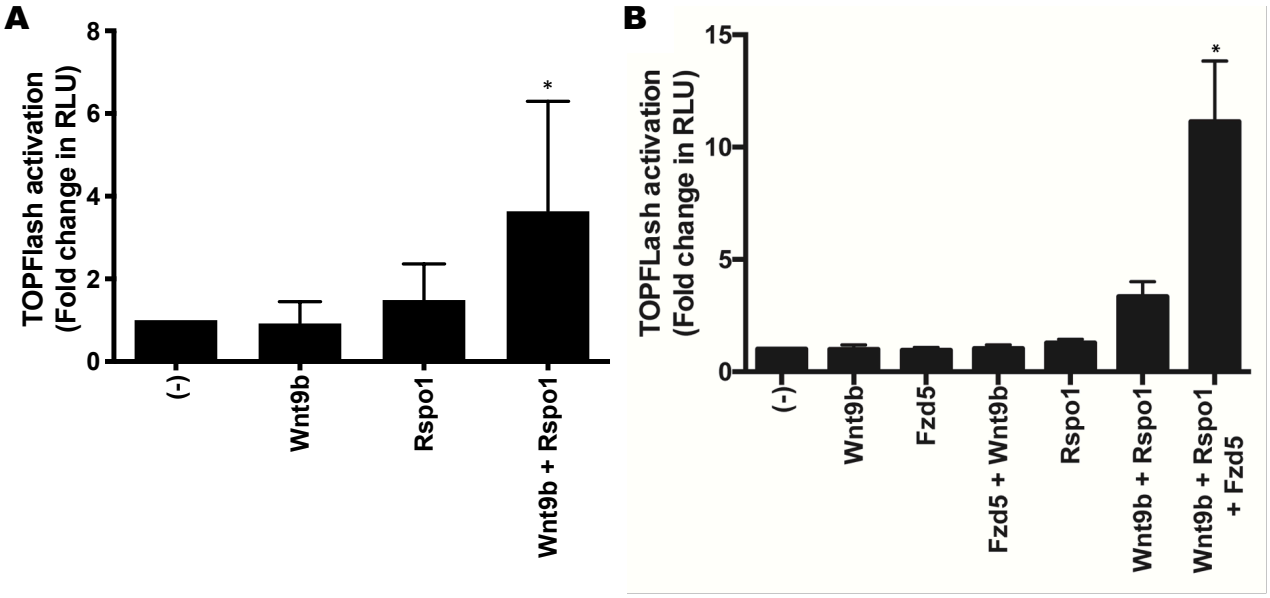

Figure A1.1. WNT-responsiveness of NPCs exposed to recombinant WNT9B and RSPO1.
UD-NPCs (A; n=2) and M15 cells (B; n=3) were transfected with TOPFlash and exposed to recombinant WNT9B (50ng/mL), recombinant RSPO1 (200ng/mL) or both. After 48 hours, TOPFlash to Renilla signal (RLU) was measured in a luminometer. A one-way ANOVA followed by a Dunnett correction for multiple comparisons was performed. Each experiment was performed in triplicate (technical replicates).

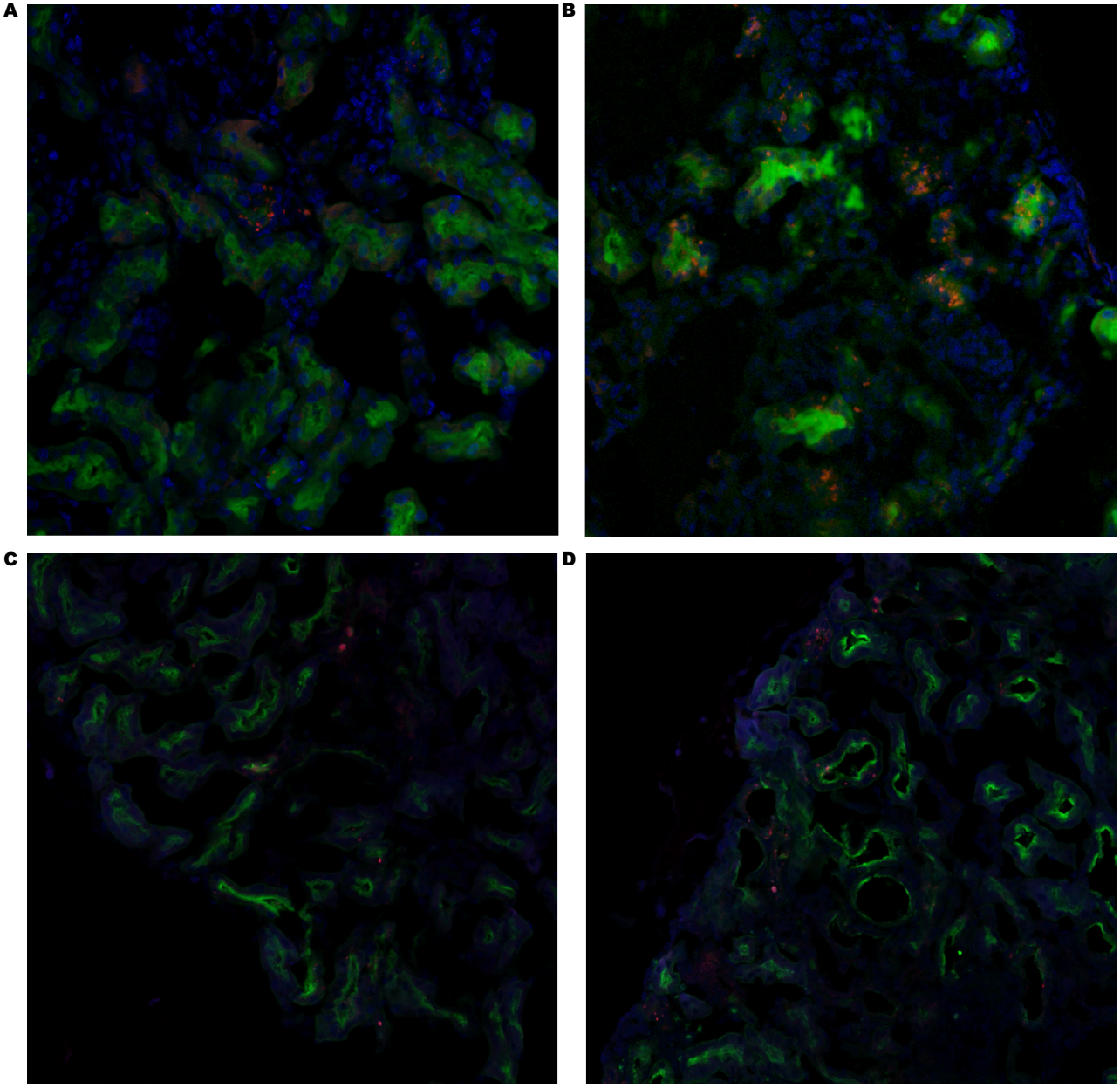

Figure A1.2. Integration of cells infused into glycerol-injured mice.
Cryosections (63X magnification) of 6-week old C57/BL6 (A and B) and NOD-SCID (C and D) mouse kidneys injured with glycerol were assessed by immunofluorescent microscopy to detect the presence of PKH26-labelled cells (red). Sections were counterstained with DAPI (blue - nuclei) and fluorescein labelled Lotus Tetragonolobus lectin (green – proximal tubule lumen marker). M15 cells treated with PBS (A) and RSPO1 (B) were tested. UD-NPCs treated with PBS (C) and RSPO1 (D) were also tested.

Pre-treating NPCs with RSPO1 increases cell integration in a model of AKI

Our next goal was to test whether increased canonical WNT-signalling activity could improve cell integration in a model of AKI. Mice were injected with glycerol to induce AKI. PKH26-stained M15 cells were treated with either recombinant RSPO1 or PBS for 24 hours and injected intravenously into C57/BL6 mice three days after glycerol injection. As seen in Figure A1.2A, PBS-treated M15 cells exhibit minimal integration into the kidney. Interestingly, an increased number of RSPO1-treated M15 cells could be detected in the kidney after injury (Figure A1.2B). These experimental conditions were repeated using UD-NPCs infused into NOD-SCID mice. As seen in Figure A1.2C, PBS-treated UD-NPCs showed minimal integration into injured kidneys. RPSO1-treated UD-NPCs exhibited a relatively minimal increase in integration compared to the PBS control (Figure A1.2D).

Discussion

Mammalian kidneys do not have the ability to generate new nephrons after the perinatal period, however, they retain the ability to repair nephrons in response to injury. Although these intrinsic repair mechanisms are often very effective in restoring renal function after injury, they can also result in incomplete/maladaptive repair, leading to the formation of fibrosis/scarring. Therefore, using an exogenous cell-based therapy to aid the intrinsic mechanisms to prevent this maladaptive process remains a promising area of investigation. Our lab previously showed that integration of embryonic NPCs was dependent on the status of the canonical WNT-signalling pathway, as inhibition of the pathway decreased cell integration (Zhang et al., 2015). Therefore, prior to injection of NPCs in this study, we assayed the ability of our cell lines to respond to a canonical WNT signal. Both M15 cells and UD-NPCs were unresponsive to a WNT9B signal. However, simply adding recombinant RSPO1 to the growth media increased responsiveness to WNT9B by nearly 3.5-fold in both cell types. This suggests that canonical WNT-signalling can be augmented using a simple method to prime cells prior to injection into a model of AKI.

As expected, pre-treatment of NPCs with RSPO1 appeared to increase the number of integrated cells in the damaged kidney. Due to time constraints, the measure of cell integration was purely qualitative. Adding quantitative measures of renal function, such as serum creatinine and blood urea nitrogen could further demonstrate the ability of integrated cells to improve renal function

after injury. Additionally, it would be of interest to test a number of different cell lines isolated from neonatal or adult urine. As described by Arcolino et al., adult UD-NPCs and neonatal UD-NPCs have different expression profiles, primarily, adult UD-NPCs lack *SIX2* expression compared to neonatal UD-NPCs (Arcolino et al., 2016). However, not all cells isolated from one individual have identical expression profiles (unpublished data). Therefore, due to the relatively minimal integration of RSPO1-treated UD-NPCs, it is possible that a more suitable/optimal UD-NPC exists and can be identified with additional characterization. In particular, UD-NPCs can be screened with a more extensive panel of canonical WNT-signalling genes prior to injection. Identification of a fully primed UD-NPC, in combination with pre-treatment with RSPO1, should increase cell integration into injured tubules. Nonetheless, use of UD-NPCs remains a promising endeavor as it provides a novel and minimally invasive source of cells to treat renal injury.

Studies into cell-based therapy of AKI also have potential implications for use in diseases with associated CKD such as Alport syndrome. Amniotic fluid mesenchymal stem cells (amMSCs) were infused into a mouse model of Alport syndrome and delayed the onset of renal fibrosis and also prolonged the lifespan of the treated mice (Sedrakyan et al., 2012). However, similar to studies of cell-therapy of AKI, the infused cells failed to integrate and differentiate into renal epithelia. Instead, these cells modulated the microenvironment to prevent activation of tissue remodeling factors. Cell-based therapy has also been tested in the setting of kidney transplantation, specifically to test the ability of infused cells to prevent chronic allograft nephropathy. In one study, allogenic MSCs were infused in a rat model undergoing a kidney transplant where treated mice showed reduced fibrosis and tubular atrophy (Franquesa et al., 2012). Similar to amniotic fluid cells, it was concluded that MSCs in this study exerted their protective effects through modulation of the immune response instead of integration and repair of damaged regions. Human kidney cells have also been investigated in the field of CKD. Primary human renal cells isolated from a discarded kidney were infused into an immunocompromised mouse with CKD. Treated mice showed improvements in kidney injury-related biomarkers and reduced fibrosis but again showed little to no integration (Yamaleyeva et al., 2012). Although these three cell types helped improve kidney injury in the short-term, little is known about the long-term effects of

peritubular localization of stem cells within the kidney. In particular, assessment of the risk of developing cancer as a result of this treatment over time. Interestingly, human fetal kidney cells from aborted fetuses consist of NPCs of the cap mesenchyme which were able to integrate and form renal tubular structures in a model of CKD (Harari-Steinberg et al., 2013). Therefore, it becomes apparent that relatively early NPCs, partially pushed toward the renal lineage allow for increased integration and differentiation into tubular structures and may improve AKI and CKD outcomes. Ensuring NPCs integrate and differentiate into tubular epithelia should minimize the long-term risk associated with other cell types that localize in peritubular regions. This highlights the importance of finding the appropriate NPC which allows for integration and also can be isolated from a minimally-invasive source.

Based on our observations, UD-NPCs can be treated with recombinant RSPO1 prior to injection to increase cell integration in a model of AKI. Although this technique requires additional optimization, it remains a highly promising area of investigation in the field of kidney injury.

Appendix II: List of Publications

Published Articles

1. Dickinson K.K., Hammond L.C., Karner C.M., Hastie N.D., Carroll T.J., and Goodyer P.R. Molecular determinants of WNT9b responsiveness in nephron progenitor cells. *PLoS ONE*. 14(4): e0215139.
2. Tokhmafshan F., Dickinson K., Akpa M.M., Brasell E., Huertas P., and Goodyer P.R. A no-nonsense approach to hereditary kidney disease. *Pediatric Nephrology*. doi: 10.1007/s00467-019-04394-5 [Epub ahead of print].

Submitted Articles

1. Dickinson K.K, Hammond L.C., Akpa M.M., Chu L.L., Goodyer P.R. WT1 Regulates Expression of the DNA-Repair Gene *Neil3* During Nephrogenesis. Submitted to PloS ONE in November 2020.

Published Abstracts

1. Canadian Student Health Research Forum - June 2016, Winnipeg, Canada - Dickinson K.K., Hammond L.C., Carroll T.J., and Goodyer P.R. Components of the canonical Wnt-signalling pathway in the developing kidney.
2. American Society of Nephrology - Nov 2016, Chicago, USA - Dickinson K.K., Hammond L.C., Carroll T.J., and Goodyer P.R. Identification of key Wnt-signalling components in the developing kidney.
3. American Society of Nephrology - Nov 2017, New Orleans, USA - Dickinson K.K., Hammond L.C., Carroll T.J., and Goodyer P.R. Determinants of Renal Progenitor Cell responsiveness to the inductive Wnt9b signal from ureteric bud.
4. International Workshop on Developmental Nephrology - April 2018, Ein Gedi, Israel - Dickinson K.K., Hammond L.C., Carroll T.J., and Goodyer P.R. Determinants of Renal Progenitor Cell responsiveness to the inductive Wnt9b signal from ureteric bud.

5. 12th International Podocyte Conference - May 2018, Montreal, Canada - Dickinson K.K., Hammond L.C., Carroll T.J., and Goodyer P.R. Determinants of Renal Progenitor Cell responsiveness to the inductive Wnt9b signal from ureteric bud.
6. Stem Cell Regenerative Medicine Network Symposium - Nov 2018, Montreal, Canada - Dickinson K.K., Hammond L.C., Carroll T.J., and Goodyer P.R. RSPO1 enhances integration of Nephron Progenitor Cells into glycerol-damaged renal tubules.