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ROLES OF THE RETINOBLASTOMA PROTEIN RB AND P53 IN THE CONTROL OF RENAL STEM AND PROGENITOR CELLS' PROPERTIES IN *VITRO* - DIRECT IMPLICATIONS ON KIDNEY DEVELOPMENT-

by
IHSAN SHADI HAMMOURA

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AN ABSTRACT OF THE THESIS OF

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Title: Roles of the Retinoblastoma protein Rb and p53 in the control of renal stem and progenitor cells' properties in *vitro* -direct implications on kidney development-

Nephron formation necessitates the presence of a tight balance between self-renewal and differentiation of NPCs (Nephron Progenitor cells). Proper nephrogenesis during embryonic development is, therefore, critical for proper renal function in the adult kidney. Thus, a better understanding of the molecular framework (genes and pathways) regulating renal cell development including nephron stem and progenitor cells (NSPCs) is of major significance and will provide a better insight about disease pathogenesis.

The Retinoblastoma protein, pRb, is known for its central role in controlling cell cycle progression at the G1-S phase checkpoint. Various studies have also highlighted other functions carried by the Rb pathway such as the regulation of progenitors' differentiation and migration as well as survival of their mature progeny in different tissues including the brain and the retina. Recent work from our laboratory revealed, for the first time, that Rb is also required for proper kidney development and control of nephrogenesis. Hence, we found that Rb negatively regulates nephron progenitors' proliferation, and is indispensable for the survival of immature nephrons *in vivo*. On the other hand, the role of the tumor suppressor gene, p53, is well investigated in kidney development. Several studies have shown that p53 is needed, in a dose-dependent manner, to regulate ureteric bud branching, self-renewal and maintenance of nephron stem/progenitor cells inside the cap mesenchyme as well as terminal maturation of nephron structures.

To gain a better understanding of the roles of Rb and p53 in the control of NSPCs' development, we have investigated here how each tumor suppressor gene regulates the properties of these cells *in vitro*. We used a well-established protocol, adapted from Brown et. 2011, to isolate and culture renal cells derived from the cap mesenchyme (by mild enzymatic digestion and purification) after inducing a deletion of Rb and p53 during mid-development (Aaron C Brown et al., 2011). Then, we assessed renal cell growth, morphology as well as their rate of proliferation and their expression of key developmental markers such as Cited1 and Six2 by immunocytochemistry after 2-3 days in culture. To delete Rb and p53, we generated pregnant females carrying Nestin-CreERT2-YFP; $Rb^{fl/fl}$ and Nestin-CreERT2-YFP; $p53^{fl/fl}$ embryos along with heterozygous

or wild type control littermates, separately. Then, we induced Rb-p53 gene deletion by single tamoxifen treatments administered to these females by oral gavage at E10.5 followed by sacrifice at E17.5.

Our results showed successful Cre recombination and gene deletion in cultured renal cells, the majority of which belonged to the cap mesenchyme lineage and expressed the NSPCs' markers Cited1 and Six2. Accordingly, more than half of the isolated renal cells co-expressed Nestin and GFP and showed similar morphology between different genotypes after 2-3 days in culture. However, we detected, in Rb-/- cultures, a significant and proportional increase in cell proliferation as assessed by cell density, Ki67 labeling and BrdU incorporation compared with controls. This indicated that Rb negatively controls proliferation of renal cells including NSPCs in primary culture and is consistent with our recent in *vivo* findings showing enhanced proliferation inside the nephrogenic zone following the loss of Rb. Moreover, we found that the loss of p53 significantly compromises renal cell growth in culture as manifested by the sharp reduction in cell density compared with p53+/+ cultures. This finding is unlikely associated with a proliferation defect given that the rate of proliferation was not affected by the loss of p53. Hence, the mechanisms mediating this effect requires further investigation, and may be linked to a survival defect among other defects as seen in our *in vivo* study.

This is the first study to assess the roles of Rb and p53 in the control of renal stem and progenitor cells' proliferation *in vitro*. Complementary work in the future may shed more light on how these genes regulate NSPCs' differentiation and survival in culture. Altogether, these findings will help provide a better understanding of the cellular and molecular pathways implicating the Rb and p53 pathways in kidney development.

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ABBREVIATIONS

Agt – Angiotensinogen

ADH- anti-diuretic hormone

ASPP 1/2- apoptosis-stimulating protein of p53 1/2

BMP - Bone morphogenic protein

BrdU - Bromodeoxyuridine

BSA - Bovine serum albumin

CDK - Cyclin-dependent kinase

Cited1 – Cbp/p300-interacting transactivator with Glu/Asp-rich C-terminal domain 1

CM – cap mesenchyme

CS - Carnegie stage

CSB –Comma-shaped body

DMEM – Dulbecco's modified Eagle's medium

DMSO - Dimethyl sulfoxide

Dvl - Dishevelled

E - Embryonic day

ENaC- apical epithelial sodium channel

EPO- Erythropoietin

ERT2 - mutated estrogen receptor

Eya1 - Eyes absent 1 protein

FBS – fetal bovine serum

FGF - Fibroblast growth factor

FGFR1/2- Fibroblast growth factor receptor 1/2

fl/fl - floxed/floxed

Fzd - frizzled

GDNF - Glial cell-derived neurotrophic factor

GFP - Green fluorescent protein

HBSS - Hank's Balanced Salt Solution

HIFs - Hypoxia Inducible Transcription Factors' (HIFs)

HIF1α- Hypoxia Inducible Transcription Factor 1 alpha

HIF2α- Hypoxia Inducible Transcription Factor 2 alpha

HIF3α- Hypoxia Inducible Transcription Factor 3 alpha

HRE – Hypoxia Inducible Response Elements

IM - Intermediate Mesoderm

Jag - Jagged

LRP- lipoprotein receptor-related protein

MAPK - Mitogen-activated protein kinase

Mdm2 -Murine Double Minute-2

MET - Mesenchymal-to-Epithelial transition

MM – Metanephric mesenchyme

NC - Nephrogenic Cord

NICD - Notch intracellular domain

ND - Nephric Duct

NPC - Nephron Progenitor Cell

NuRD - Nucleosome remodeling and deacetylase

NZC – nephrogenic zone cells

Osr1 - Odd-Skipped Related Transcription Factor 1

P - Postnatal day

PA - Pre-tubular aggregates

Pax2 - Paired box gene 2 transcription factor

PBS – phosphate buffered saline

PCR - Polymerase chain reaction

PCP- Planar cell polarity

PFA – Paraformaldehyde

PHD - Prolyl Hydroxylase Domain proteins

qRT-PCR – quantitative real-time PCR

RAAS - Renin-Angiotensin-Aldosterone System

Rb - Retinoblastoma protein

REPs - Renal erythropoietin-producing cells

RSPC - Renal Stem and Progenitor cells

RPCs - Renal Progenitors Cells

RV - Renal Vesicles

Sall1 - Spalt Like Transcription Factor 1

SEM- Standard error of the mean

Six2 - Sine oculis homeobox homolog 2

Slc12a3 - Solute Carrier Family 12 Member 3

SSB - S-shaped bodies

TCF/LEF - T cell-specific transcription factor/lymphoid enhancer factor-1

UB - ureteric bud

V – Vasopressin Receptor

wt – Wild type

WT1 - Wilm's tumor 1

YFP - Yellow fluorescent protein

CHAPTER I

INTRODUCTION

A. The adult mammalian kidney

1. The structure of the adult kidney: human versus mouse

The human kidneys are enclosed by a fibrous capsule and located retroperitoneally at both sides of the vertebral column extending from the last thoracic vertebra (T12) to the third lumbar vertebra (L3) with the left kidney located slightly higher than the right kidney (Little, 2015). The renal parenchyma of the kidney consists of an outer region - the cortex - and an inner region - the medulla-. The human renal parenchyma is divided into a set of lobes known as renal pyramids. At the papilla, the base of renal pyramid, urine flows into minor calyces that join to form major calyces to connect with the renal pelvis. The renal pelvis in turn narrows down and joins with the ureter (Little, 2015). The number of the renal papillae vary widely in humans ranging from to 4 to 18. However, unlike the human kidney that is multi-papillate, the mouse kidney consists of a single renal papilla that extend into a renal pelvis, which in turn opens into a ureter (**Figure 1**). Moreover, both human and mouse kidneys are made up of superficial and juxtamedullary nephrons while only the human kidney has mid-cortical nephrons.

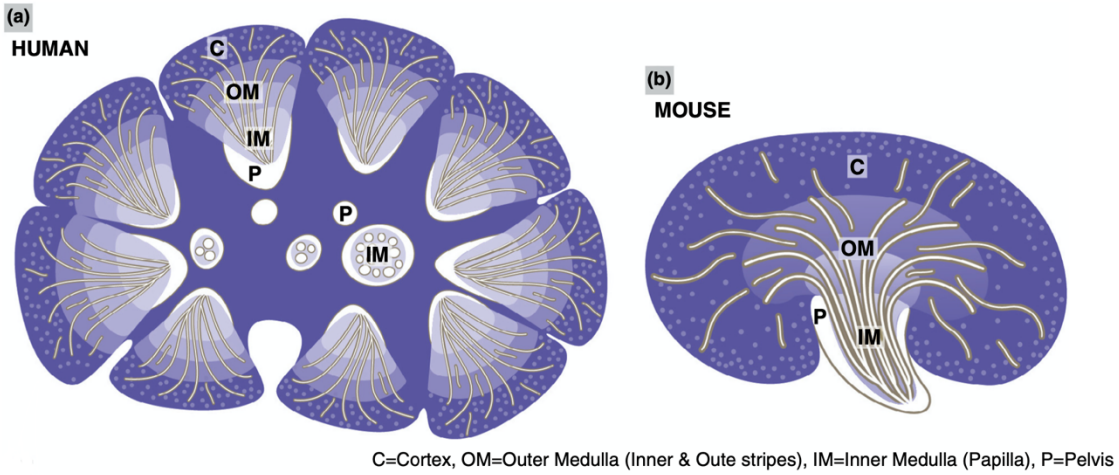


Figure 1. Structure of the adult kidney in human versus mouse
Diagrams depicting the structures of the human (a) and the mouse (b) adult kidneys with the anatomical differences between the two organs. The human kidney is multi-papillate made of 8–15 lobes while the mouse kidney is uni-papillate. C, cortex; OM, outer medulla, IM, inner medulla; P, pelvis (as modified from Little, 2015).

2. The physiological functions of the adult kidney

The kidney has various functions one of which is the maintenance of the level of water and electrolytes in the body. It keeps the level of water content constant by regulating the output of water in the urine. Moreover, it controls the rate of excretion of minerals to balance its level (C Eaton, 2009). Another important function of the kidney is the excretion of metabolic wastes such as urea, uric acid and creatinine, and bioactive substances such as drugs. It is also responsible of regulating the arterial blood pressure through controlling the blood volume (see next section). Furthermore, the kidney synthesizes the active form of vitamin D (1,25-dihydroxyvitamin D_3) and is considered a site of gluconeogenesis in case of prolonged fasting (C Eaton, 2009).

In addition, the kidney is well known for its role in the regulation of red blood cell production. Renal erythropoietin-producing cells (REPs), situated in the renal cortex, the outer medulla and around peritubular capillaries, are fibroblast-like cells known for the secretion of erythropoietin (Epo), an indispensable peptide hormone that controls the production of erythrocytes by the bone marrow. The role of these cells is indispensable since an insufficient production of EPO results in anemia (Souma, Suzuki, & Yamamoto, 2015). Studies have also shown that, in chronic kidney disease, REPs tend to transform into scar forming myofibroblasts that produce inflammatory cytokines. Thus, when dysfunctional, these cells are at the origin of renal fibrosis and anemia as seen in chronic kidney diseases. However, the disease pathophysiology can be reversed once REPs recover to a normal state (Souma et al., 2013).

Knowing that the *Epo* gene possess hypoxia inducible transcription factors' (HIFs) and response elements (HREs), one of the most important regulators of the synthesis of erythropoietin is the oxygen supply to the kidneys (Ratcliffe, 2013). HIFs are heterodimeric complexes consisting of an alpha subunit (HIF1α, HIF2α or HIF3α) and a beta subunit. In the presence of adequate oxygen levels, the proline residue of the alpha subunit is hydroxylated via prolyl hydroxylase domain proteins (PHD), which leads to the proteasomal degradation of the latter via von Hippel Lindau protein-mediated ubiquitination (Tanimoto, Makino, Pereira, & Poellinger, 2000). On the other hand, in case of oxygen deficiency, PHD are inhibited and HIFα proteins are not degraded. This would eventually induce the alpha and beta subunits to dimerize and form a complex that binds to HREs in the regulatory region of the *Epo* gene in order to activate its transcription.

Another important endocrine function of the kidney is its role in Renin-Angiotensin-Aldosterone System (RAAS). The RAAS controls the blood pressure and the level of sodium in the body, thus maintaining a circulatory homeostasis. The major player of this system is angiotensin II that is formed from the sequential cleavage of angiotensinogen. Angiotensinogen (Agt), the solely known renin substrate, is produced by the liver and secreted into the blood with a half-life of five hours (Lewicki, Printz, & Printz, 1983). Agt is part of a large superfamily of protease inhibitors known as Serpin A8 proteins and characterized by a distinct structure made up of three β sheets and nine α helices (Law et al., 2006).

Renin is an aspartyl protease that cleaves 10 amino acids from the N-terminus of Agt in order to form angiotensin I. Renin is secreted by the juxtaglomerular cells under three physiological conditions: a drop in the arterial blood pressure sensed by the baroreceptors of the afferent arterioles, a reduction in the concentration of sodium chloride at the macula densa and/or an increase in the level of catecholamines activating beta adrenergic receptors on juxtaglomerular cells. Eventually, the produced angiotensin I is converted to angiotensin II by the action of angiotensin-converting enzyme. This process eventually leads to the secretion of aldosterone, the reabsorption of sodium, the retention of water, an increase in the production of the anti-diuretic hormone (ADH) and the constriction of vascular smooth muscles (Sparks, Crowley, Gurley, Mirotsou, & Coffman, 2014).

Aldosterone is a steroid hormone possessing a mineralocorticoid activity manifested through binding to the cytosolic mineralocorticoid receptor present in the renal

distal convoluted tubule. Aldosterone ensures the activation of the apical epithelial sodium channel (ENaC) and the basolateral (Na+, K+ - ATPase) pump leading to an increase in the reabsorption of sodium. It enhances the activity of ENaC through various mechanisms one of which is the induction of the expression of the alpha subunit of this channel. Another mechanism of regulation of aldosterone is mediated by the modulation of various protein kinase pathways including the serine/threonine protein kinase, the serum and glucocorticoid regulated kinase1 (Sgk1) and ubiquitin ligase Nedd4-2 (Bhalla, Soundararajan, Pao, Li, & Pearce, 2006). Moreover, aldosterone relieves the negative regulation of ENaC by the mitogen-activated protein kinase (MAPK) by inducing the expression of an inhibitor of this pathway the glucorticoid-induced leucine zipper (Gilz) (Loffing & Korbmacher, 2009).

Another player of the RAAS system is the antidiuretic hormone (ADH). ADH is a nonapeptide formed in the hypothalamus and secreted from the posterior pituitary during a hypovolemic state. ADH manifests its role by binding to V receptors on cells of the distal tubule and the collecting duct. Upon binding to these receptors, the adenylate cyclase pathway is activated, which promotes the insertion of aquaporin-2 (AQP2) storage vesicles into the apical membrane of the tubules and the reabsorption of water along with the production of a concentrated urine. ADH also plays a role in stimulating the contraction of vascular smooth muscles leading to an increase in blood pressure (Boone & Deen, 2008).

B. Kidney development in mammals

1. Overview of mouse kidney development

During development, the mammalian kidney originates from the intermediate mesoderm (IM) that extends along the rostral–caudal axis to form three organs; the

pronephros, the mesonephros and the metanephros (Wingert & Davidson, 2008) (**Figure 2**). In mice, the pronephros, a non-functional transitory organ, develops at embryonic day 8.5 (E8.5), and consists of 6 to 10 tubules that open distally into the pronephric duct and proximally into the coelom (Little, 2015). At E9, the pronephros regress while the mesonephros develops at the thoracolumbar region. The mesonephros also degenerate while the metanephros, the final excretory organ, forms at E10.5 giving rise to the permanent kidney (Mugford, Sipilä, Kobayashi, et al. 2008).

At E11.5, the ureteric bud (UB) forms at the caudal end representing the early phases of development of the metanephros. The metanephric blastema aggregates around the ureteric bud stimulating this epithelium to proliferate and branch through the secretion of glial derived neurotrophic factor (GDNF). GDNF in turn activates the Ret and GFRα1 receptors expressed on the tip cells of the ureteric bud which facilitates the branching and the invasion of the UB (Mukherjee, Fogarty, Janga, & Surendran, 2019). In turn, the ureteric bud secretes Wnt9b, which leads to the induction of the metanephric mesenchyme (MM). These induced mesenchymal cells then produce Wnt4 and fibroblast growth factor 8 (Fgf8) that initiate mesenchymal to epithelial transition (MET) (Costantini & Kopan, 2010). Subsequently, the metanephric mesenchyme gives rise to the nephrons including the nephric proximal and distal tubules and glomeruli, as well as to the stroma, while the ureteric bud develops into the collecting duct system (**Figure 2**).

The process of nephron formation, known as **nephrogenesis**, commences at E11 and ends around post-natal day 4 (P4) (Cebrian, Borodo, Charles, & Herzlinger, 2004). In order to generate enough nephrons, a tight balance must be maintained between self-

renewal and differentiation of nephron progenitor cells. This event is characterized by the aggregation and condensation of mesenchymal cells near the tip of the ureteric bud. After the release of ureteric inductive signals, these cells undergo mesenchymal to epithelial transition to form renal vesicles (RV). Then, renal vesicles elongate and form a cleft at the proximal end known as coma-shaped bodies (CSB), which in turn develop another cleft at the distal end to give rise to the S-shaped bodies (SSB). The distal region of the S-shaped body then connects with the ureteric bud and undergoes a series of elongation and twisting events in order to form the distal convoluted tubule of the nephron. The loop of Henle and the proximal convoluted tubule develop from the central region of the S-shaped body while the renal capsule develop from the lower region through a process known as **glomerulogenesis** (Bard, Gordon, Sharp, & Sellers, 2001) (**Figure 2).**

The earliest transcription factor expressed in the intermediate mesoderm (IM) is the Osr1 (Odd skipped related1) (James, Kamei, Wang, Jiang, & Schultheiss, 2006). Osr1+ cells give rise to three distinct renal lineages including the stromal lineage, the nephric ureteric lineage and the nephron lineage (Mugford, Sipilä, McMahon, & McMahon, 2008). Renal cell fate is controlled by spatial and temporal regulation and developmental conditions whereby the anterior portion of IM gives rise to the nephric duct lineage whereas the posterior part generates the nephron and the stromal lineages. Once the UB starts to branch into the MM, the expression of Osr1 becomes restricted to the CM, but is essential to ensure the expression of key genes required for the maintenance of nephron progenitor cells including *Six2*, *GDNF* and *Pax2* (refer to the next sections for details) (Mugford, Sipilä, McMahon, & McMahon, 2008). The MM is characterized by the

presence of three major progenitor cell populations: the (Six2+;Cited1+) epithelial cells that form the epithelial components of the nephron, the Foxd1+ stromal progenitors that give rise to the mesangial and interstitial components, and the (flk1+;CD146+) endothelial progenitors that form the vasculature of the nephron (Kobayashi et al., 2008)

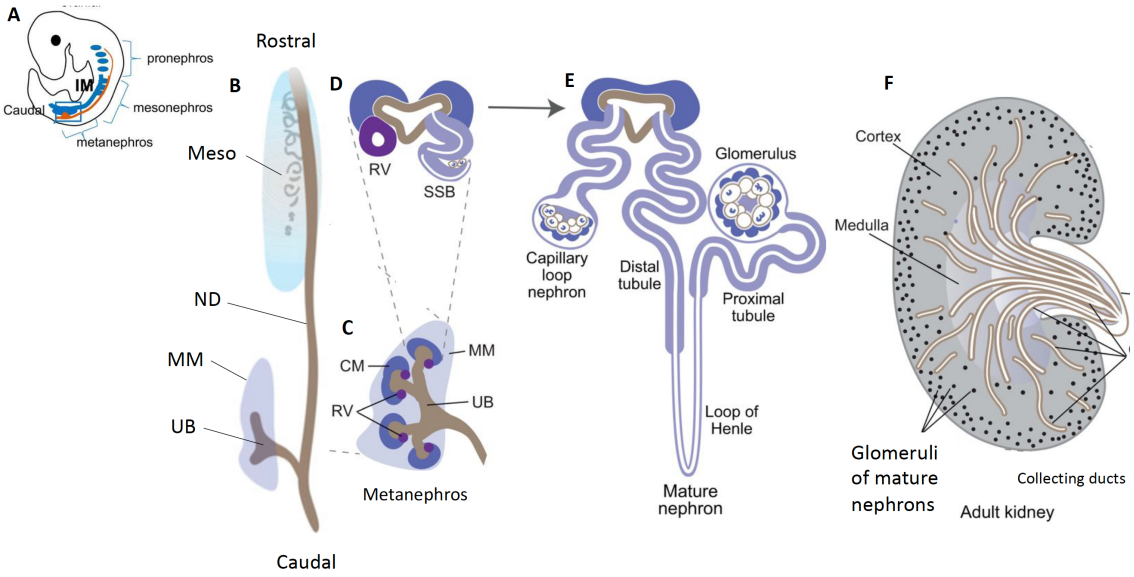


Figure 2. Schematic illustration of the mouse kidney development (A) Drawing of a mouse embryo at E9.5. The intermediate mesoderm (IM) that extends along the rostral–caudal axis forms three renal organs: the pronephros, the mesonephros and the metanephros. The dorsal IM (red line), forms the nephric duct (ND) while the ventral IM (in blue) forms the nephrogenic cord (NC). (**B**) The mesonephros (Meso) forms posterior to the pronephros and develops nephrons that drain directly into the nephric duct. The mesonephros is closely associated with the developing gonad (light blue). Both the mesonephros and the pronephros eventually degenerate. **(C)** The metanephros arises from the ureteric bud (UB, brown), an epithelial branch from the nephric duct and the metanephric mesenchyme (MM, light blue). As the UB branches within the MM, those MM cells closest to the UB form the cap mesenchyme (CM, dark blue) from which the nephrons arise via a mesenchyme-to-epithelial transition (MET). Within each ureteric tip/cap mesenchyme niche, renal vesicles (RVs, purple) develop at the edges of the CM and further elongate and segment into more differentiated structures, the S-shaped bodies (SSBs). These elongate further to establish a capillary loop before finally maturing into a nephron, which is comprised of a proximal tubule and a distal tubule separated by the loop of Henle, and a glomerulus. The adult mouse

kidney has an external cortex, containing the glomeruli, a medulla and a single papilla, through which the collecting ducts drain (as modified from Takasato and Little 2015).

2. Human versus mouse kidney development

The process of nephrogenesis in human begins around the 4th week of gestation and terminates at the 35th week forming around 1 million nephrons whereas in mouse it initiates at E10.5 and ends at P4 forming around 16000 nephrons (Little 2015). Moreover, unlike in mouse where the UB branches at E12.5 (around mid-gestation), the first UB invasion in human begins at the Carnegie stage 13 (CS13) and the first UB branch appears at CS16 (Little 2015). Furthermore, unlike in mouse where a single renal lobule is present, the human kidney possesses several interlobular and peripheral regions consisting of SIX2+ nephron progenitor cells (NPCs) aggregated in caps around the UB tips at CS19. These interlobular regions are surrounded by an interstitial stroma similar to that found around the CM in mouse. Another temporal difference identified between renal development in mouse and human is the duration of the transition phase from the PA stage to the SSB stage, being around 8 days in human compared with 24 hours in mouse (Gao et al., 2005; Short et al., 2014). Another main difference is the expression pattern of *SIX1* gene, which is stable throughout kidney development in human whereas its expression is only transient and appears around E10.5 in mouse. Despite these developmental variations, the overall cellular organization of the nephrogenic niche as well as its function are identical and follow similar paths of development in both species.

C. Mechanisms of regulation of kidney development

1. Inductive signals and signaling pathways that trigger nephrogenesis

a. Notch signaling pathway

Notch signaling pathway is an evolutionary conserved pathway that functions in the determination of cellular identity. It has been shown to play a critical role in embryonic development of various organs including the kidney. The pathway consists of four notch receptors (Notch 1-4) and five ligands Delta-like (Dll) 1, 3, 4 and Jagged (Jag) 1, 2 (Kopan & Ilagan, 2009). Upon ligand binding, the receptor undergoes various modifications such as proteolytic cleavage by metalloproteases of the ADAM family and γ-secretase complex. The action of the gamma secretase complex leads to the release of the notch intracellular domain (NICD), which forms along with Rbpj and Mastermind a transcriptional complex to activate various notch target genes (Sirin & Susztak, 2012). Of these genes are the basic helix loop helix (b-HLH) genes that belong to the Hes and Hey family of transcription factors. Notch signaling elicits various responses in different cell types ranging from proliferation to apoptosis, depending on its interaction with various signaling pathways such as NFkβ and distinct types of enhancers that respond to notch activity (Shin et al., 2006).

In the developing kidney, specific components of the notch pathway are expressed in unique patterns. Hence, Notch 2 receptor is expressed in nephron progenitor cells while both Notch 1 and Notch 2 receptors are expressed in peritubular aggregates and renal vesicles. Cells of the renal vesicles closest to the UB tip express the notch ligands Jagged 1 and delta-like 1, which are also expressed in the middle region of the S-shaped body (SSB)

(L. Chen & Al-Awqati, 2005). Notably, their expression overlaps with that of lunatic fringe (Lfng) that modulate the response of the notch receptor to Jag1 and Dll1 through post-translational modification. On the other hand, cells of the collecting duct lineage express Notch 1, 2 and 3 as well as Jag 1 and Dll1 (L. Chen & Al-Awqati, 2005).

Nephron formation necessitates the presence of a tight balance between self-renewal and differentiation of NPCs. This balance is regulated by various pathways including Notch pathway, which represses the expression of *Six2* in NPCs, thus resulting in exist from the progenitor state and induction of differentiation. Studies have shown that inactivation of notch receptors (notch 1 and 2) results in a sustained expression of *Six2* (Chung, Deacon, & Park, 2017), whereas forced expression of *NICD*1 and 2 leads to premature differentiation of nephron progenitor cells (Callahan & Egan, 2004). Studies using Pax3-Cre;Notch2 $^{flox/flox}$ mice have shown that Notch 2 is required to maintain the specific compartmentalization of gene expression at the renal vesicle stage (Cheng et al., 2007). One of these genes is the *Lhx1* whose segment specific expression is established by Notch 2.

Studies using Six2-Cre; Notch1$^{fl/fl}$; Notch2$^{fl/fl}$ mice have also shown that notch signaling is essential for nephron segmentation and maintenance of the proximo-distal axis of the renal vesicle (Chung, Deacon and Park, 2017). Moreover, defective nephrogenesis with failure to form S- shaped bodies along with a reduction in the expression of mature nephron markers (e.g. *Slc12a3, Wt1*) was reported in Wnt4-Cre; Notch1$^{fl/fl}$; Notch2$^{fl/fl}$ mice (Cheng et al., 2003). The above studies have shown that notch signaling is required for the proper transition of renal vesicles to S-shaped bodies by sustaining the expression of *Lhx1, Hnf1b* associated with the proliferation of Jag1+ cells. In addition to its role in early

nephrogenesis, notch pathway is required during late stages of nephrogenesis to suppress any aberrant cellular growth and maintain normal morphogenesis of the proximal tubules (Surendran et al., 2010).

Furthermore, the formation of the renal duct network requires the branching and the growth of the ureteric bud. This is accompanied with differentiation of cells into either intercalated cells essential for pH balance, or, principle cells required for water homeostasis. It has been shown that notch signaling is required for proper patterning of cells of the collecting duct; hence, inactivation of notch pathway favors the selection of intercalated cells whereas ectopic expression of Notch1 promotes the formation of principal cells (Zhang, 2019). Moreover, alteration of the notch signaling impaired ureteric branching or budding while overexpression of Jag1 resulted in unilateral aplasia and hypoplastic kidneys (Jeong et al., 2009) (Refer to **table 1** for a summary of notch signaling functions).

b. Wnt signaling pathway

Inductive signals from the UB are required to ensure proper differentiation of the nephron (Grobstein, 1953). These signals could be divided into two events; a primary event that is characterized by the separation of stromal and nephron progenitor cell populations, followed by a secondary event whereby MET of the CM forms the nephron. One of the factors responsible for these inductive features is a member of the Wnt family. In 1982, the *Wnt-1* gene was identified and shown to be a homolog of the *Wingless*(wg) gene in *Drosophila*, that is known to play an essential role in the formation of the body axis during development. Since then, 19 additional Wnt ligands have been identified in the mammalian

genome. The Wnt signal is characterized by the binding of Wnt ligands to the extracellular domain of the frizzled (Fzd) receptor and its co-receptor, the low-density lipoprotein receptor-related protein 5 and 6 (LRP5 and LRP6). This leads to the downstream activation of either the canonical (β-catenin-dependent) Wnt pathway or the β-catenin-independent/non-canonical Wnt pathway(s) (Logan & Nusse, 2004).

The canonical β-catenin-dependent pathway is characterized by the binding of the Wnt ligand(s) to Fzd and LRP 5/6 receptors, which activates the cytoplasmic protein dishevelled (Dvl). Dvl, in turn, inhibits the β-catenin destruction complex, which is made up of adenomatous polyposis coli (APC), glycogen synthase kinase 3 β (GSK-3 β), casein kinase 1 (CK1), and the scaffold protein Axin. This leads to the accumulation of unphosphorylated form of β-catenin in the cytoplasm that enters the nucleus and regulates the expression of various Wnt target genes through its interaction with a family of transcription factors, the T cell factor (TCF) /lymphoid enhancer binding factor (LEF). Another important factor for the signal transduction of canonical Wnt signaling pathway is the transmembrane prorenin receptor (PRR) (Reya & Clevers, 2005).

On the other hand, one example of non-canonical/β-catenin-independent pathway is the Wnt;PCP pathway that is known to play a role in embryonic tissue patterning and cellular polarization. The interaction of the Wnt/PCP ligands such as Wnt 5,7, 11 with the Fzd receptor allows the recruitment of scaffold protein Dvl along with various other components including Van Gogh homolog 2 (Vangl2), Prickle and Celsr1(Logan & Nusse, 2004; Sokol, 2015). A second non-canonical Wnt signaling pathway is the Wnt; Ca2+ pathway that is characterized by an elevation in intracellular calcium, which activates phospholipase C, the subsequent production of protein kinase C, and activation of the

phosphatase calcineurin. The latter dephosphorylates the nuclear factor of activated T cells (NFAT). This pathway has been known to be involved in dorsal/ventral patterning but is not directly implicated in kidney development (Logan & Nusse, 2004).

However, the Wnt/β-catenin and Wnt/PCP pathways have been involved in kidney development. Activation of Wnt signaling was shown to occur in the epithelia of the metanephric mesenchyme and the ureteric bud with specific β-catenin expression in renal vesicles (Iglesias et al., 2007). Several Wnt ligands have been shown to be expressed in the developing kidney including Wnt2b, Wnt4, Wnt5a, Wnt6, Wnt7b, Wnt9b and Wnt11(Merkel, Karner, & Carroll, 2007).

Notably, Wnt4 is expressed in renal mesenchymal cells and shown to play a role in the early stages of the kidney development through the canonical and non-canonical pathways. Moreover, Wnt4 regulates the fate of the smooth muscle cells in the medullary stroma through its interaction with bone morphogenetic protein 4 (BMP4) (Itäranta et al., 2006; Tanigawa et al., 2011). In comparison, Wnt5a acts via the non-canonical pathway to induce renal morphogenesis, and loss-of-function mutation(s) in this pathway results in the disruption of tissue differentiation during kidney development (Yun et al., 2014). Wnt7b is another essential factor in renal development given that Wnt7b−/− null mice were shown to form kidneys without a medullary zone (Yu et al., 2009). Another Wnt member with key role in early renal development in Wnt9b; cells expressing Wnt9b are needed for the inductive response in the metanephric mesenchyme (**Figure 3**). Hence, studies have shown that Wnt9b signaling in Six2-positive cells is essential for cellular renewal and proliferation (Karner et al., 2011).

Moreover, Wnt9b plays a role in planar cell polarity of the renal epithelium and thus, any alternation in this signaling pathway could affect the tubular diameter (Carroll, Park, Hayashi, Majumdar, & McMahon, 2005). Wnt11 is also expressed in the nephric duct and the tips of the ureteric bud in a Wnt4-independent manner. A deficiency in the Wnt11 gene results in renal hypoplasia along with a disruption in the branching of the ureteric bud. Thus, Wnt11 functions in a synergistic manner with the GDNF/Ret signaling pathway to regulate ureteric branching (Majumdar, Vainio, Kispert, McMahon, & McMahon, 2003).

One of the signaling effectors of the Wnt pathway that plays a role in kidney development is the co-receptor LRP6. Loss of this co-receptor results in renal defects since LRP6 was shown to mediate UB inductive signaling through the Ret signaling pathway. Studies have also indicated that mutations in Fzd4 and/or 8 result(s) in the development of smaller kidneys and disrupt the growth of the UB. β-catenin, the main mediator in the canonical Wnt signaling pathway, has been shown to play a role in kidney development. Importantly, β-catenin-induced differentiation along with Six2-dependent self-renewal maintain the balance between the renewal of nephron progenitor cells and nephrogenesis (Bridgewater et al., 2008). Moreover, using conditional knockout experiments, studies have further demonstrated the importance of β-catenin in renal development given that its deletion from the Wolffian duct epithelium using the Hoxb7-Cre system resulted in renal hypoplasia and branching defects (Marose, Merkel, McMahon, & Carroll, 2008). Similarly, deletion of β-catenin using the Pax8-Cre system was associated with formation of a thin renal cortex with hypoplastic renal parenchyma. These results highlighted a central role of β-catenin starting at the S-shaped body stage and ending at late stages of nephrogenesis (Bridgewater et al., 2008) (Refer to **table 1** for a summary of Wnt signaling functions).

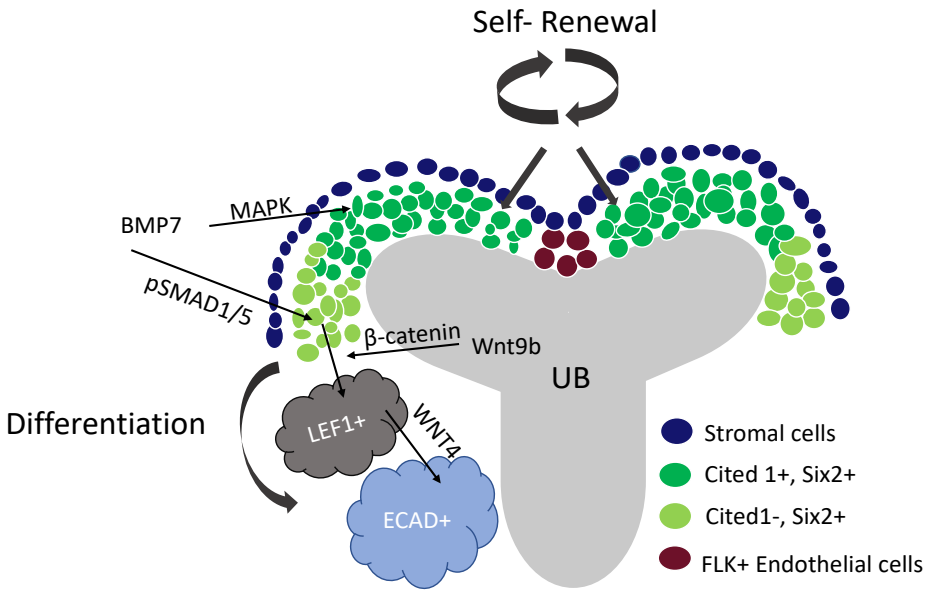


Figure 3. Compartmentalization inside the cap mesenchyme
The (Cited1+/Six2+) cells (dark green) are self-renewing nephron stem/progenitor cells, resistant to Wnt9b/β-catenin-induced differentiation. Bmp7 promote their proliferation through the activation of MAPK pathway, however, phosphorylation of SMADs induce the transition of (Cited1+/Six2+) cells to a Six2+/Cited1- (light green) compartment, that is inducible for differentiation. Eventually, these cells enter the LEF+ (Lymphoid enhancer binding factor) compartment characterized by a more differentiated peritubular aggregate primed for epithelial transition. Under the action of Wnt4, the latter group of cells epithelialize by expressing the tubular and epithelial marker E-Cadherin (ECAD). Two other progenitor populations residing within this niche: Foxd1+ stromal progenitors (dark blue) and Flk1+ endothelial cells corresponding to vascular progenitors (dark red).

2. Control of CM maintenance and proliferation

a. Bmp signaling pathway

Proper nephron endowment is ensured through the regulatory action of niche signals in the nephrogenic zone. One of the essential signaling pathways that play a role in the maintenance of the CM is the BMP signaling pathway. In fact, Bmp-7 was shown to be secreted by both the CM and the UB (Gonçalves & Zeller, 2011). Germline deletion of *Bmp-7* results in perinatal lethality of mice due to the presence of mal-developed kidneys. Moreover, studies have shown that the renal hypoplasia seen in $Bmp7^{-/-}$ mice is caused by a premature arrest of nephrogenesis and increased cellular death in the nephrogenic zone resulting in the depletion of the progenitor cell population (Dudley, Lyons, & Robertson, 1995; Luo et al., 1995). Thus, *Bmp-7* is required to support nephrogenesis, being an important survival factor for progenitor cells of the metanephric mesenchyme. Another important role of *Bmp-7* is to suppress the ureteric bud's inductive signals and thus, inhibit differentiation of the metanephric mesenchyme (Godin, Takaesu, Robertson, & Dudley, 1998). Bmp-7 mediates its function through activating the SMAD independent and JNK-dependent pathways (Blank, Brown, Adams, Karolak, & Oxburgh, 2009). *In vitro*, Bmp7 tends to act via the MAPK-Jnk signaling pathway to enhance proliferation of Cited1+ cells (Blank et al. 2009). On the other hand, activation of the SMAD pathway by Bmp7 promotes the differentiation of Cited1+ progenitors (Aaron C Brown et al., 2013) (**Figure 3)**.

Bmp4 is another member of the Bmp family that plays a role in kidney development. It is expressed in the mesenchyme around the ureteric stalk, and ensures the

inhibition of ectopic budding of ureteric tips by promoting the elongation of ureteric stalk (Miyazaki, Oshima, Fogo, Hogan, & Ichikawa, 2000) (Refer to **table 1** for a summary of the Bmp signaling functions).

b. FGF signaling pathway

FGF signaling has been shown to be essential for the maintenance and the survival of NPCs. In fact, FGF9 and FGF20 ligands are both secreted by the UB and their loss causes renal agenesis (Barak et al. 2012). The receptors for these ligands are FGFR1 and FGFR2, respectively. They are expressed in NPCs and possess redundant functions given that a single deletion of one receptor using the Six2-Cre system does not cause any detectable renal defects. In contrast, combined inactivation of both receptors results in premature loss of the Cited-1+ population of stem/progenitor cells (Di Giovanni et al. 2015).

Another important protein that partially mediates the function of the FGF receptor(s) is the adaptor protein FSR2α (FGFR substrate 2α). This protein mediates the interaction between FGFR and downstream signaling molecules such as the PI3K/AKT pathway (Di Giovanni et al., 2015). Of note, there exists a synergistic role between *Bmp7* and *Fgf9* (Barak et al. 2012). In fact, the two pathways converge to activate the FOS and JUN, components of the AP1 complex. This in turn leads to the transcription regulation of various target genes including *Myc* and *p53*. Myc acts along with β-catenin to promoter the transcription of genes essential for self-renewal of NPCs (Pan, Karner, & Carroll, 2017). The role p53 in the context of kidney development will be described in detail in **Section G** (Refer to **table 1** for a summary of Fgf signaling functions).

Summary of major signaling pathways regulating kidney development

	Functions	References
Notch pathway	1. Maintains the specific compartmentalization of gene expression in the RVs 2. Is required for the proper transition to the S-shaped phase 3. Maintains normal morphogenesis of proximal tubules 4. Is required for proper patterning of cells of the collecting ducts	(Chung, Deacon and Park, 2017; Cheng et al., 2007; Surendran et al., 2010; Zhang, 2019)
Wnt pathway	5. Wnt4 regulates the fate of the smooth muscle cells in the medullary stroma 6. Wnt5a acts via the non-canonical pathway to induce renal morphogenesis 7. Wnt9b is essential for the inductive response in the metanephric mesenchyme 8. Wnt9b signaling in Six2-positive cells is required for	(Itäranta et al., 2006; Majumdar, Vainio, Kispert, McMahon, & McMahon, 2003; Karner et al., 2011;(Bridgewater et al., 2008)

	cellular renewal and proliferation	
	9. Wnt9b plays a role in planar cell polarity of the renal epithelium	
	10. Wnt11 regulates ureteric branching	
	11. β-catenin-induced differentiation along with Six2-dependent self-renewal maintain the balance between the renewal of nephron progenitor cells and nephrogenesis	
Bmp pathway	12. Bmp7 is an important survival factor for progenitor cells of the metanephric mesenchyme	(Miyazaki et al., 2000)
	13. Bmp7 inhibits differentiation of the metanephric mesenchyme	
	14. Bmp4 inhibits ectopic budding of ureteric tips and promotes the elongation of ureteric stalk.	

FGF Pathway	15. Essential for the maintenance and survival of NPCs.	(Di Giovanni et al. 2015 ; Barak et al. 2012)

Table 1 Summary of the roles played by major signaling pathways during kidney development.

3. Transcription factors with important roles in kidney development and nephrogenesis

a. Role of WT1

An important transcription factor for the specification MM is the Wilm's tumor suppressor gene (WT1). Studies have demonstrated that loss of Wt1 expression in the MM results in the failure of recruitment of the UB (Kreidberg et al. 1993). Moreover, it was shown that Wt1 directly binds to the promoter region of *Gas1* (Growth arrest-specific 1) activating its expression. Gas1 in turn activates the PI3K/AKT pathway to positively control proliferation inside the CM (Kann et al. 2015). Wt1 also plays a role in late kidney development; hence, it is expressed in S-shaped bodies and mature podocytes and is required in the process of nephron differentiation. In conclusion, Wt1 plays a role in the specification of the MM and cell fate determination in the CM (Refer to **table 2** for a summary of the functions of this transcription factor).

b. Role of *Sall1*

Three *Sal*-related genes have been identified in humans: *SALL1, SALL2* and *SALL3*. *SALL1* is located on chromosome 16q12.1 and codes for a multi zinc finger transcription factor that is expressed in renal progenitor cells co-expressing *Cited-1* and

Six2, as well as in more mature structures including peritubular aggregates, renal vesicles, comma- and S-shaped bodies (Boyle et al., 2008). Loss of *Sall1* was shown to induce differentiation of progenitor cells instead of promoting self-renewal/proliferation. Thus, *Sall1* is essential to maintain the stem cell-like proprieties of renal progenitor cells by opposing differentiation. This function is manifested through the modulation of the expression level of *Six2,* which modulates the level of responsiveness of nephron progenitors to the Wnt9b-β-catenin pro-differentiation signal (Basta, Robbins, Kiefer, Dorsett, & Rauchman, 2014).

Moreover, *Sall1* was shown to play an essential function during the initial outgrowth of the ureteric bud into the metanephric mesenchyme, and can regulate the UB inductive signal through influencing the expression of *Wnt9b* (Osafune, Takasato, Kispert, Asashima, & Nishinakamura, 2006). Heterozygous mutations in the human form of *SALL1* results in Townes Brocks syndrome (TBS), an autosomal dominant disease that is characterized by defects in multiple organs including the kidney (Kohlhase, Wischermann, Reichenbach, Froster, & Engel, 1998). It was also suggested that Sall1 might interact with the nucleosome remodeling and deacetylase chromatin remodeling complex (NuRD) to repress the transcription of β-catenin (Reynolds et al., 2012). Altogether, the above studies concluded that *Sall1* is an essential nuclear factor in the regulation of UB branching and the maintenance of the population of nephron progenitor cells (Refer to **table 2** for a summary of the functions of this transcription factor).

c. Role of Pax proteins

The Pax family of proteins is a group of nine transcription factors that act as essential regulators of organogenesis. They are characterized by a DNA binding paired domain, a complete homeodomain in *Pax3, Pax4, Pax6* and *Pax7*, and a partial homeodomain in *Pax2, Pax5,* and *Pax8* (Mansouri, Hallonet, & Gruss, 1996). Of the Pax genes, only *Pax2* and *Pax8* are expressed in the developing kidney. Loss of *Pax2* results in complete loss of the metanephric kidney while its heterozygous inactivation results in renal hypoplasia (Saifudeen et al., 2012). On the other hand, germline deletion of *Pax8* does not show any major defects in kidney development. However, the inactivation of both *Pax2* and *Pax8* results in exacerbated renal defects suggesting the presence of partial functional redundancy between these genes (Bouchard, Souabni, Mandler, Neubüser, & Busslinger, 2002). Moreover, *Pax2/8* positively regulate the transcription factor Gata3, which in turn drives the guidance of the nephric duct and its responsiveness to inductive signals (Grote et al., 2008). Moreover, it has been shown that in the metanephric mesenchyme *Pax2* binds directly to the promoter region of *Gdnf* to positively regulate its expression (Boualia et al., 2013). Gdnf is secreted by the MM and induces the branching of the UB after activating Ret receptors on ureteric epithelial cells (Sainio et al. 1997; Costantini and Shakya 2006) (Refer to **table 2** for a summary of the functions of this transcription factor).

d. Role of *Cited1*

Cited1, Cited2 and Cited4 belong to a family of non-DNA binding transcriptional co-factors (Cbp-P300 Interacting Trans-activators with E/D rich tails). *Cited1* and *Cited2* are expressed in the metanephric mesenchyme at E11 while *Cited4* is expressed in the ureteric bud (Boyle, Shioda, Perantoni, & de Caestecker, 2007). During hypoxia, the expression of *CITED2* is positively regulated by HIF-1α. However, CITED2 itself competes with HIF-1α binding site, thus negatively regulating the hypoxia driven response (Freedman et al., 2003). No kidney phenotype is detected following the knockout of *Cited2* in mice. However, one study suggested a possible role of CITED2 in the regulation of the activity of HIFs in podocytes (Freedman et al., 2003).

Cited1 is specifically expressed in nephron stem cells inside the CM (early population of renal stem cells) and thus, is considered a specific marker of this population. It is also co-expressed with Six2 in the most cortical-least differentiated self-renewing population of NPCs. Unlike Cited2, Cited1 expression is downregulated as cells undergo mesenchymal to epithelial transition. Despite the unique expression pattern of Cited1, its deletion alone or in combination with Cited2 did not surprisingly result in major disruption in kidney development (Boyle et al., 2008). Thus, these factors do not seem to play a unique role in the process of nephrogenesis and their loss may be dispensable or compensated for by unknown mechanisms (Boyle et al., 2007) (Refer to **table 2** for a summary of the functions of this transcription factor).

e. Role of *Six2*

The *Six* gene family of transcription factors is homologous to *Drosophila* sine oculis (so) and includes six members (Six1-6). These proteins are comprised of two conserved domains including a homeodomain and specific Six-domain, the latter being essential for protein to protein interaction (P.-X. Xu et al., 2003). One of these homeobox transcription factors, Six2, plays a key role during nephrogenesis and kidney development. Of note, Six2 can regulate its own transcription through binding to its own promoter (Kobayashi et al. 2008). Six2 is expressed throughout the cap mesenchyme and its co-expression with other genes demarcate specific cellular compartments. The most cortical least differentiated self-renewing compartment of the cap mesenchyme is characterized by the presence of a Six2+/Cited1+ population of nephron progenitors. These cells are resistant to Wnt9b/β-catenin induced differentiation. With time, they become responsive to a Bmp7-Smad signaling and transition to a Six2+/Cited1- compartment that can be induced into differentiation. Eventually, the latter population enters the LEF+ (Lymphoid enhancer binding factor) compartment characterized by a more differentiated peritubular aggregate primed for epithelial transition (Aaron C Brown et al., 2013) (**Figure 3**).

Studies have shown that Six2 plays an essential role in the maintenance of the nephron progenitor population throughout kidney development. Thus, its deletion results in ectopic formation of renal vesicles (pre-mature differentiation) and premature depletion of the progenitor pool (Self et al., 2006). Similarly, loss of Six2 activity from cap mesenchymal cells led to ectopic formation of nephron tubules as mediated by the inductive signal of Wnt9b. Thus, downregulation of Six2 by Notch signaling is essential to

trigger differentiation of progenitor cells and formation of nephron segments (Kobayashi et al., 2008).

In addition, Six2 controls the expression of several target genes in order to ensure a proper balance between NPCs' self-renewal and commitment. For example, Six2 along with TCF/LEF factors silence the expressions of the *Fgf8* and *Wnt4* genes by binding to their *cis*-regulatory elements, which prevents commitment of NPCs. Notably, Wnt9b promotes self-renewal of NPCs when combined with high level of Six2, and differentiation of progenitor cells with low level of Six2 (Karner et al. 2011). Moreover, at high level of expression, Six2 competes with β-catenin in binding to the TCF/LEF complex, thus favoring self-renewal rather than pre-differentiation of NPCs (J. S. Park et al. 2012).

Eya1 is a transcriptional co-activator of Six2 since its inactivation results in reduction in Six2 expression, leading to a premature differentiation of the CM. Moreover, Six2 induces the translocation of Eya1 protein into the nucleus where it alters the phosphorylation state of Myc and help induce proliferation of nephron progenitor cells (Jinshu Xu et al., 2014). Six2 also associates with Osr1 to repress the activity of target genes that are essential for Wnt-mediated differentiation of renal cells. Similar to the phenotype seen after loss of Six2 activity, inactivation of Osr1 results in renal hypoplasia that is characterized with a premature depletion of the progenitor population (J. Xu, Liu, Park, Lan, & Jiang, 2014).

In summary, Six2 is expressed in undifferentiated multipotent nephron progenitors, and has been implicated in the regulation of NPCs' proliferation,

differentiation, migration and apoptosis (Xia et al., 2017) (Refer to **table 2** for a summary of the functions of this transcription factor).

Transcription factors with important roles in kidney development

	Functions	References
Wt1	1. Specification of the MM 2. Control of cell proliferation and differentiation of the CM	(Kann et al. 2015; Kreidberg et al. 1993)
Sall1	3. Regulation of UB branching 4. Maintenance of NPCs population	(Basta et al., 2014; Reynolds et al., 2012)
Pax proteins	5. Pax 2/8 play a role in the guidance of the nephric duct and its responsiveness to inductive signal	(Boualia et al., 2013; (Miyazaki et al., 2000)
Six2	6. Major regulator implicated in NPCs' proliferation, differentiation, migration and apoptosis	(Xia et al., 2017)
Cited1	7. A major marker of nephron stem cells and early progenitor cells	(Boyle et al., 2008)

Table 2 Summary of the major functions played by key transcription factors during kidney development and nephrogenesis.

D. In vitro properties of renal stem and progenitor cells

During murine development, the cap mesenchyme's population of renal stem and progenitor cells is maintained for around 10 days starting around mid-gestation. However, *in vitro,* these cells tend to undergo apoptosis after 48 hours (Perantoni, Dove, & Karavanova, 1995). Hence, several studies were conducted with the goal of better understanding the factors controlling the properties of these cells in order to design optimized culture conditions and protocols and mimic their native -*in vivo*- environment. In order to study the signaling events controlling the properties of renal stem and progenitor cells, Brown et al. developed a primary culture system using mild enzymatic digestion to derive these cells from the nephrogenic zone in the developing kidney. The dissected kidneys were placed in a mixture of collagenase A and pancreatin at 37°C on a nutator. Following mild digestion, the residual kidney tissues are left behind, and the isolated cells are recovered (for a detailed protocol, refer to the section on materials and methods). The collected nephrogenic zone cells (NZCs) consisted of a mixture of various cell types including ~35% cortical stroma cells (stromal lineage) and ~52% cap mesenchyme cells (mesenchymal-epithelial lineage), and had a survival rate of ~95% up to two days in culture (A. C. Brown et al., 2011).

Few years later, the same group developed an optimized *in vitro* protocol to specifically select and propagate nephron progenitor cells (NPCs) from murine embryonic kidneys and human embryonic stem cells by modulating the *FGF, BMP*, and *WNT*

signaling pathways (A. C. Brown, Muthukrishnan, & Oxburgh, 2015). FGF9 ensures the maintenance and proliferation of isolated NPCs (but not other cell types), BMP7 facilitates the renewal of NPCs through the activation of the JNK pathway, and WNT signaling is essential for the renewal and proliferation of the (CITED1+; SIX2+) progenitors.

In related studies, other research groups treated murine embryonic stem cells' aggregates with activin A, retinoic acid and BMP7 (Kim & Dressler, 2005). BMP7 was used to maintain survival of the MM cells whereas activin A and retinoic acid induced the expression of intermediate mesoderm markers *Pax2* and *Wt1*. These primed stem cells were shown to be capable of undergoing tubulogenesis and forming glomeruli once grafted *in vivo* (Osafune et al., 2006; Vigneau et al., 2007). Using a similar approach, Takasato et al. was able to differentiate mouse embryonic stem cells by inducing the primitive streak with an opposing gradient of Bmp4 and activin A along with the addition of Fgf9. The latter factor triggered differentiation in the intermediate mesoderm. Inductive signals were then used to induce the formation of metanephros including Fgf9, Bmp7 and retinoic acid. Several of the differentiated cells expressed various metanephric genes such as *Six2* and *Wt1*, and, eventually formed nephron-like structures *in vitro* (Takasato et al., 2014).

Knowing that the posterior mesoderm precursors expressing Brachyury (T) give rise to progeny of the metanephric mesenchyme, investigators maintained these T+ precursors by treatment with high levels of Wnt agonist. Adding retinoic acid and FGF9, they were able to form renal stem and progenitor cells (RSPCs) capable of undergoing tubulogenesis (Mari & Winyard, 2015). Moreover, Hendry et al. over-expressed various genes such as *SIX1, SIX2*, and *OSR1* in adult human proximal tubule cell lines by infection

with lentiviruses. This resulted in the generation of RSPCs capable of forming the cap mesenchyme structure (Hendry et al., 2013).

In summary, a better understanding of the developing kidney's signaling environment has allowed scientists to create an *in vitro* niche in order to isolate and culture primed NPC that are capable of further differentiation. This is moving one step further toward the ultimate goal of tissue engraftment.

E. Adult kidney regeneration

1. Glomerular regeneration

The glomerulus is comprised of the Bowman's capsule that is derived from the parietal epithelial cells (PEC), the central glomerular tuft made of endothelial and mesangial cells, and perivascular cells that form the slit diaphragm known as the podocytes (Scott & Quaggin, 2015). Podocytes are terminally differentiated cells found in the G0 phase of the cell cycle. Glomerular regeneration is still controversial given that few studies have investigated this process and further evidence in favor of this process is still needed. In fact, after glomerular injury in humans, podocytes were shown to remodel their actin cytoskeleton, and undergo increased cell size, cellular detachment, effacement of the foot processes and, finally, cellular death (Lasagni, Lazzeri, J Shankland, Anders, & Romagnani, 2013).

One study has suggested that, following glomerular injury, the lost podocytes could be potentially replaced for by intrarenal progenitors. These renal progenitors cells (RPCs) are present at the urinary pole of the Bowman's capsule and known to express CD24 -a marker of renal embryonic cells- and CD33 -a marker of adult stem cells-

(Ronconi et al., 2009). Moreover, these cells manifest the properties of renal stem cells including self-renewal potential, high clonogenic efficiency, a multi-differentiation ability and, do not express any lineage specific markers. Hence, they seem to form a heterogeneous population of pluripotent and undifferentiated stem cells located in the urinary pole, next to differentiated cells at the vascular pole.

Of note, among these RSCs, a podocyte progenitor subpopulation has been identified that expresses podocyte-specific markers and can differentiate into podocytes only. The potential role of renal progenitor cells (RPC) as a source of podocyte replacement has been investigated by various studies (Daniel Appel et al., 2009; Eng et al., 2015; Wanner et al., 2014). One study has shown that injection of RPCs into an immune-deficient mouse model of focal segmented glomerulosclerosis resulted in cell differentiation into podocytes, thus replacing lost podocytes and improving the disease outcome (D. Appel et al., 2009).

Also, it was shown that the cellular lesions detected in the glomerular disease, crescentic glomerulonephritis, are comprised of cells co-expressing CD24 and CD133, and believed to be derived from the aberrant proliferation of RPCs (Bart Smeets et al., 2009). Therefore, these renal progenitor cells could be potentially a major source for the regeneration of podocyte leading to glomerular disease remission, however, further evidence is still needed (B. Smeets et al., 2011).

In conclusion, the regenerative potential associated with RPCs is very promising and essential for the repair mechanism after injury; however, a better understanding of the various factors that control the balance between renal injury and renal regeneration is yet to be fully unlocked.

2. Tubular regeneration

Transient ischemia results in renal tubular injury, which is at the basis of acute kidney injury. However, due to the high regenerative potential of renal tubules, kidney function can be restored after acute kidney injury with mild tubular injury. The regenerative potential of the renal tubules is associated with the presence of a tubular progenitor subpopulation among the stem cell population that co-expresses the CD24 and CD133 markers. However, this subpopulation is distinct from the 'podocyte' progenitor population and does not express the surface marker CD106. The committed tubular CD133+ CD24+ CD106- progenitors are resistant to death and mostly localized in the S3 segment of the proximal tubule, the distal convoluted tubule and the connecting segment (Lindgren et al., 2011). They represent the only tubular progenitors to be detected so far. Further characterization of the specific markers and properties of these progenitors is essential in order to target these cells and manipulate their potential to boost tubular regeneration.

F. Nestin expression in the embryonic and adult kidney

Nestin is a cytoskeleton associated class VI intermediate filament and a widely known cellular marker for neural stem and progenitor cells. Its structure consists of an alpha helical domain with hydrophobic motifs, a short N terminal domain and a long C terminal domain. (Bernal & Arranz, 2018).

Dubois et al. was the first to report Nestin expression in the adult and developing kidney using Nestin-Cre1 transgenic mice (Dubois, Hofmann, Kaloulis, Bishop, & Trumpp, 2006). Cellular lineage studies using the same line have subsequently shown that Nestin

was expressed in the metanephric mesenchyme but was not been detected in the ureteric bud. Hence, in the developing kidney, Nestin was shown in Cited1+ cells as early as E11.5, in the vascular cleft of the S shape body, and in the capillaries of immature glomeruli, specifically in (CD31+;Flk1+) endothelial cells (J. Chen et al., 2006). In the adult kidney, Nestin's expression becomes restricted to the glomerular podocytes where it is essential to maintain their shape (Sun et al., 2014). In the adult and developing kidney, the expression of Nestin is regulated by *Wt1* (Wagner, Wagner, Scholz, Kirschner, & Schedl, 2006).

Furthermore, studies have shown the presence of dynamic changes in the level of Nestin expression following renal injury, and this was associated with repair mechanisms. Hence, during renal damage, the upregulation in Nestin's expression is believed to stimulate proliferation of mesangial cells and migration of proximal tubular cells in order to induce repair (Daniel, Albrecht, Lüdke, & Hugo, 2008; Wen et al., 2012). Moreover, Nestin-positive renal stromal cells have been detected in the postnatal murine kidney where they mediate the rescue from acute ischemic kidney injury by helping reduce the levels of serum creatinine and blood urea nitrogen. The same cells were also shown to provide a paracrine protective effect through the vascular endothelial growth factor signaling to help maintain the glomerular basement membrane (M. H. Jiang et al., 2015; Z. Jiang et al., 2010).

Therefore, Nestin as an intermediate filament protein plays a structural role in the developing kidney and is involved in repair mechanisms in the adult kidney.

G. Role of the p53-Mdm2 pathway during kidney development

TP53 is a tumor suppressor gene that was identified in 1979, and shown to code for as a simian virus 40 (SV-40) large T antigen binding protein in virally transformed cancer cells (Kamada, Toguchi, Nomura, Imagawa, & Sakaguchi, 2016; Smith, Smith, & Paucha, 1979). Later on, more than half of all human cancers were shown to carry mutation(s) in the *TP5*3 gene and/or its pathway, most of which are missense mutations that account for loss of its tumor suppressor's function. In addition to its central role in cell cycle control, p53 plays major roles in regulating various vital cellular processes including maintaining genomic integrity, inducing DNA repair, apoptosis/autophagy, senescence as well as controlling stem cell self-renewal and cell fate (Solozobova & Blattner, 2011).

The human p53 protein is made of 393 amino acids that undergo alternative splicing leading to the formation of twelve different isoforms (Khoury & Bourdon, 2011). In mice, this short-lived protein is ubiquitously expressed during early embryogenesis until mid-gestation. After this time-point, p53 expression becomes restricted to specific tissues and cell types (Molchadsky, Rivlin, Brosh, Rotter, & Sarig, 2010), and is primarily regulated by post-translational modifications (PTMs), which include phosphorylation, acetylation, and methylation among other modifications. The transcriptional activity of p53 is regulated by Murine Double Minute-2 (Mdm2), which binds to N-terminal end of p53 leading to the blockage of the latter transcriptional activity (Meek & Anderson, 2009).

P53 regulates the level of transcriptional of various genes including those implicated in the control of cell fate, and self-renewal of tissue-specific stem cells such as

those of the mesenchymal and neuronal lineages (Meletis et al., 2006). The role of p53 has been widely investigated during kidney development. In the developing kidney, the full length p53 is only expressed in Six2+ nephron progenitor cells (Li et al. 2015). However, alternate isoforms of this protein may be expressed in other cellular lineages. Of note, the level of p53 expression changes during development whereby a fourfold decrease in its mRNA level was reported between E15.5 until adulthood.

Germline deletion in *p53* gene led to the formation of double ureters and renal hypoplasia along with a reduction in the metanephric mesenchyme in mice (Saifudeen et al., 2009). A similar duplex-ureter phenotype with an incomplete penetrance, was observed upon epithelial-specific deletion of *p53* using the Hoxb7-Cre system (Saifudeen et al., 2009). Studies have also shown that loss of p53 function in the Wolffian duct leads to hypersensitivity to low doses of GDNF, which caused increased activity of phosphatidylinositol-3 kinase and ectopic budding of the UB. In contrast, inhibition of p53 degradation through inhibition of its interaction with Mdm2, prevented budding despite the presence of high levels of GDNF. Thus, the above study showed that p53 plays an antagonistic role on the GDNF → c-Ret → PI3k pathway in the control of the outgrowth of the UB (Saifudeen et al. 2009).

On the other hand, deletion of p53 in the cap mesenchyme resulted in cellular disorganization and kidney hypoplasia. This phenotype was associated with a decrease in the number of immature nephrons and glomerular dysfunction (Li et al. 2015). Moreover, $Six2^{p53-/-}$ kidneys showed decreased levels of expression of the Neural Cell Adhesion Molecule (NCAM1), that is required for cell-to-cell and cell-to-matrix interactions, as well

as Pax2, a direct target gene of p53 (Saifudeen et al., 2009; Saifudeen et al., 2012). The same mice also displayed a decrease in stem/progenitor cell proliferation with no detectable change in the levels of apoptosis or senescence according to the same study (Li et al. 2015). The cellular disorganization and decreased cellular proliferation reported above were linked to downregulation in the levels of expression of various genes responsible for cell adhesion and migration such as collagens on the one hand, and genes controlling oxidative respiration and glucose metabolism on the other hand (Li et al., 2015). In summary, this study highlighted the presence of reduced metabolic fitness coupled to decreased self-renewal capacity in p53-deficient nephron stem/progenitor cells.

In vitro assays performed with isolated $Six2^{p53-/-}$ cells showed deficits in mesenchymal-to-epithelial transition and cell differentiation in the presence of inducers of the Wnt/β-catenin pathway (Li et al., 2015). These findings suggest that the nephron deficit observed in $Six2^{p53-/-}$ kidneys *in vivo* is likely linked to defects in ureteric bud branching, disruption of the microenvironment of the cap mesenchyme and/or impairment of the self-renewal of nephron progenitor cells.

Conditional deletion of Mdm2 from the epithelium of the UB has led to an expected overexpression of p53 with severe renal hypoplasia and perinatal lethality. $UB^{Mdm2-/-}$ kidneys also showed various defects in UB branching, reduction in cell proliferation, enhanced cell death and reduction in the expressions of various genes including *Wnt9b, Pax2* and *Lhx1*, which resulted in underdeveloped nephrogenic zone (Hilliard, Aboudehen, Yao, & El-Dahr, 2011). A similar phenotype was reported upon the deletion of Mdm2 in Six2+ cells; hence, the mutant cap mesenchyme had significantly less

Six2+ cells with reduced expression in various progenitor markers such as *Cited1, Pax2* and *Sall1* (Hilliard et al., 2011). Notably, the above phenotypes observed in $NPC^{Mdm2-/-}$ and $UB^{Mdm2-/-}$ mice could be rescued, and normal renal development could be partially restored by the deletion of p53 (El-Dahr, Hilliard, & Saifudeen, 2017).

In summary, the above studies have shown that inhibition of Mdm2 function resulted in constitutive activation of p53, which led to the depletion of nephron progenitor cells along with enhanced apoptosis. Therefore, the fine-tuning of p53 level of activity by Mdm2 is a critical requirement for normal kidney development. Moreover, p53 dysfunction resulted in depletion of the nephron progenitor cell population due to the disruption of the cellular metabolic fitness (in case of p53 loss), and reduction in cellular proliferation accompanied with an increase in apoptosis (in case of p53 overexpression).

H. Role of the Rb-E2F pathway during development

1. Role of Rb in the control of the G1-S phase checkpoint

The Retinoblastoma gene, *Rb*, encodes a universal cell cycle protein that controls the commitment of a cell to initiate DNA replication and therefore, divide. Rb manifests this regulatory role by controlling cell cycle progression at the G1-S phase restriction point through its interaction with members of the E2f family of transcription factors (Conklin, Baker, & Sage, 2012). *Rb* is the first tumor suppressor gene to be identified in human with various pediatric and adult tumors showing loss of function of this gene or the Rb pathway (T'ang, Varley, Chakraborty, Murphree, & Fung, 1988).

The Rb family of pocket proteins consists of three members: pRb (or p105), p103 (or Rb2) and p107 (or Rb1) (Graña, Garriga, & Mayol, 1998). Despite the homology of the

pocket domains found in these proteins, they manifest unique binding affinities to distinct E2Fs (Trimarchi & Lees, 2002). Hence, pRb binds to E2Fs 1 through 5, while p103 and p107 primarily interact with E2Fs 4 and 5. This differential binding affinity allows for the sequential regulation of cell cycle progression where p130-E2F4/5 complexes act in G0 phase, p107-E2F4 in G1, and pRb-E2F4 during S phase (Dimova & Dyson, 2005).

Rb is inactivated by cyclin-dependent kinases and cyclin complexes that alter the phosphorylation status of Rb. Thus, CDK4/6-cyclin D complex binds to Rb and phosphorylates it. CDK2-cyclin E complex further phosphorylates Rb, thus forming hyper-phosphorylated inactive form of Rb (Kitagawa et al. 1996). On the other hand, when hypo-phosphorylated, Rb remains active and interacts with E2F1-3s (transcriptional activators) to inhibit their transcriptional activity and block the G1-S phase transition. Moreover, activation of p53 mediates the inhibition of the Cdk2-cyclinE complex by p21, thus stabilizing Rb in its active hypo-phosphorylated form to block S phase entry (Sherr & McCormick, 2002).

Loss of Rb is generally associated with increased cell proliferation, uncontrolled cell division, which often leads to cell transformation and progression to a cancer state in many tissues (Burkhart & Sage, 2008). However, various studies have also highlighted additional roles for Rb in the control of stem/progenitor cells development that extend beyond cell cycle control. For example, deletion of Rb in muscle satellite cells led to defective cell differentiation in addition to cell cycle re-entry and expanded cell proliferation (Conklin et al., 2012). Loss of Rb in the developing retina resulted in defective differentiation of cholinergic neurons (D. Chen et al., 2004). During brain development, the

Rb-E2F pathway was shown to directly control transition into neuronal differentiation via the modulation of the *Dlx1-2* gene expression in the telencephalon (Ghanem et al., 2012). Moreover, deletion of Rb caused ectopic proliferation of cortical neuroblasts and immature olfactory sensory neurons as well as blocked neuroblast's migration and increased apoptosis (Ghanem et al., 2012; Jaafar et al., 2016); (Ferguson et al., 2002); (Andrusiak, Vandenbosch, Park, & Slack, 2012).

Hence, in addition to its role in cell cycle progression, the Rb-E2f pathway is essential in the regulation of progenitor differentiation, maturation and/or survival in various cell lineages of different tissues. However, the role of Rb during kidney development was recently investigated for the first time in our laboratory.

2. Role of Rb during kidney development in vivo

During kidney development, the expression levels of CDKs and cyclins vary with time (S. K. Park et al., 1997), and are also found dysregulated in various renal diseases (Thomasova & Anders, 2015). Moreover, one study has shown a modification in the phosphorylation status of Rb during proliferation of mesangial and tubular cells under normal and pathological conditions (Shankland, 1997). Besides these few reports, the role of Rb and other cell cycle regulators during renal development remained largely unknown.

Over the past four years, our laboratory has investigated and reported for the first time a critical requirement for Rb during kidney development. A study conducted by Jaafar et al. in the laboratory has recently demonstrated that Rb negatively controls NPCs proliferation, and is essential for nephron survival around birth (Jaafar C, Hammoura I and Ghanem N; unpublished data). An inducible *Rb* deletion was generated during mid-

gestation to induce loss of Rb in Nestin-positive renal progenitors and their progeny. Compared with Rb littermate controls, Rb mutant mice showed increased and ectopic proliferation of (Cited1-; Six2+) progenitor population, but no major change(s) in self-renewal of (Cited1+; Six2+) stem cells, or their subsequent differentiation into a nephron lineage. However, this enhanced nephrogenesis was accompanied with a severe compromise in cell survival inside the tubule epithelia of immature nephrons at late developmental stages. Moreover, combined deletions of both *Rb* and *p53* exacerbated the survival defects observed in Rb mutant embryos and was manifested by a more severe renal hypoplasia with almost complete loss of immature glomeruli at birth (Jaafar C, Hammoura I and Ghanem N; unpublished data).

In summary, this is the first study to highlight a critical requirement for Rb during kidney development, and the presence of a crosstalk between the Rb and p53 pathways in this context, particularly with respect to renal cell growth and survival.

E. Rationale, Hypothesis and Specific aims

In a previous work of our lab, in an attempt to study the role of Rb, and combined roles of Rb and p53 in kidney development, we have induced Nestin-Cre-ERT2-YFP specific deletion of *Rb* and/or *p53* by administering tamoxifen to pregnant Nestin-CreERT2-YFP; $Rb^{fl/fl}$ and/or $p53^{fl/fl}$ females at E10.5. Embryonic kidneys were collected and analyzed in single and double mutant embryos, as well as wild type and heterozygote controls at distinct developmental stages. Histological and gene expression analyses showed that loss of Rb caused increased renal progenitor proliferation and extensive BrdU

incorporation inside the nephrogenic zone as well as in the medullary region of the kidney at E15.5. This, in turn, led to enhanced nephrogenesis that was offset by massive cell death inside the tubular epithelium around birth. Loss of p53 resulted in massive renal hypoplasia and clear enlargement of Bowman's capsule, which is consistent with previous literature (Saifudeen et al., 2012). Moreover, combined deletion of *Rb* and *p53* further exacerbates the observed defects by causing a more severe reduction in kidney size, paucity of immature glomeruli, loss of normal tubular structure and increased cell death around birth.

Given all of the above data, we **have hypothesized** that Rb actively controls the developmental properties of renal stem and progenitor cells to ensure a proper level of nephrogenesis and is needed to maintain survival of nephron lineage. To test this hypothesis, we have aimed in this project to assess and compare how Rb and p53 regulate renal stem/progenitor cells' properties *in vitro* and have set **two aims**. The first aim is to investigate the role of Rb in the control of the proliferation of renal stem/progenitor cells' *in vitro* using a well-established experimental protocol, which is based on isolating and culturing these cells from the cap mesenchyme following loss of Rb *in vivo*. The second aim is to investigate how p53, in turn, regulates renal stem/progenitor cells' proliferation using the same cell culture system.

After 2-3 days in primary culture, we fixed and stained renal stem/progenitor cells by immunohistochemistry using specific markers to assess cell proliferation (BrdU, Ki67), and commitment to renal lineage(s) including the nephron lineage (Cited-1 and Six2). We also assessed cell density, shape and survival. Phenotypic analyses were complemented by cells counts and statistical analyses. The observed phenotype(s) in Rb and p53 single

knock-out cultures were compared to those found in wild type and/or Rb heterozygote controls.

CHAPTER II

MATERIALS AND METHODS

A. Generation of Rb and p53 conditional knockout Mice

All animal procedures including animal handling, maintenance and treatments were performed according to protocols approved by the "the Institutional Animal Care and Use Committee" –IACUC- at AUB.

Nestin-CreERT2/Rosa26R-YFP/YFP mice (designed by Lagace et al., 2007) were mated with Rb floxed/floxed (fl/fl) (Marino et al., 2000) and p53 floxed/floxed $^{(fl/fl)}$ mice (The Jackson Laboratory, B6.129P2-*Trp53*^{tm1Brn}/J) to generate conditional Rb and p53 deletions, respectively. Crosses were designed to obtain mixed litters of $Rb^{fl/fl}$ and $Rb^{fl/+}$ embryos carrying the Nestin-CreERT2 cassette, thereafter referred to as Rb-/- (Rb conditional knock-out, cKO) and Rb+/- (Rb heterozygotes littermate controls), or lacking the Nestin-Cre cassette and called Rb+/+ (wild type littermate controls). Similarly, p53 crosses were designed to obtain mixed litters of $p53^{fl/fl}$ and $p53^{fl/+}$ embryos carrying the Nestin-CreERT2 cassette thereafter referred to as p53-/- (p53 conditional knock-out, cKO), or lacking the Nestin-Cre cassette and called p53+/+ (wild type littermate controls). $Rb^{fl/fl}$ females were bred and maintained on a mixed FVBN- C57/Bl6 genetic background, while the $p53^{fl/fl}$ females carried the Sv/126 background. The Rb floxed allele has exon 19 flanked by two LoxP sites (Cre-specific sites) (Marino et al., 2000). p53 floxed mice have LoxP sites flanking exons 2-10 (The Jackson Laboratory, B6.129P2-*Trp53*^{tm1Brn}/J).

The inducible Nestin-CreERT2-YFP cassette is comprised of the Nestin promoter combined with exons 1-3 of the Nestin gene, which controls the expression of the Cre recombinase enzyme fused with mutated estrogen receptors (ERT2). Plus, the YFP reporter gene separated by a stop codon. Upon treatment with tamoxifen, an estrogen analog, tamoxifen binds to ERT2 leading to the translocation of Cre to the nucleus where it excises the genes flanked by two LoxP sites including the Rb or p53 floxed alleles, and the stop codon located ahead of the YFP reporter gene.

A single dose of freshly prepared Tamoxifen was administered by oral gavage to pregnant females at E10.5 (180mg Tamoxifen/1kg mouse of body weight) with E0.5 corresponding to the day when a vaginal plug was observed. Tamoxifen Citrate (abcam cat# ab120656) was prepared at a final concentration of 44.8 mg/ml in 60% sunflower oil and 40% DMSO. Animals were anesthetized by a mixture of 1.5µl/g Ketamine and 0.25µl/g Xylazine followed by cervical dislocation. Embryos were harvested at E17.5. Kidneys were dissected out and placed in HBSS until genotyping, then processed for cell culture protocol.

B. Genotyping

DNA extraction was performed using Sodium Chloride-Tris-EDTA (STE) buffer prepared from 0.1M NaCl, 10 mM Tris-HCl pH=8 and 1mM EDTA pH=8. Embryonic tail pieces were placed in an Eppendorf tube and homogenized using a piston. Then, 100 µL of STE buffer was added for each tailpiece and samples placed on a hotplate incubator at 95°C for 15 minutes. They were centrifuged for 5 minutes at room temperature at 14500 rpm.

The supernatant including the extracted the DNA was quantified using NanoDrop™ 2000/2000c Spectrophotometer and ~2-3 µl were used for screening by PCR.

Proof of recombination was performed on primary renal cells after 2 days in culture. DNA was extracted from scraped renal progenitor cells and suspended in an Eppendorf tube with 70µl of H2O. 500µl lysis buffer and 10µl Proteinase K were added to the suspension, which was then placed on a hotplate incubator at 55°C overnight. The next day, phenol chloroform (1:1 ratio) was added to the lysed cells. After centrifugation, the aqueous layer was removed. Then, 1µl glycogen, 48 µl of Sodium Acetate (5M) and 1000µl of 100% cold ethanol were added and mixed sequentially with this layer. The tubes were then placed at -80°C for 30 minutes, then centrifuged at maximum speed and the pellet washed with 70% cold ethanol. The final DNA pellet was suspended and allowed to homogenize in 10µm Tris solution pH=8.

Screening for the Rb wt, floxed and recombined alleles was done using Rb-18 forward primer 5'GGCGTGTGCCATCAATG 3' and Rb-19 reverse primer 5'AACTCAAGGGAGACCTG 3'.

Screening for p53 floxed versus wt alleles was done using p53 floxed primers T008 5'CACAAAAACAGGTTAAACCCA 3' and T009 5'AGCACATAGGAGGCAGAGAC 3'.

To screen for recombined p53 allele(s) we used T008 and p53-intro-rev primer 5'GAAGACAGAAAAGGGCCA 3'.

To screen for the Nestin-Cre transgene, we used Cre-T2A primer forward 5' ATTTGCCTGCATTAC CGGTC 3' and Cre-T2B primer reverse: 5' ATCAACGTTTTCTTTTCG G 3'.

To screen for R26R-YFP alleles we used as forward primers 5'AAAGTCGCTCTGAGTTGTTAT 3', and 5' GCGAAGAGT TTGTCCTCAACC 3', and reverse primer 5' GGAGCGGGAGAAATGGATATG 3'.

C. Isolation and culture of renal progenitor cells from the nephrogenic zone

The isolation and culture of renal progenitor cells from the nephrogenic zone of the embryonic mouse kidney was done using a protocol adopted from Brown et al. (A. C. Brown et al., 2011). After sacrifice of the pregnant females, embryos were removed and placed in DPBS with calcium and magnesium (Sigma). Kidneys were dissected and pooled according to their genotype in HBSS (Sigma). HBSS was then removed and replaced with 1.5 ml of 0.25% collagenase A (Roche Group, cat# 10103578 001) and 1% Pancreatin (Sigma) digestion solution. Tubes containing the enzyme/kidney digest were then placed on a nutator (with gentle shaking) at 37°C for 15min. Next, the tubes were removed and 75 µl of 10% FBS (Sigma 14A173) diluted in DMEMF12 was added. 1.4 ml of each digest was transferred to a new tube to which 3µl of Dnase (1U/ml; Thermo-scientific) was added, leaving the kidneys behind. The tubes were then placed on a nutator at 37°C for 10 min. The mix was the centrifuged for 5 minutes at 1648 rpm. The supernatant was removed, and the pellet re-suspended in 1ml of 5% FBS:HBSS mix by gentle pipetting 6 times. This was followed by another centrifugation at 1648 rpm and elimination of the supernatant. Finally, the pellet was re-suspended with 500µl KSFM (GIBCO, by Life Technologies) and the

suspension filtered twice. Cells were counted and plated on fibronectin-coated (BD Biosciences) culture plates at a density of 300,000 cells per well. Fibronectin was added at a concentration of 5mg per cm^2. Cells were incubated at 37°C in a humidified incubator with 5% CO2. After day 2 or day 3 in culture, cells were treated for 2 hours with BrdU solution added at 0.02mg/ml (Sigma B5002- 250MG) and prepared using 0.9% NaCl and 1N NaOH. Finally, cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes at 4°C, washed in 1x PBS and stored at 4C for analysis later.

D. Immunocytochemistry

After fixation, cells were incubated for 1 hour in blocking solution (BS) [1% BSA (amresco 0332-25G), 0.3% Triton X, 5% donkey serum in 0.1M PBS]. Primary antibodies were added to each well and left overnight at 4C. The following day, three rounds of 10 min gentle washes were done in cold 1xPBS; this was followed by 1-hour incubation in secondary antibodies (diluted at 1:400 in BS) and Hoescht stain (diluted at 1:10000 in BS). Finally, cells were gently washed for 3 consecutive 10min washes in cold 1xPBS. Mounting with cover slips was done using Thermo Scientific™ Shandon™ Immu-Mount™ (cat# 9990402).

We used the following primary antibodies: chicken anti-GFP (1:1000; ab13970), chicken anti-Nestin (1:300; ab134017), rabbit anti-ki67 (1:500, Cell Marque SP6), rabbit anti-Six2 (1:300; Proteintech 11562-1-AP), rabbit anti-Cited1 (1:200, Thermo-fisher), and mouse anti-Brdu (1:500, Santa Cruz Biotechnology). The following secondary antibodies (from Jackson Immunoresearch) were used: Alexa Fluor donkey anti-mouse 596, donkey

anti-rabbit Cy3, donkey anti-rat Cy5, Alexa Fluor donkey anti-rabbit 488, Alexa fluor donkey anti-chicken 488, and donkey anti-chicken Cy3.

E. Imaging and cell counts

Fluorescent images were visualized and acquired using the Upright Fluorescence Microscope by Leica (DM6B) at the Central Research Science Laboratory (CRSL) facility at AUB. Cell counts were performed using ImageJ software from a tile scan image of the entire coverslip taken at x10 magnification. ImageJ was also used for picture analyses and overlapping. Statistical analysis was done using t- tests and ANOVA tests in excel.

CHAPTER III

RESULTS

A. Isolation and culture of nephron stem/progenitor cells (NSPCs) from the embryonic nephrogenic zone

To investigate a potential role for Rb and p53 in renal stem/progenitor cells' development *in vitro,* we generated Nestin-CreERT2-YFP; $Rb^{fl/fl}$ and Nestin-CreERT2-YFP; $p53^{fl/fl}$ embryos by crossing Nestin-CreERT2-YFP/YFP mice with $Rb^{fl/fl}$ and $p53^{fl/fl}$ mice, respectively (Lagace et al. 2007; Marino et al. 2003). We induced the deletion of *Rb* or *p53* by single tamoxifen treatments administered to pregnant females by oral gavage at E10.5. Crosses were designed to obtain mixed litters of $Rb^{fl/fl}$ and $Rb^{fl/+}$ littermate embryos carrying the Nestin-CreERT2-YFP cassette, thereafter referred to as Rb-/- (Rb conditional knock-out cKO) and Rb+/- (Rb heterozygotes littermate controls), or lacking the Nestin-Cre cassette and called Rb+/+ (wild type littermate controls). $p53^{fl/fl}$ embryos possessing the Nestin-CreERT2-YFP cassette were referred to as p53-/- (p53 conditional knock-out cKO) and those lacking it as p53+/+ (wild type littermate controls). Embryos were harvested at E17.5 and the kidneys were dissected and pooled according to their genotype. Using a protocol adapted from Brown et al. 2011, we isolated and cultured renal stem/progenitor cells derived from the nephrogenic zone of the embryonic kidneys (refer to material and methods for experimental detail) (**Figures 1 and 2**). Cells were plated with the same cellular density of 300,000 cells per well. 50-72% of cultured cells expressed Nestin while 44-56% were GFP-positive across different genotypes, indicating successful isolation and

Cre recombination of Nestin-derived progeny (**Table 1**). Moreover, around 61.8-69% of the total cells expressed Cited1 whereas 85-90.3% expressed Six2 across different genotypes, indicating that the vast majority of isolated cells in our primary culture are nephron stem and/or progenitor cells that originate from the cap mesenchyme lineage.

B. Proof of Cre recombination and Rb-p53 gene deletion in primary cultures of NSPCs

Nestin is a cytoskeleton associated class VI intermediate filament that is known as a cellular marker for neural stem and progenitor cells. It was also shown to be expressed in the developing and adult kidneys (Dhaliwal and Lagace 2011; Dubois et al. 2006; J. Chen et al. 2006). In fact, cell lineage studies revealed Nestin expression in the metanephric mesenchyme but not the ureteric bud. Moreover, Nestin was found to be co-expressed with Cited1- and Six2-positive cells as early as E11.5, and detected in the vascular cleft of the S shape body and capillaries of immature glomeruli specifically in CD31+ and Flk1+ endothelial cells (J. Chen et al., 2006).

Our *in vitro* results from primary cultures of NSPCs derived from the Nestin-CreERT2-YFP line were consistent with the above studies. Accordingly, we detected high overlap between Nestin and GFP expressions in isolated renal cells; hence, the percentages of recombined cells [characterized by the number of (Nestin;GFP) double positive cells divided by the total number of Nestin-positive cells] were 78.9 -81% and 77.8-78% in Rb-/- and p53-/- cultures, respectively.

To verify whether Cre recombination was successful, PCR screening was performed on genomic DNA extracted from cultured cells at day 2 (for p53 screening), and,

gDNA derived from the brain tissues of embryos whose kidneys were used for primary cultures (for Rb screening). Specific pairs of primers were used to amplify the recombined band for each PCR screening (refer to material and methods for detail). One strong recombined band was detected in each screening, confirming a successful Cre recombination and cleavage of the Rb and p53 floxed alleles (**Figure 3;** 3A; a recombined band of 320bp for the Rb-PCR, and, 3B; 612bp band for p53-PCR).
Moreover, we detected a significant decrease in Rb protein expression by Western blot analysis that was performed on E15.5 protein lysates derived from whole embryonic kidneys treated at E10.5 (data not shown).

The above results showed that we have successfully used the Nestin-CreERT2-YFP line to induce Rb-p53 gene deletion and derive primary renal cell cultures that are enriched in NSPCs (cap mesenchyme lineage) during kidney development.

C. Enhanced proliferation of renal stem/progenitor cells *in vitro* following loss of Rb *vitro*

To investigate the role of Rb in the control of renal stem/progenitor cells' properties *in vitro,* we performed a series of histological experiments on Rb+/+, Rb+/- and/or Rb-/- primary cultures derived from the nephrogenic zone after 2 and 3 days in culture as described earlier (**Figure 2**). All cells were plated at equal cellular density of 300,000. Our results showed a significantly higher cellular density in Rb-/- cultures compared Rb+/+ and Rb-/+ cultures at day 2 and day 3, with no major difference in cell morphology among the three cultures. Hence, at day 2, the average total number of cells

was 86 591.5 ± 22 576.5 in Rb-/- cultures versus 35 946.5 ± 5364.1 and 26 893.8 ± 7796.3 in Rb+/- and Rb+/+, respectively (p= 2.2691 x 10^{-6} for Rb-/- vs Rb-/+, and p= 7.76778 x 10^{-7} for Rb-/-, Rb+/+, n=2 cultures) (**Figure 4I, A-A'' and, Graph 1a**; Hoechst counts). There was no significance difference in cell density between the two control groups. Notably, Rb-/- cells frequently formed aggregates compared with the other genotypes (**Figure 4I,** black arrows in Rb-/- panel). Consistent with the increased cell density in the absence of Rb, we detected significant increase in the number of Nestin+ cells in Rb-/- compared to Rb-/+ and Rb+/+ (Rb-/-: 61 269 ± 4772.8, Rb+/-: 35 946.5 ± 5364.1, Rb +/+: 16497.5± 3193.5, p (Rb-/-, Rb-/+) = 0.000571597, p (Rb-/-, Rb+/+) = 0.000277286, n=2 cultures) (**Figure 4I, B-B'' and Graph 1a**).

Immunostaining for Ki67, a general proliferation marker, revealed that there is significant increase in renal cell proliferation upon loss of Rb compared with Rb+/- and Rb+/+ cultures (Rb-/-: 49936.3 ± 16294.4, Rb+/-: 17774 ± 325, Rb +/+:13606.3 ± 1662.9, p (Rb-/-, Rb-/+) = 0.032371959, p (Rb-/-, Rb+/+) = 0.006744547, n= 2 cultures) (**Figure 4I, C-C'' and Graph 1a**). This increase was further confirmed with BrdU incorporation (an S-phase marker) in Rb-/- versus Rb+/+ cultures at day 2 (Rb-/-: 325 ± 70 vs Rb +/+: 81.5 ± 6.5, p(Rb-/-, Rb+/+) = 0.037098175, n= 2 cultures) (**Graph 1b**). Remarkably, we did not find any significant difference in the proliferation ratio [ratio = the number of (Nestin+; Ki67+) cells over the total number of Nestin+ cells] between Rb-/- and Rb+/- cultures (Rb-/-: 0.63 ± 0.058 vs Rb +/-: 0.54 ± 0.13, p(Rb-/-, Rb+/-) = 0.285208567, n= 2 cultures) (**Graph 1c**), indicating that loss of Rb leads to a proportional increase in cell proliferation. Similar results were obtained at day 3 in culture (**Graph 2**).

To assess whether the increase in cell division affected the population of NSPCs, we immuno-stained for Cited1 and Six2. Our results showed that the number of cells expressing each marker alone as well as those co-expressing each marker with GFP were significantly higher in Rb-/- cultures compared with heterozygote littermate controls (**Figure 5).** Hence, the total number of Cited1+ cells were Rb-/- 54936 ± 14495.5 vs Rb+/- 16 445± 2377.5, Rb +/+: 11690, p (Rb-/-, Rb-/+) = 0.031869163, n=2 cultures. (**Figure 5C-C'** and g**raph 3**), and, Six2+ cells were 78 548 in Rb-/- vs 35 716 in Rb+/-, n=1 culture (**Figure 5H-H' and graph 3**).

These results indicate that Rb negatively controls proliferation of renal cells including NSPCs in primary cultures; however, whether this regulation affects stem cells or progenitors or both types remains unknown. Moreover, these results are consistent with our recent in *vivo* work showing enhanced and proportional proliferation inside the nephrogenic zone following loss of Rb (Jaafar C and Ghanem N unpublished data). Future work should aim at investigating whether NSPCs can exit properly the cell cycle and differentiate into immature nephron structures in culture, and importantly, examine whether Rb is required for their survival as seen *in vivo*.

D. Loss of p53 negatively affects growth and proliferation of renal stem/progenitor cells *in vitro*

To study the role of p53 in the control of renal stem/progenitor cells' properties *in vitro,* we used the same experimental approach to isolate and culture renal cells derived from the cap mesenchyme following loss of *p53 in vivo.* While we did not detect any major

change in cell morphology (data not shown), we noted a significant reduction in the overall cellular density in p53-/- cultures compared with p53+/+ cultures after 2 and 3 days in culture. Hence, at 2 days, the total number of Hoechst+ cells in p53-/- cultures was unexpectedly lower by ~3 folds compared to p53+/+ cultures (p53+/+: 95529 ± 5245.1 vs p53-/-: 34710.8 ± 2677.2, p= 7.44663 x 10^{-5}, n=3 wells) (**Figure 6A-A' and Graph 4a**). This reduction was paralleled by significant decreases in the total number of Nestin+ cells (p53+/+: 24 395.7 ± 7422.6, vs p53-/-: 5 581.3 ± 2677.2, p = 0.002513443, n=3 wells) (**Graph 4a, Figure 6B-B'**) as well as Ki67+ cells (Figure 6C-C' and graph 4a; p53+/+: 60138 vs p53-/-: 26647), and, Cited1-expressing cells (**Figure 7C-C' and graph 5;** p53+/+: 55027 vs p53-/-: 27991) in the absence of p53.

Similar results were obtained at day 3 in culture (**Figure 8, Graphs 6 and 7**). This is in addition to a pronounced reduction in the total number of Six2+ (p53+/+: 38 473 vs p53-/-: 19 524) (**Figure 8E-H' and Graph 7**), and reduced BrdU incorporation (**Graph 6b;** p53+/+: 110.5 ± 12.5 vs p53-/-: 59 ± 23, p = 0.039499, n=3 wells) in p53 null cultures compared with WT cultures at day 3. Despite the remarkable decreased in cell density in the absence of p53, there was no significant change in the proliferative ratio between the two genotypes (**Graph 6c**; p53+/+: 0.00201± 0.0003 vs p53-/-: 0.00224 ± 0.00016, p = 0.299600175, n= 3 wells).

Given that p53 is a tumor suppressor gene, the above data is counterintuitive and therefore, suggest that p53 loss does not seem to directly affect cell growth and proliferation. Rather, p53 may be needed for cell survival (as seen with our *in vivo* data) and/or metabolic fitness as shown in previous studies (Saifudeen et al. 2012; Y. Li et al.

2015). In fact, our culture results are consistent with the in *vivo* work conducted in our lab and showing severe renal hypoplasia, massive cell death but no significant difference in the rate of proliferation inside the nephrogenic zone in p53-/- embryos compared with littermate Wt controls (Jaafar C and Ghanem N, unpublished data). Similarly, our data is consistent with a previous study where *in vivo* deletion of p53 using the Six2-Cre driver led to the formation of a less compact cap mesenchyme and 30% decrease in the number Six2+ cells with premature depletion of Cited1+ cells at E14.5-15.5 (Li et al., 2015).

Altogether, our *in vitro* data clearly indicate that loss of p53 negatively affects renal stem/progenitor cell growth and proliferation. While the mechanisms mediating this effect remain unknown, they are likely to be linked to survival defects and/or decreased metabolic fitness in the absence of p53. Future studies should aim at examining these possibilities.

FIGURES

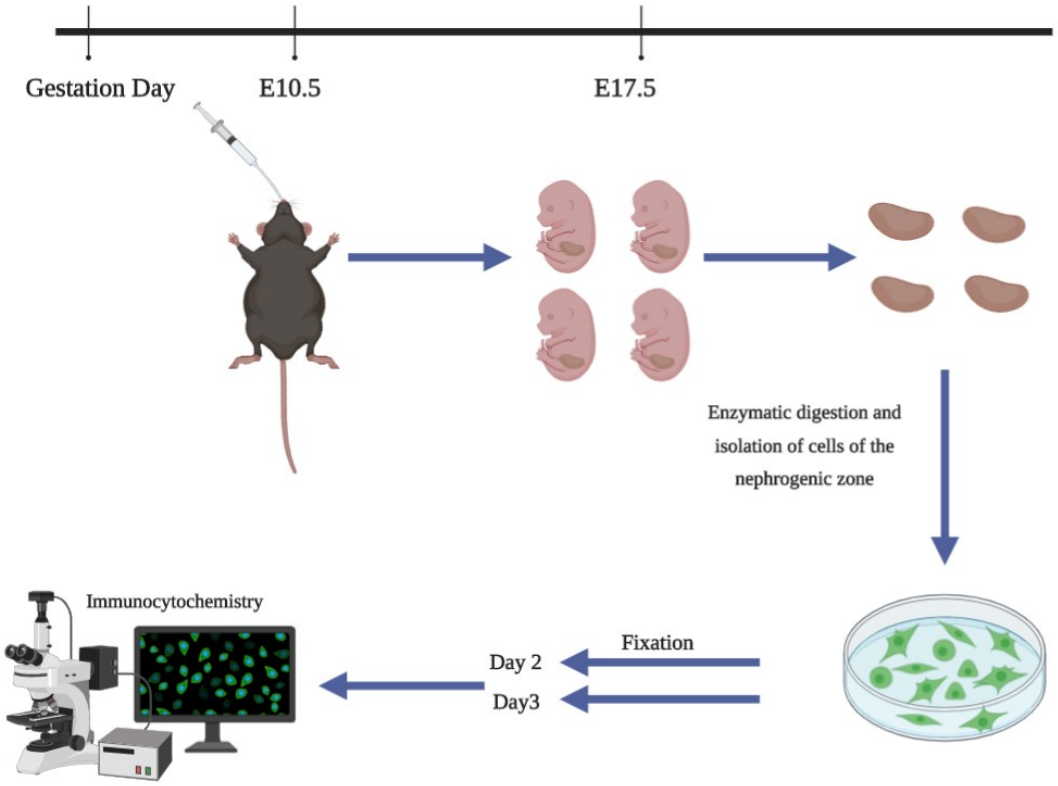


Figure 1. Isolation and culture of cells from the nephrogenic zone
Illustration showing the experimental steps followed to isolate and culture renal stem and progenitor cells using a protocol developed by Brown et al. 2011. *Rb* or *p53* deletion was induced by single tamoxifen treatments (by oral gavage) administered to pregnant Nestin-CreERT2-YFP; $Rb^{fl/fl}$ or $p53^{fl/fl}$ females at E10.5. Embryos were harvested at E17.5 and the kidneys dissected and pooled according to their genotype. Mild enzymatic digestion was performed to isolate renal cells from the nephrogenic zones, then cells were purified and plated (see figure 2 for culture detail), fixed and immuno-stained after 2 and 3 days in culture.

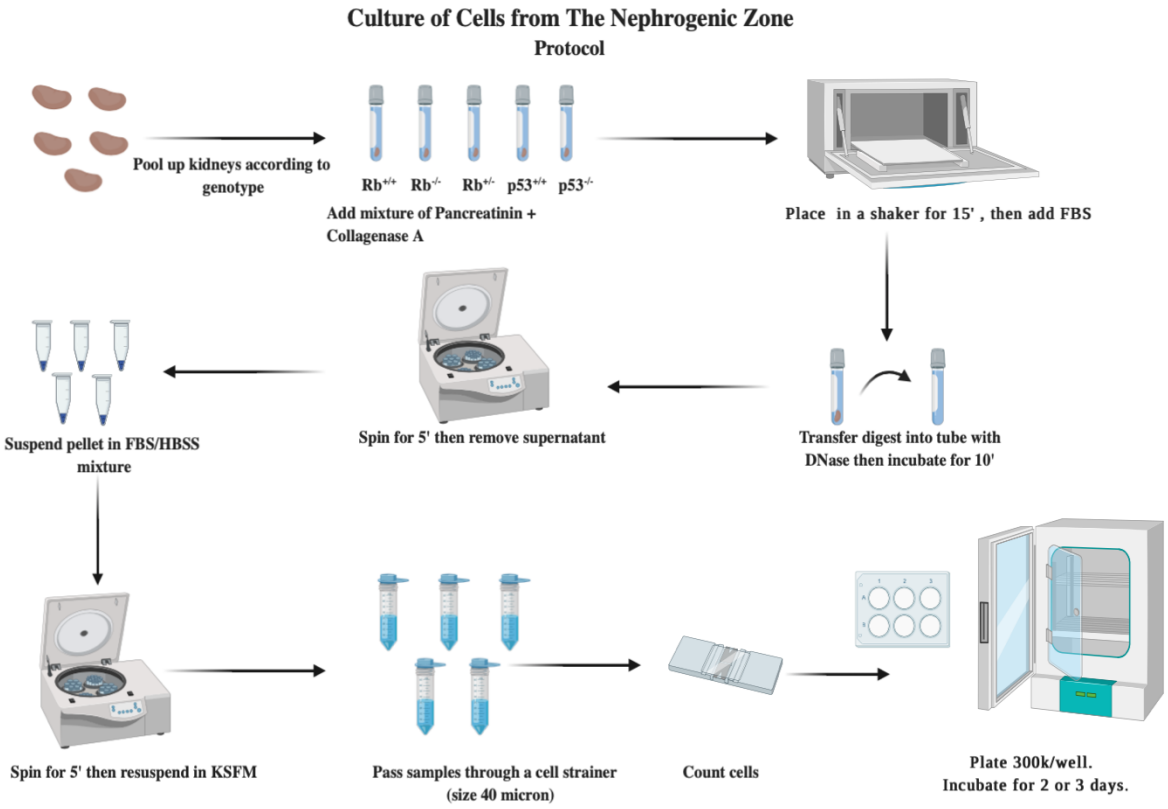
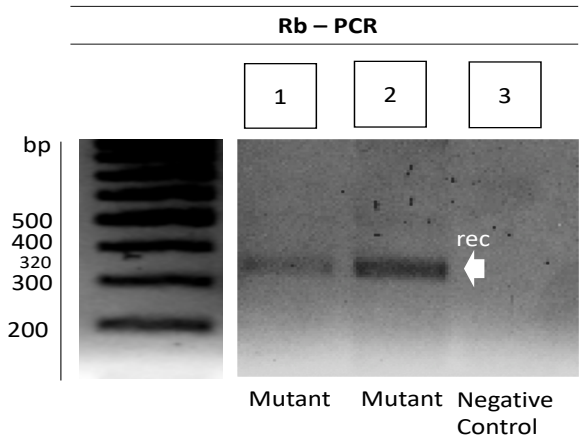


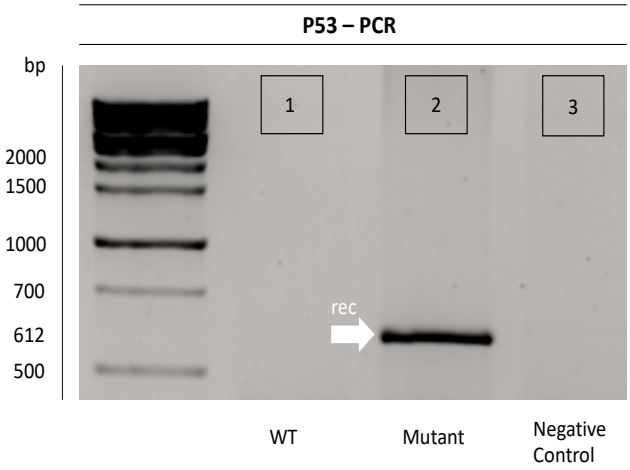
Figure 2. Culture of renal stem and progenitor cells isolated from the embryonic nephrogenic zone Depiction of the experimental steps performed to isolate, purify and plate renal cells from the nephrogenic zones following loss of Rb or p53 in vivo. The culture protocol was adopted from Brown et al. (A. C. Brown et al., 2011).

Percentages of Nestin+ cells and GFP+ cells of the total number of isolated cap mesenchymal cells					
	Rb +/+	Rb+/-	Rb-/-	p53+/+	P53-/-
% Nestin^{+} cells	50	65	69	58	72
% GFP^{+} cells	NA	56	55	NA	44

Table 1. Table showing the percentage of Nestin+ cells and GFP+ cells of the total number of isolated cap mesenchymal cells in various genotypes and ranging between 50-72% and 44-56%, respectively.

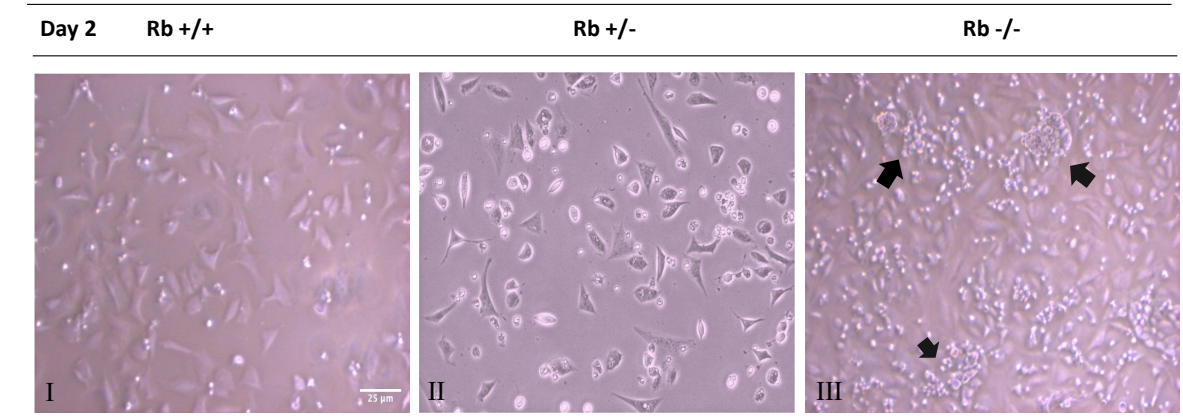


Panel A:

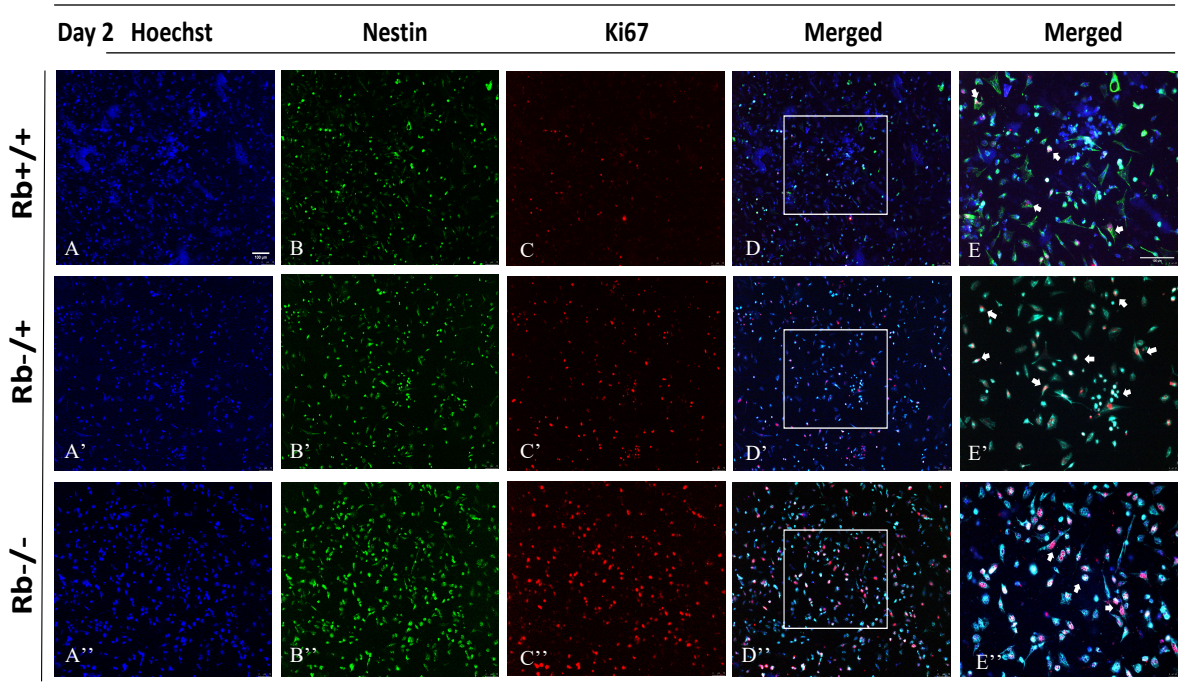


Panel B:

Figure 3. Proof of Cre recombination and deletion of Rb and p53 genes
(**A**) Proof of Cre recombination and Rb deletion: PCR analysis was done on genomic DNA extracted from the brains of the dissected embryos whose kidneys were cultured at E17.5. Lanes 1 and 2; two different Rb-/- samples showing a recombined band at 320bp, and lane 3; negative control (water). (**B**) Proof of Cre recombination and p53 deletion; PCR analysis was performed on genomic DNA (gDNA) extracted from isolated cells of the cap mesenchyme after 2 days in culture. Lane 1: p53+/+ sample, lane 2; p53-/- sample showing a recombined band at 612bp and lane 3; negative control (water).



I



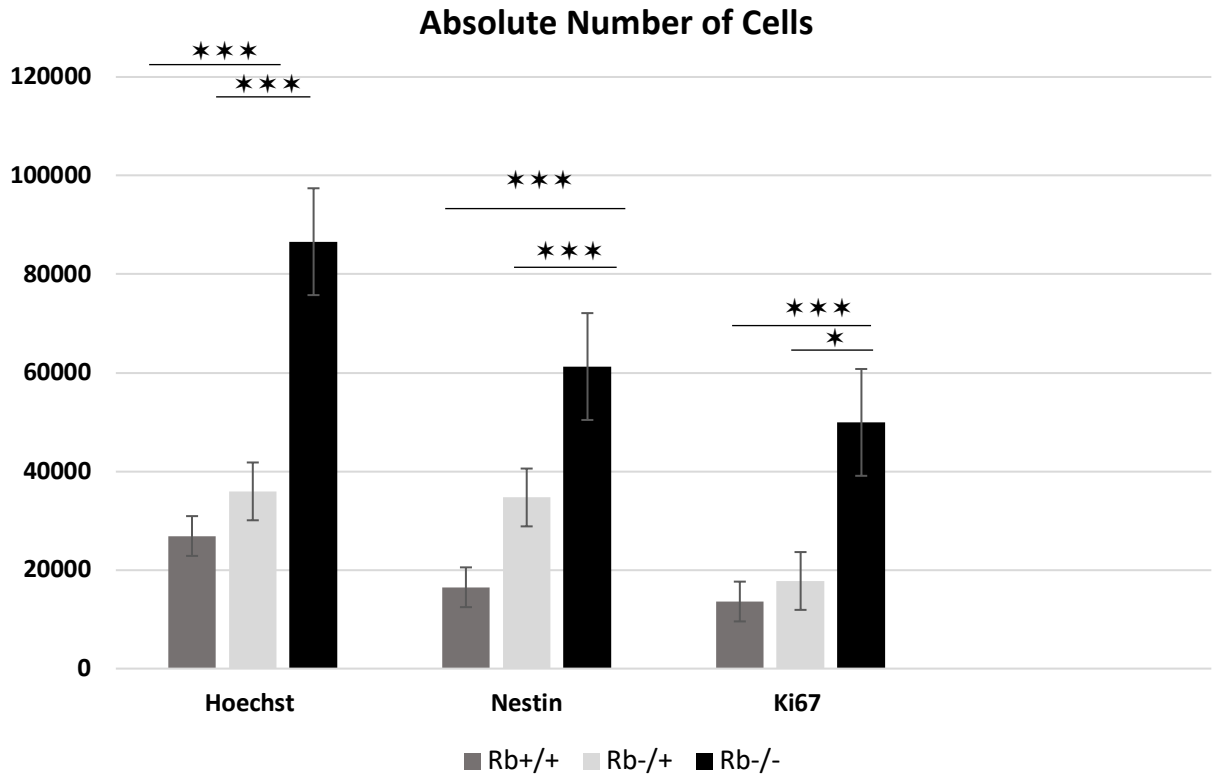
II

Figure 4. Rb negatively controls proliferation of renal stem/progenitor cells *in vitro*.

(I) Bright field images of renal stem/progenitor cells isolated from the nephrogenic zones at day 2 in culture showing similar morphology but higher density in Rb-/- culture compared to Rb+/- and Rb+/+ cultures. Note that Rb-/- cells tend to form aggregates compared with controls (black arrows). Scale bar = 25 μm.

(II) Double immunostaining was performed on isolated renal cells using antibodies against Nestin (green; B-B'') and ki67 (red; C-C'') in Rb+/+ (A-E), Rb+/- (A'-E') and Rb-/- (A''-E'') cultures. (E-E'') higher magnification pictures showing the overlap between Nestin and ki67 expressions (white solid arrows). Note the

presence of significantly high numbers of Nestin+ and Ki67+ cells in the absence of Rb compared with both controls. Scale bars (A-D” = 75μm; E-E’’=100μm).


Absolute Number of Cells
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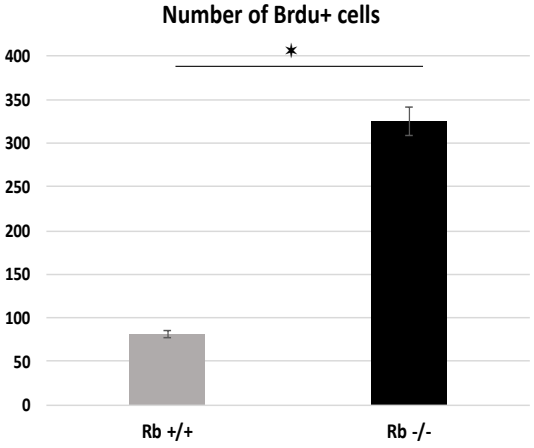
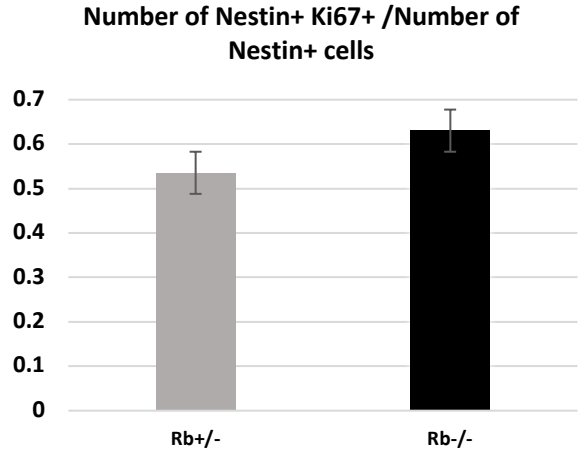
100000
80000
60000
40000
20000
0
Hoechst
Nestin
Ki67
Rb+/+
Rb-/+
Rb-/-

1a

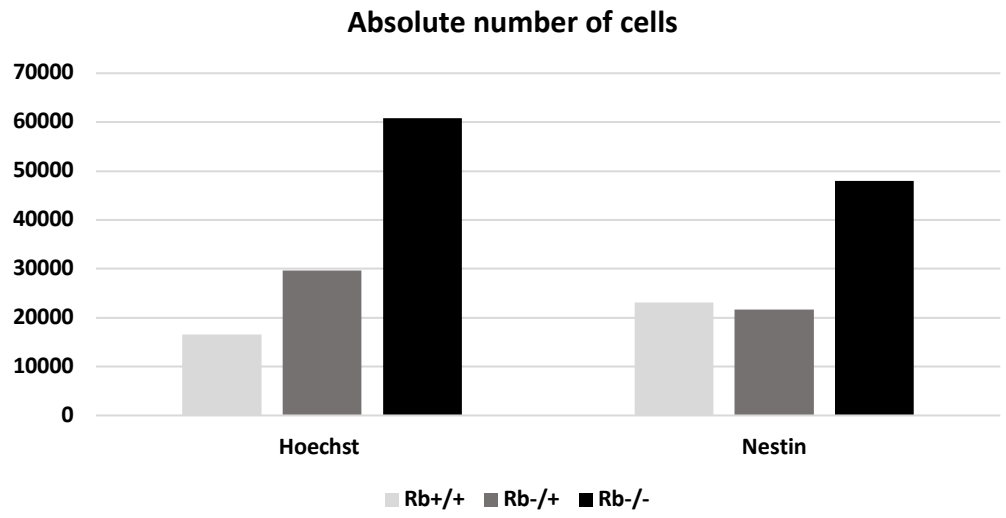

Number of Brdu+ cells
400
350
300
250
200
150
100
50
0
Rb +/+
Rb -/-

1b

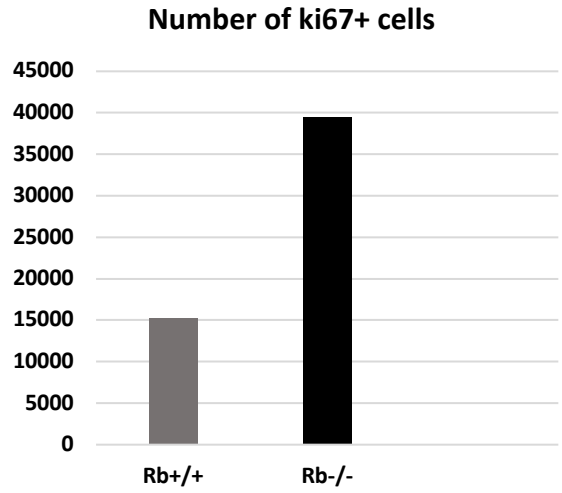


1c

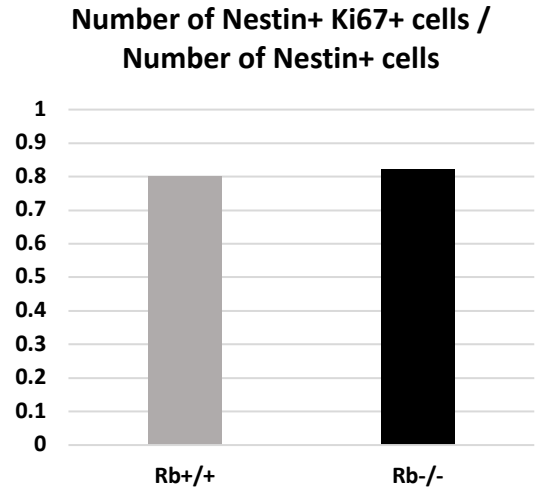
Graph1. Quantification of the total numbers of renal cells including BrdU+, Nestin+ and Ki67+ cells in Rb+/+, Rb+/- and Rb-/- cultures after 2 days *in vitro*.
Results showed that, compared with wild type and heterozygous controls, there is a significant but proportional increase in cell proliferation in the absence of Rb as manifested by the total numbers of Hoechst positive cells (86 591.5 ± 22 576.5 in Rb-/- cultures versus 35 946.5 ± 5364.1 and 26 893.8 ± 7796.3 in Rb+/- and Rb+/+, respectively ($p= 2.2691 \times 10^{-6}$ for Rb-/- vs Rb-/+, and $p= 7.76778 \times 10^{-7}$ for Rb-/-, Rb+/+), Nestin+ cells (Rb-/-: 61 269 ± 4772.8, Rb+/-: 35 946.5 ± 5364.1, Rb +/+: 16497.5± 3193.5, p (Rb-/-, Rb-/+) = 0.000571597, p (Rb-/-, Rb+/+) = 0.000277286) and Ki67+ cells (Rb-/-: 49936.3 ± 16294.4, Rb+/-: 17774 ± 325, Rb +/+:13606.3 ± 1662.9, p (Rb-/-, Rb-/+) = 0.032371959, p (Rb-/-, Rb+/+) = 0.006744547) **(Graph 1a).**
This is also true for BrdU+ cells (Rb-/-: 325 ± 70 vs Rb +/+: 81.5 ± 6.5, p (Rb-/-, Rb+/+) = 0.037098175, n= 2) **(Graph 1b).**
However, no significant difference was detected in the ratio of (Nestin+; ki67+) cells over the total number of Nestin+ cells (Rb-/-: 0.630307585 ± 0.058765595 vs Rb +/-: 0.535451708 ± 0.128164551, p (Rb-/-, Rb+/-) = 0.285208567) (**Graph 1c**).
Error bars represent SEM of measurements from n=2 cultures and asterisks indicate a statistically significant difference between genotypes using t-tests, (*) represents $p<0.05$, (**) represents $p<0.01$ and (***) represents $p<0.001$.



2a.



2b.



2c.

Graph 2. Quantification of the total numbers of renal cells including Nestin+ and Ki67+ cells in Rb+/- and Rb-/- cultures after 3 days *in vitro*.
Consistent with the data obtained on day 2, and compared with Rb heterozygous controls, we detected a significant but proportional increase in cell proliferation in Rb-/- cultures as manifested by the total numbers of Hoechst positive cells (60792 in Rb-/- cultures versus 29703 and 16497.5 ± 1773.5 in Rb+/- and Rb+/+, respectively), Nestin+ cells (48015 in Rb-/- cultures versus 21614 and 23100.25± 4845.5 in Rb+/- and Rb+/+, respectively) (**Graph 2a**) and Ki67+ cells (39489 in Rb-/- cultures versus 15286 in Rb+/+) **(Graph 2b).** However, there was no significant difference in the ratio of (Nestin+; ki67+) cells over the total number of Nestin+ cells (Rb-/-: 0.821805686 vs Rb +/+: 0.800041975) (**Graph 2c**). Measurements from n=1 culture.

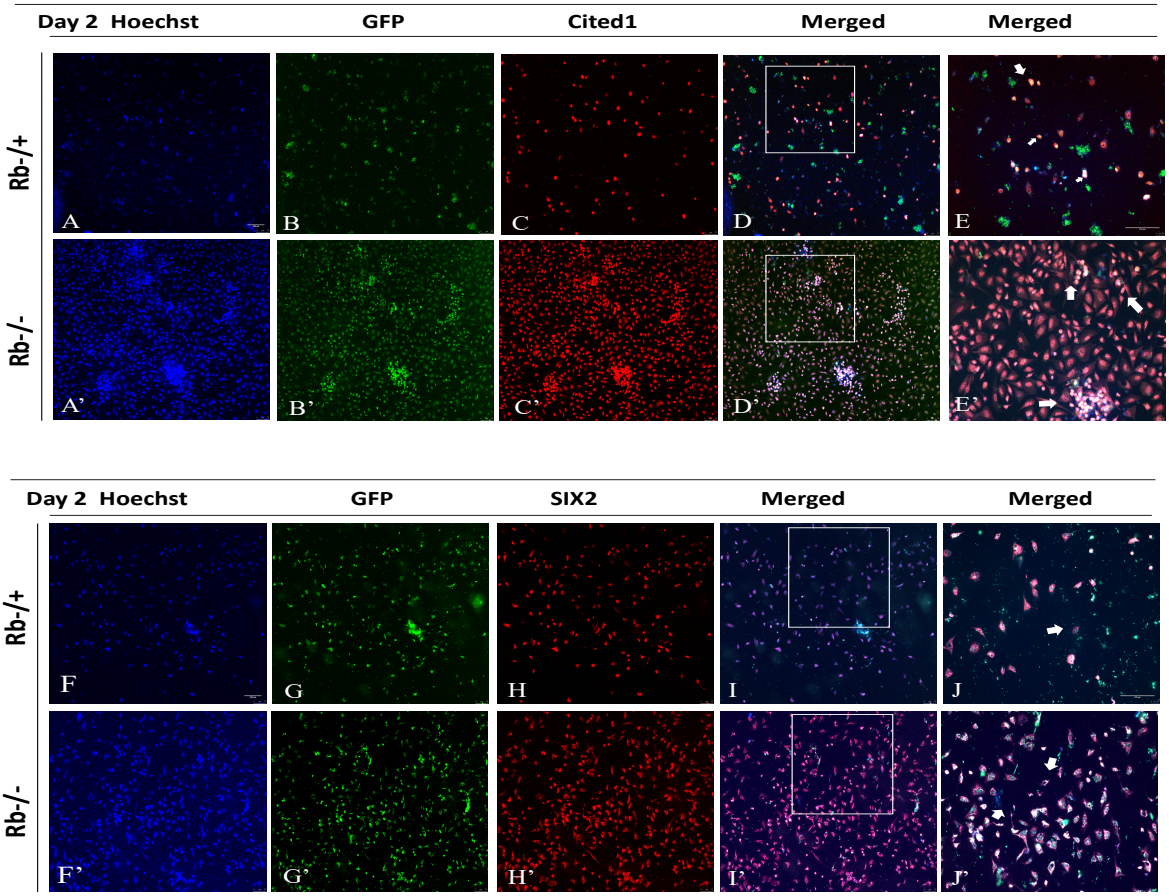
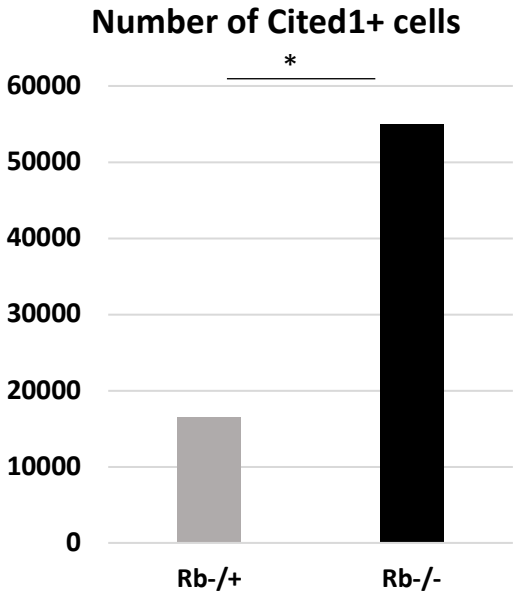
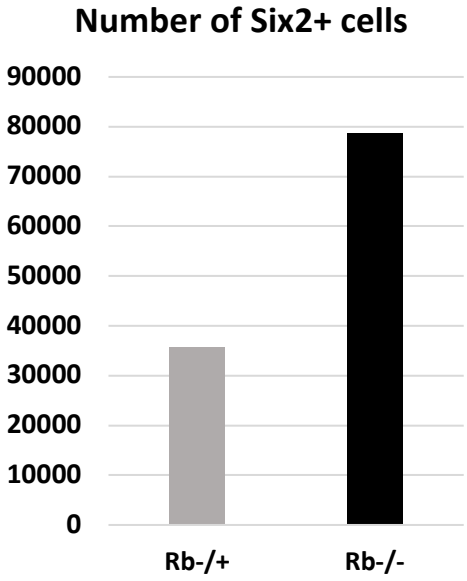


Figure 5



Graph 3a.



Graph 3b.

Figure 5 Enhanced renal progenitor proliferation in the absence of Rb *in vitro*.
(A-E') Double immunostaining for GFP (green) and Cited-1 (nephron stem cell marker; red) in Rb-/+ (A-E) and Rb-/- (A'-E'). (E-E') are higher magnification and overlapping images of the boxes shown in D and D', respectively (white arrows; double positive cells). (F-J') Double immunostaining for GFP (green) and Six2 (nephron progenitor cell marker, red) in Rb-/+ (F-J) and Rb-/- (F'-J'). (J-J') are higher magnification and overlapping images of the boxes shown in I and I', respectively. Compared with Rb-/+ littermate controls, we detected significantly higher numbers of Cited1+ and Six2+ cells (note the cell aggregates in Rb null cultures in A'-E') indicating an expansion in the pool of nephron stem and/or progenitor cells upon loss of Rb.

Graph 3: Quantification of the total numbers of Cited1+ and Six2+ cells in Rb+/- and Rb-/- cultures after 2 days in *vitro*
Quantification of the data shown in figure 5 confirming the significant increase in the numbers of Cited-1 (Rb-/- 54936 ± 14495.5 vs Rb+/- 16 445± 2377.5, p(Rb-/-, Rb-/+) = 0.031869163, n=2 cultures) (Graph 3 a) and Six2 (78548 in Rb-/- vs 35716 in Rb+/-, n= 1 culture) (Graph 3b) in the absence of Rb. Scale bar (A-D' = 75μm; E-E'=100μm). Error bars represent SEM of measurements from n=2 cultures and asterisks indicate a statistically significant difference between genotypes using t-tests, (*) represents $p<0.05$.

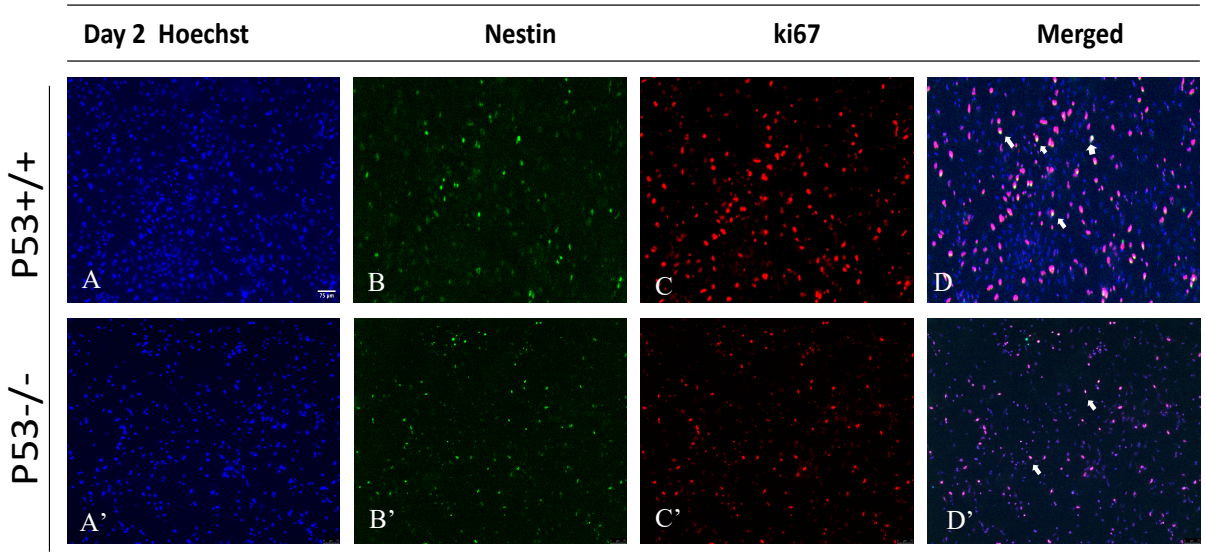
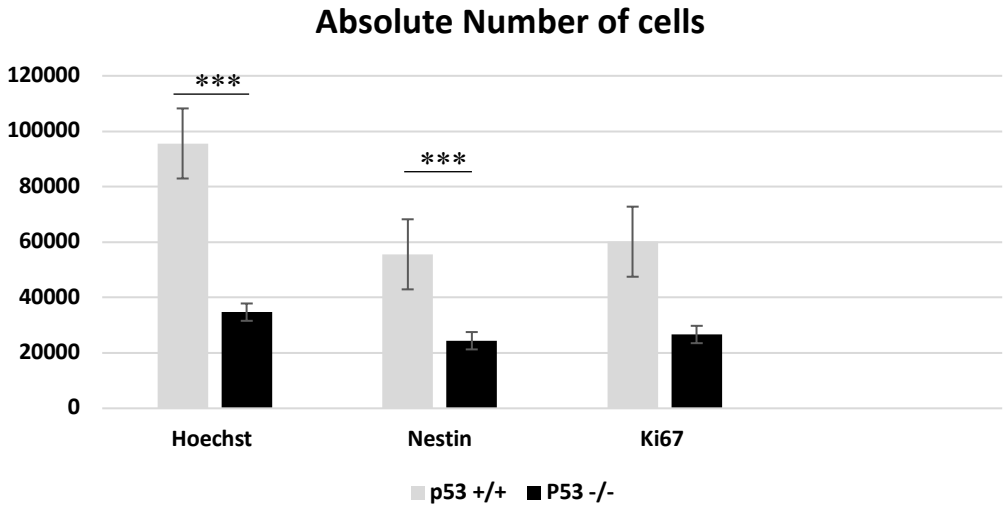
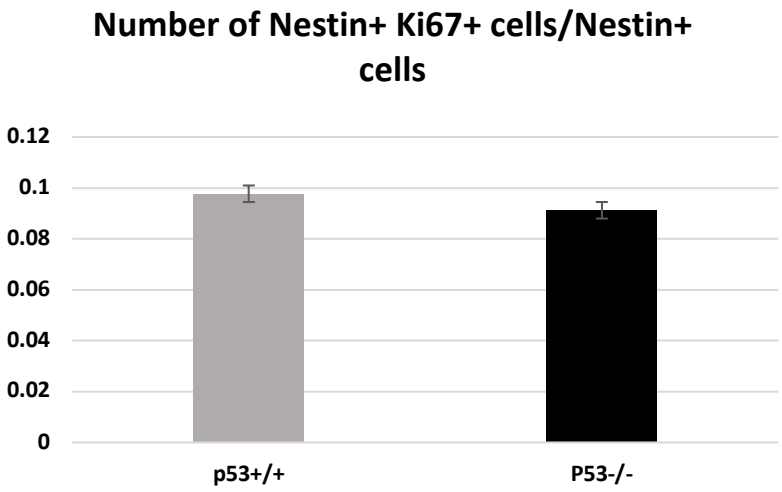


Figure 6



Graph 4a.



Graph 4b.

Figure 6: Loss of p53 negatively affects renal stem/progenitor growth and proliferation *in vitro*

Graph 4: Quantification of the total numbers of renal cells including Nestin+ and Ki67+ cells in p53-/- and p53+/+ cultures after 2 days in vitro

(A-D') Double immunostaining for Nestin (green) and ki67 (red) in p53+/+ (A-D) and p53-/- (A'-D') cultures. (D-D') are merged images showing the overlap between Nestin and ki67 expressions (white solid arrows). Compared with p53+/+ littermate controls, p53-/- renal cultures show lower cell density overall (p53+/+: 95529 ± 5245.1 vs p53-/-: 34710.8 ± 2677.2, p= 7.44663 x 10^{-5}; A-A' and **Graph 4a**) including significantly lower counts of Nestin+ cells (B-B'; p53+/+: 24 395.7 ± 7422.6, vs p53-/-: 5 581.3 ± 2677.2, p = 0.002513443) and Ki67+ cells (C-C'; ; p53+/+: 60138 vs p53-/-: 26647) (**Graph 4a**). Scale bar (A-D' = 75μm). However, no significant difference was noted in the ratio of (Nestin+; ki67+) cells over the total number of Nestin+ cells between genotypes suggesting the absence of a proliferation defect upon loss of p53 (p53+/+: 0.097710248 vs p53-/-: 0.091213091) (**Graph 4b**). Error bars represent SD of measurements from n= 3 wells and asterisks indicate a statistically significant difference between genotypes using t-tests, (***) represents p<0.001.

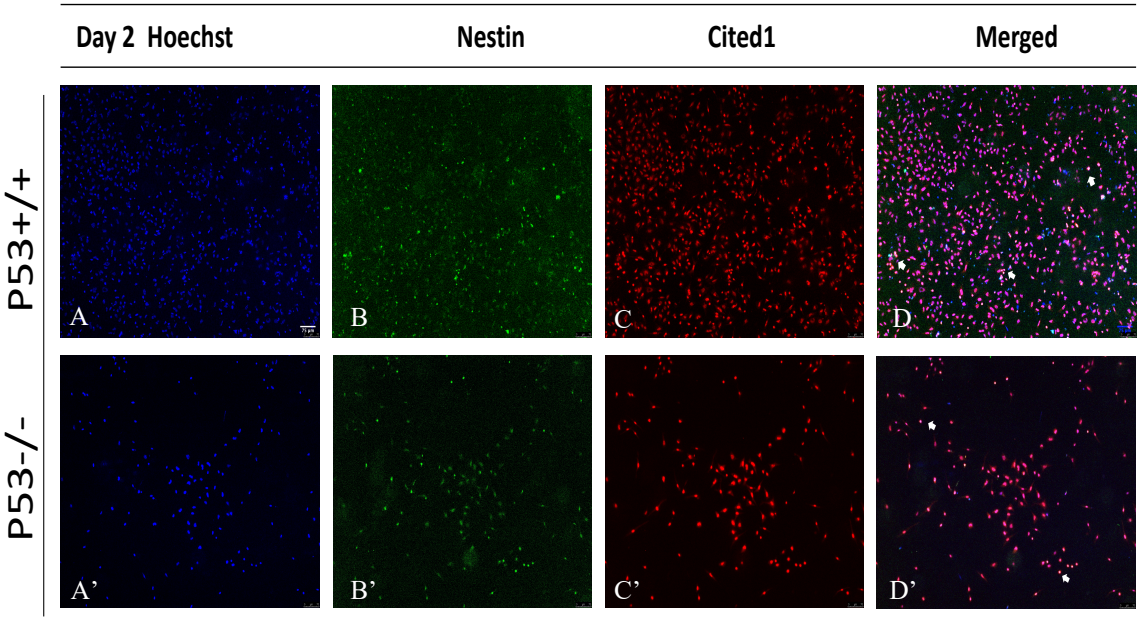
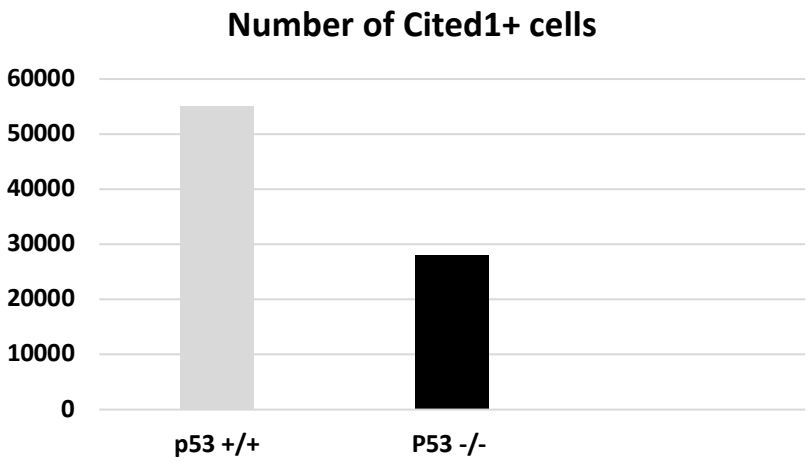


Figure 7

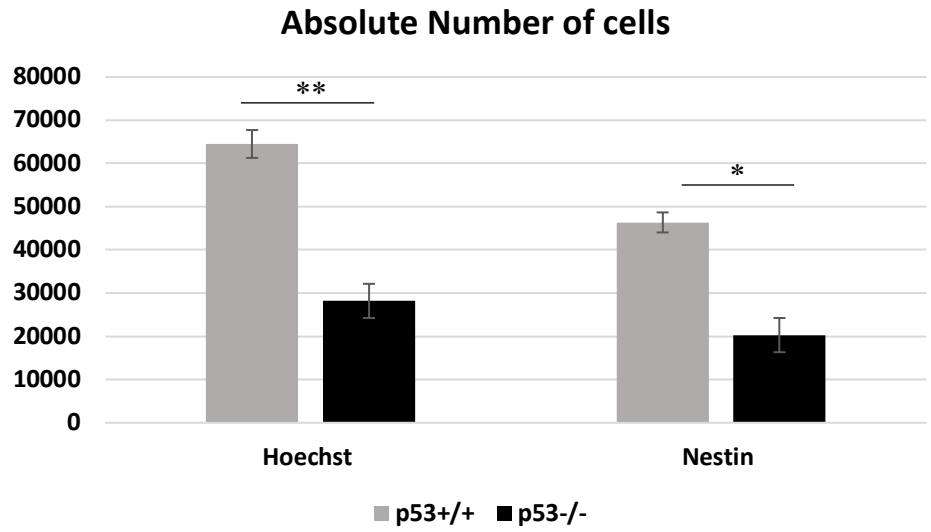


Graph 5

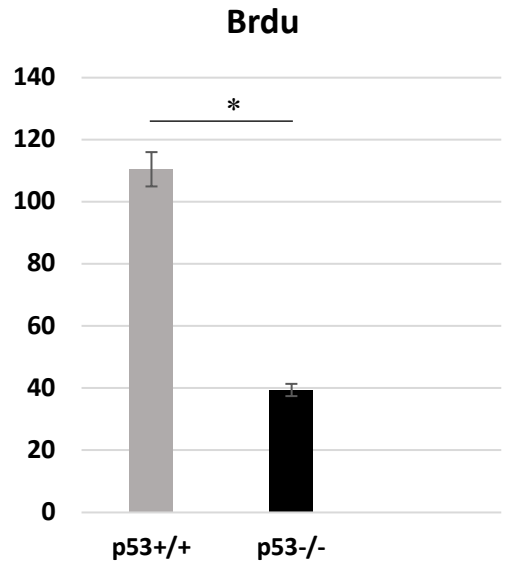
Figure 7: Reduction in the population of Cited1+ stem/progenitor cells in the absence of p53 *in vitro*.

Graph 5: Quantification of the total numbers of Cited1+ cells in p53-/- and p53+/+ cultures after 2 days in *vitro*

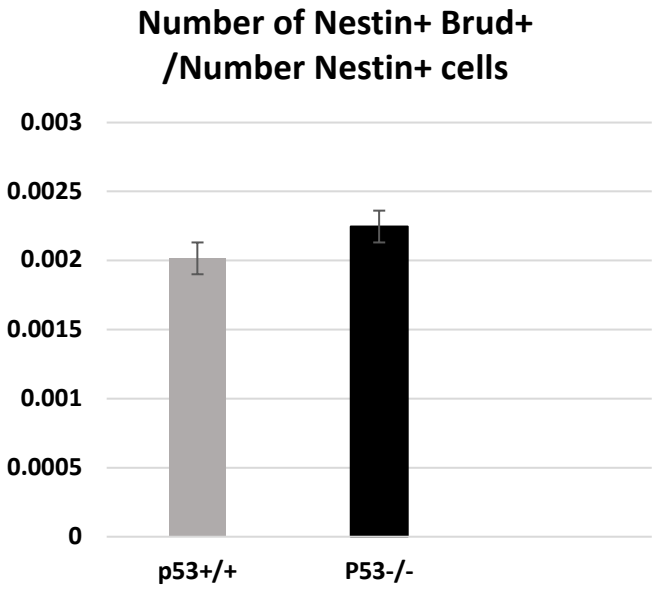
(A-D') Double immunostaining for Nestin (green) and Cited1 (red) in p53+/+ (A-D) and p53-/- (A'-D') cultures. (D-D') are merged images showing the overlap between Nestin and Cited1 expressions (white solid arrows). Compared with p53+/+ littermate controls, p53-/- renal cultures showed a significantly lower numbers of Cited1-positive cells (C-C'; p53+/+: 55027 vs p53-/-: 27991) (**Graph 5**). Scale bar (A-D' = 75μm). Measurements from n=1 culture.


Absolute Number of cells
80000
70000
60000
50000
40000
30000
20000
10000
0
**
*
Hoechst
Nestin
p53+/+
p53-/-

6a.


Brdu
140
120
100
80
60
40
20
0
*
p53+/+
p53-/-

6b.



6c.

Graph 6 The rate of renal stem/progenitor proliferation does not seem to be affected by loss of p53 after 3 days in culture. After 3 days in culture, we detected a significant decrease in the absolute counts of renal cells expressing Hoechst (Graph 6a; p53+/+: 64499 ± 11095.2 vs p53-/-: 28196 ± 6309.3, p= 0.007916624), Nestin (Graph 6a; ; p53+/+: 46347.5 ± 11095.2 vs p53-/-: 20284.7 ± 6309.3, p= 0.013167952) and BrdU (Graph 6b; p53+/+: 110.5 ±12.5 vs p53-/-: 59 ± 23, p = 0.039499) in p53-/- cultures compared with p53+/+ controls. Despite this decrease, there was no significant change in the rate of cell proliferation between genotypes suggesting that loss of p53 indirectly affects renal cell proliferation and is likely to be needed to maintain their survival (Graph 6c; p53+/+: 0.00201± 0.0003 vs p53-/-: 0.00224 ± 0.00016, p = 0.299600175). This data is consistent with the one obtained at day 2. Error bars represent SEM of measurements from n=3 wells and asterisks indicate a statistically significant difference between genotypes using t-tests, (*) represents $p<0.05$, and (**) represents $p<0.01$.

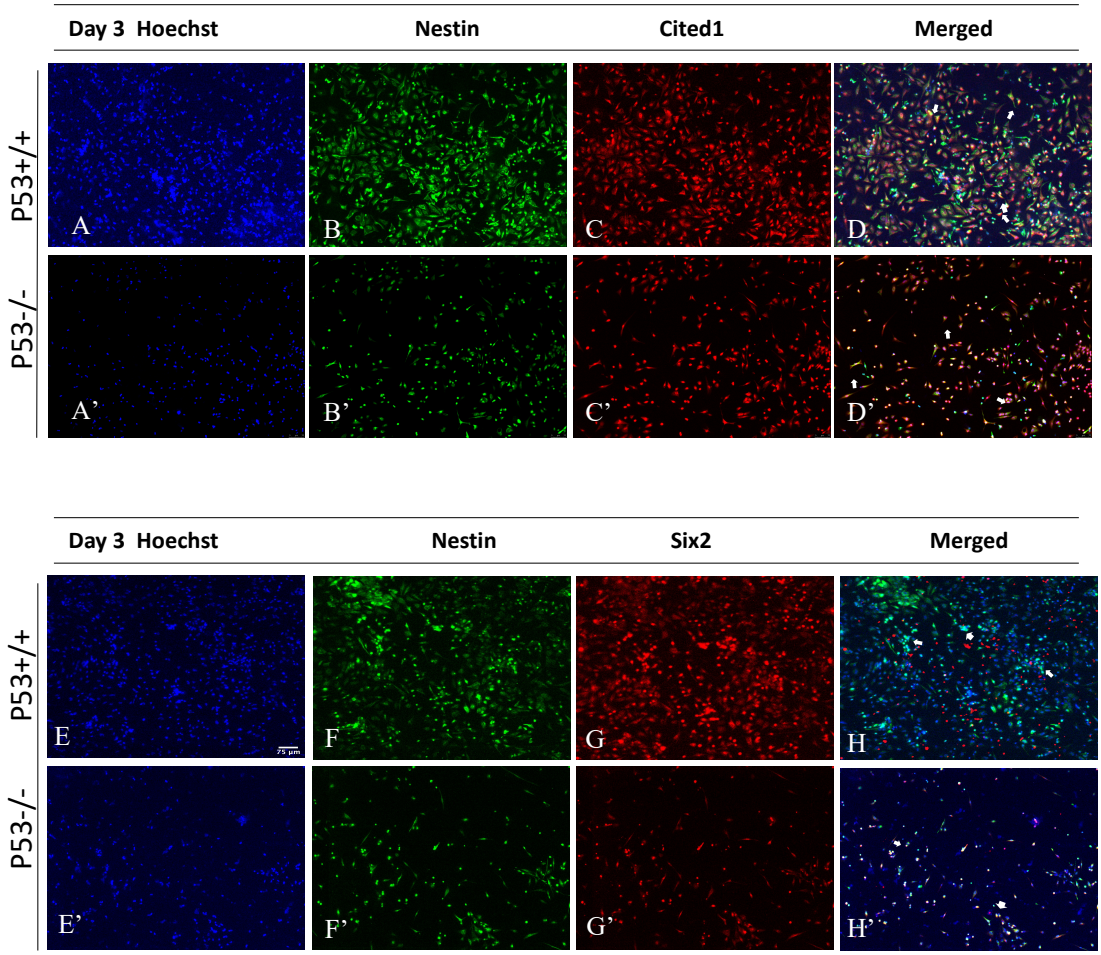
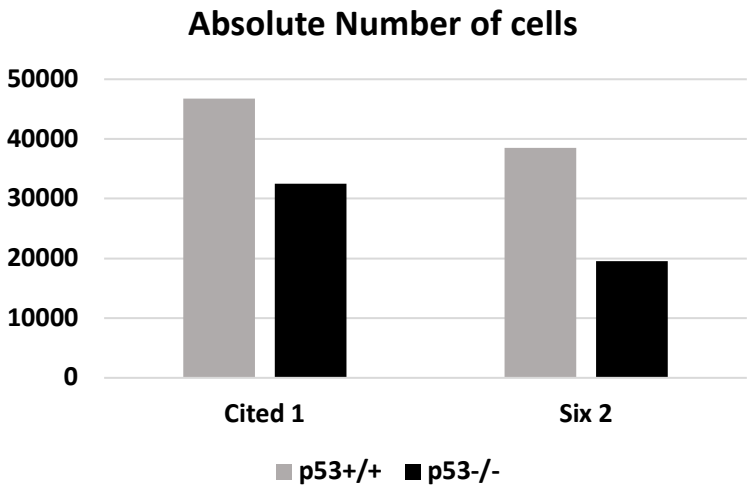


Figure 8



Graph 7

Figure 8: Significant reduction in the population size of stem/progenitor cells in the absence of p53 after 3 days in culture.

Graph 7: Quantification of the total numbers of Cited1+ an Six2+ cells in p53-/- and p53+/+ cultures after 3 days in *vitro*

(A-D') Double immunostaining for Nestin (green) and Cited1 (red) in p53+/+ (A-D) and p53-/- (A'-D') after 3 days in culture. (D-D') are merged images showing the overlap between Nestin and Cited1 expressions (white solid arrows). Compared with p53+/+ littermate controls, p53-/- renal cultures showed a significantly lower numbers of Cited1-positive cells (C-C'; p53+/+: 46813 vs p53-/-: 32491) (**Graph 7**). (E-H') Double immunostaining for Nestin (green) and Six2 (red) in p53+/+ (E-H) and p53-/- (E'-H') cultures. (H-H') are merged images showing the overlap between Nestin and Six2 expressions (white solid arrows). Compared with p53+/+ littermate controls, p53-/- renal cultures showed a significantly lower numbers of Six2-positive cells (G-G'; p53+/+: 38473 vs p53-/-: 19524) (**Graph 7**). Measurements from n=1 culture. Scale bar (A-D' = 75μm).

Chapter IV

DISCUSSION

To study the roles of *Rb* and *p53* genes in the control of the developmental properties of renal stem and progenitor cells *in vitro*, we utilized a primary culture system to isolate renal cells from the nephrogenic zone of the developing kidney. This protocol relies on mild enzymatic digestion of the collected kidneys, and was shown to successfully isolate various cell types including ~35% of cells that belonged to the stromal lineage and ~52% from the cap mesenchyme (mesenchymal-epithelial lineage) (A. C. Brown et al., 2011). Accordingly, our results revealed that the majority of the isolated renal cells were derived from the mesenchymal lineage with 62-69% expressing Cited1, and 85-90% expressing Six2 among the total number of cells. We did not examine the proportions of cells in the stromal lineage e.g. Foxd1-positive or endothelial lineage e.g. Flk+ cells although this should be taking into consideration in future studies. Moreover, as reported by Brown et al., we detected a high cell survival rate up to three days in primary culture, the period after which cells started to gradually die. Therefore, we restricted our histological analysis to two time points, day 2 and day 3.

Despite the efficacy of this protocol, we should highlight some drawbacks. First, as stated above, this system yields a mixture of various renal cell progenitors; second, the system is not suitable to maintain and/or propagate cells over long periods; and, third, it may not be adaptable to induce NSPCs differentiation and maturation/survival of the

nephron progeny. In fact, more recently, Brown et al. developed and optimized a new protocol that allows specifically selecting for and propagating nephron stem and progenitor cells (NSPCs) from murine embryonic kidneys and human embryonic stem cells by modulating the *FGF, BMP*, and *WNT* signaling pathways (A. C. Brown, Muthukrishnan, & Oxburgh, 2015). This new method is based on two main factors: **1)** negative selection by the utilization of specific magnetic beads carrying stromal and endothelial markers to eliminate these progenitors from the renal cells' mixture, and, **2)** the addition of FGF9 to ensure maintenance and proliferation of isolated NSPCs, BMP7 to facilitate their self-renewal through the activation of the JNK pathway, and, WNT to target the (CITED1+; SIX2+) progenitors 'population. Future studies could make use of this new protocol to further examine the roles of Rb and p53 in later stages of nephrogenesis *in vitro*.

To our knowledge, our laboratory is the first to use the Nestin-CreERT2-YFP recombination system to delete Rb and study its role in the developing kidney (Lagace et al., 2007; Morano et al. 2000). Moreover, the current study is the first to use this system to isolate and culture Rb-null and p53-null renal stem/progenitor cells from the nephrogenic zone during development. Moreover, based on GFP reporter gene expression, we previously found high Nestin expression in the metanephric mesenchyme lineage as early as mid-gestation as well as in immature nephrons and podocytes at later developmental stages. Our *in vitro* results are in line with these findings. Hence, we have successfully detected a strong overlap between Nestin and GFP expressions in our primary cultures, in addition to a high recombination rate ranging between 78.9 -81% and 77.8-78% in Rb-/- and p53-/- cultures, respectively [rate of recombination = the number of (Nestin; GFP)

double-positive cells divided by the total number of Nestin-positive cells]. This successful Cre recombination was further confirmed by PCR screening for the recombined p53 and Rb alleles from the corresponding gDNAs. This should also be complemented by western blot analysis using from protein lysates extracted from these cultures to confirm loss (or reduction in the level of expression) of both proteins.

Thus, our *in vitro* study demonstrated that the Nestin-CreERT2-YFP model is suitable and highly reliable for studying gene function in the developing metanephric mesenchyme, particularly cell growth and proliferation. The primary cultures could also be useful in other applications such as gene expression and molecular studies.

Another important aspect of this model is the importance of the Cre driver used. In fact, Nestin-expressing cells were shown to be potentially involved in repair mechanism(s) following renal injury in the adult kidney. Hence, dynamic changes in Nestin's expression have been reported, and it is believed that the upregulation in Nestin's expression stimulates the proliferation of mesangial cells and migration of proximal tubular cells to induce repair (Daniel, Albrecht, Lüdke, & Hugo, 2008; Wen et al., 2012). Moreover, Nestin-positive stromal cells have been implicated in the rescue from acute ischemic kidney injury. The same cells were also shown to provide a paracrine protective effect through the vascular endothelial growth factor signaling to help maintain the glomerular basement membrane (M. H. Jiang et al., 2015; Z. Jiang et al., 2010). In the future, it would be interesting to examine further a potential role for Nestin in renal repair and injury, particularly in the context of growth and regeneration of NSPCs, using the same model system used here.

Although well-known for its role as a universal cell cycle protein that controls a cell's commitment to divide as well as cell survival and senescence (Conklin et al., 2012), the role of Rb has not been investigated in the context of kidney development and disease. Our laboratory was the first to identify a critical requirement for Rb in the control of nephrogenesis and maintenance/survival of immature nephrons (Jaafar C and Ghanem N; unpublished data). In fact, Nestin-driven loss of Rb inside the developing kidney was associated with increased and ectopic proliferation of the Cited1-, Six2+ population of progenitors with subsequent enhanced nephrogenesis *in vivo* (without major differentiation defects). Yet, this was offset by massive cell death inside the renal tubules and immature glomeruli in Rb-/- kidneys compared to littermates' controls.

In comparison, we obtained consistent results *in vitro* showing that Rb negatively controls the proliferation of renal cells including NSPCs in primary cultures. We detected an increase in the numbers of Six2+, Cited1+, and ki67+ single cell populations in Rb-/- cap mesenchyme cultures compared to Rb-/+ and Rb+/+ cultures. However, we were technically not able to co-stain for Cited1 and Six2 for the lack of suitable antibodies, and therefore, could not examine a differential effect for the loss of Rb among the distinct populations (stem cells versus progenitor cells). Moreover, we did not find any significant difference in the proliferation ratio [ratio = the number of (Nestin+; Ki67+) cells over the total number of Nestin+ cells] between Rb-/- and Rb+/- cultures indicating that loss of Rb led to a proportional increase in cell proliferation as seen *in vivo*. However, it remains to be determined whether such loss is associated with cell cycle exit and/or differentiation defects in culture.

In fact, the role of Rb in cell division control and survival of mature cell is consistent in various types of progenitors. For instance, deletion of Rb in intestinal progenitors resulted in increased proliferation along with defects in cell cycle exist (Yang & Hinds, 2007). Similar observations were reported after loss of Rb in retinal and neural progenitor cells during development (MacPherson et al. 2004; Zhang et al. 2004; Ferguson et al. 2002). This was also true upon the deletion of *Rb* in adult neural stem/progenitor cells inside the adult subventricular zone and the subgranular zone; however, it was coupled to severe long-term survival defects of adult-born neurons inside the olfactory bulb and the hippocampus (Naser et al. 2016, Vandenbosch et al. 2016). Due to the technical limitations described earlier, we were not able to address the role of Rb in cell differentiation and survival in our primary cultures after longer incubation periods. This could be overcome in the future when alternative cell culture protocol(s) become available and enable the maintenance and propagation of renal stem/progenitor cells over time.

With respect to the control of NSPCs' proliferation by Rb, it is tempting to hypothesize that the Rb-E2F pathway could be directly modulating the expression level of Six2, the main transcriptional regulator found in nephron progenitor cells. In fact, qRT-PCR studies performed in our laboratory revealed a 4.5-fold increase of Six2 expression inside the nephrogenic zone in the absence of Rb (Jaafar C and Ghanem n, unpublished data). This increase in Six2 expression could, in turn, induce upregulation in the expressions of Six2 direct target genes that stimulate cell proliferation such as *Osr1* and *Gdnf.* It could also be implicated in inhibiting the expression of pro-differentiation markers as *Wnt4* via the formation of repressor complex between Six2, Osr1 and TCF-Groucho-related repressor protein as described earlier (J.-S. Park et al., 2012). Therefore, future

studies should examine potential changes in protein levels of Six2 and its target genes in culture before and after cell differentiation. On the other hand, upon signaling with the Wnt9b/β-catenin pathway, β-catenin competes with the Groucho related repressor proteins forming an activator complex which upregulates the expression of Wnt4 (Jingyue Xu et al., 2014). An earlier study have shown that there is crosstalk between the Rb/E2f pathway and the Wnt/β-catenin pathway; hence, in human colorectal cancer, E2F1 activates an inhibitor of β-catenin (Jingyue Xu et al., 2014). A similar interaction could be mediating, at least partially, the role of Rb/E2F pathway in the control of nephron progenitors' proliferation both *in vivo* and *in vitro*.

Besides cell cycle control, various studies have shown that the Rb-E2F pathway is actively required for cell cycle exit and cell differentiation in many organs including the developing telencephalon. Hence, this pathway is involved in inducing the expression of differentiation markers such as *Dlx* genes and securing proper transition from proliferation to differentiation (Ghanem et al. 2012; Ferguson et al. 2002). Our recent study reported enhanced nephrogenesis (nephron differentiation) in the absence of Rb *in vivo*. This was characterized by an increase in the expression levels of various differentiation markers as Slc12a3, Slc34a3, and Podxl along with an increase in the number of differentiating nephrogenic structures such as S-shaped bodies at various developmental time points (unpublished data; Jaafar C and Ghanem N). While this phenotype could be associated with deregulated proliferation of Rb-null nephron progenitor cells (secondary consequence to the loss of Rb), it does not rule out a potential/direct role for the Rb-E2F pathway in the control of nephron differentiation. Therefore, future work should aim to test this possibility using

the protocol optimized by (A. C. Brown et al., 2015) to prime nephron progenitor cells and induce them to differentiate for example.

In addition, Rb is well known for its anti-apoptotic role in both embryonic and adult tissues via the repression of E2F1 apoptotic function. E2F1 has been shown to trigger apoptosis through a p53 dependent manner (Hershko, 2005); hence, E2F1 upregulates the expression of apoptosis-stimulating proteins of p53 (ASPP1/2) which activate p53 and trigger the expression of pro-apoptotic genes as Bax (Fogal et al., 2005). Moreover, E2F1 can manifest its apoptotic role in a p53-independent manner, which is mediated by p73 that activates common pro-apoptotic genes (Polager, Ofir, & Ginsberg, 2008). Moreover, various studies have shown that Rb is essential for maintaining a post-mitotic state in mature cell types, thus preventing cell cycle entry (Knudsen & Knudsen, 2008). Our in *vivo* work revealed that, indeed, loss of Rb in the developing kidney resulted in massive apoptosis inside the tubular epithelia compared to Rb+/- littermate controls. These results are consistent with the findings from other studies conducted in the laboratory and showing increased apoptosis in olfactory sensory neurons in the developing olfactory epithelia as well as compromised long-term survival of adult-born neurons following loss of Rb (Jaafar et al., 2016; Naser et al., 2016). Thus, another important feature to investigate in the future is the effect of loss of Rb on the maintenance and survival of nephron progenitor cells' and their subsequent progeny e.g. this could be done by TUNEL assay.

Altogether, our *in vitro* data uncovered an essential role for Rb in the control of the properties of nephron stem and progenitor cells, particularly during cell proliferation. While this data is consistent with our *in vivo* findings, more experiments are still needed to better understand the role of the Rb pathway at later stages of nephrogenesis.

In addition to its central role in cell cycle control, p53 plays major roles in regulating various vital cellular processes including maintaining genomic integrity, DNA repair, apoptosis/autophagy, senescence as well as controlling stem cell self-renewal and cell fate (Solozobova & Blattner, 2011). Our current study showed that loss of p53 in primary cultures of isolated renal cells negatively affects cell growth given that it led to a significant decrease in the total number of cultured cells after 2-3 days in culture. This decrease affected the numbers of Nestin+, ki67+, Cited1+ and Six2+ cells in p53-null cultures in comparison to p53+/+ littermate controls. Moreover, while we clearly identified reduced cell proliferation by BrdU incorporation and Ki67 labeling, the rate of proliferation of p53-/- renal cells was remarkably not changed and therefore, matched the control level. This indicates that, rather than being associated with proliferation defect (which is counterintuitive in the case of loss p53 tumor suppressor function), this phenotype is likely due to a role of p53 in cell survival. Strong evidence in support of such role comes from our in *vivo* study showing severe renal hypoplasia and loss of mature nephrogenic structures including irregular formation of glomeruli in the absence of p53 (unpublished data; Jaafar C and Ghanem N).

Moreover, other studies highlighted the need for p53 in the control of metabolic fitness inside nephron stem/progenitor cell, which is a critical feature for the maintenance and self-renewal of these cells (Li et al 2015). In fact, using a Six2-Cre driven deletion of *p53*, Li et al. showed that loss of p53 resulted in premature depletion of the Cited1+; Six2+ population. This phenotype was presumably associated with decreased stem cell self-renewal capacity and downregulation in the expression level of various genes that are essential for cellular adhesion, migration as well as oxidative respiration and glucose

metabolism (Saifudeen et al. 2009; Yuwen Li et al. 2015). However, it was not linked to aberrant increase in apoptosis, which is in direct contrast with our *in vivo* findings, and, our *in vitro* data showing no change in proliferation rates between genotypes. This discrepancy is probably due to the fact that Li et al only examined the absolute number of proliferating cells *in vivo* without taking into account the relative rate of proliferation.

While the role of p53 in metabolic fitness is strongly backed-up with scientific evidence, its positive regulation of renal stem cells' self-renewal is counterintuitive, less convincing and warrants further investigation. In fact, in the adult brain, p53 was shown to negatively regulate self-renewal of neural stem cells without directly affecting proliferation of progenitor cells. In fact, loss of p53 led to a remarkable increase in stem cells' proliferation and self-renewal as well as accelerated differentiation both *in vivo* and *in vitro* (Gil-Perotin et al. 2006 ; Halaby N, Saliba A and Ghanem N, unpublished data). Therefore, we argue that the decreased cell proliferation reported in our culture system (and the study by Li et al.) is due to compromised cell survival and/or other defects e.g. lower metabolic fitness, rather than decreased self-renewal potential. More experiments are surely required to confirm this scenario. Finally, it would be interesting to also examine potential crosstalk between the Rb and p53 pathways in future experiments given that their combined loss resulted in exacerbated renal defects *in vivo* e.g. more severe renal hypoplasia and pronounced cell death compared to the phenotypes observed with single knock-out mice.

Taken together, our study is the first to investigate the roles of Rb or p53 in the control of renal stem and progenitor cells' properties in *vitro*. Our results uncovered novel roles for these proteins in culture and complemented previous work done *in vivo*. While more experimentation is still needed to fully dissect the various functions of these

pathways, our findings will help contribute to a better understanding of the molecular mechanisms controlling kidney development, and potentially, kidney regeneration in the long run.

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